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eIF2α Phosphorylation in Cellular Stress Responses and Disease

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PHOSPHORYLATION OF THE α SUBUNIT OF EUKARYOTIC translation initiation factor 2 (eIF2α) is a highly conserved regulatory event activated in response to diverse stresses (Chapter 12). It elicits translational reprogramming as its primary consequence and secondarily affects the transcriptional profile of cells (Chapter 9). Together, these two strands of the eIF2α phosphorylation-dependent integrated stress response (ISR) broadly affect gene expression, amino acid and energy metabolism, and the protein-folding environment in the cell. Rare human mutations and transgenic mice, in which components of the ISR have been severely altered, reveal the pathway's importance to mammalian pathophysiology. Here, we review the components of the mammalian ISR and consider their function in the context of the cellular adaptation to protein misfolding, nutrient deprivation, and other stresses. We address the potential importance of the ISR to such common human diseases as diabetes mellitus, the metabolic syndrome, osteoporosis, neurodegeneration, and demyelination. Special emphasis is placed on instances suggesting that failure of homeostasis in the ISR contributes to disease, and these are considered in the context of the hypothetical therapeutic opportunities they present.

BACKGROUND

Molecular and Physiological Principles That Determine the Consequences of eIF2α Phosphorylation

Phosphorylation on serine 51 of its α subunit converts eIF2 from a substrate to an inhibitor of its guanine nucleotide exchange factor, eIF2B. Thus, the level of phosphorylated eIF2α regulates the rate at which eIF2 can be recycled to the GTP-bound form to join in a ternary complex with charged initiator methionyl-tRNA and promote the initiation of mRNA translation (Chapter 4). Consequently, the reversible phosphorylation of eIF2α has evolved as a potent means for regulating translation initiation rates. This phosphorylation event is purely regulatory; it has no direct effect on the function of eIF2 as an initiation factor. Indeed, the side chain of residue 51 is relatively unimportant, as translation initiation proceeds very well with an eIF2α^{S51A} mutant protein, a point we return to shortly.

Experiments in yeast suggest that the measurable effects of eIF2α phosphorylation can be suppressed by mutations in components of eIF2B (Chapter 9). Therefore, the consequences of eIF2α phosphorylation are likely mediated through their effects on translation. Despite this narrow convergence on a single molecular target, the moderating effect of eIF2(αP) on protein synthesis has surprisingly broad biological consequences.

Protein synthesis consumes much energy and competes with other metabolic processes for the utilization of free amino acids. For example, it is estimated that more than half the energy consumed by a prominent secretory cell such as the hepatocyte is devoted to protein synthesis (Pannevis and Houlihan 1992). Thus, the ability to reversibly attenuate protein synthesis is an important adaptation of cells to diverse stressful conditions that threaten energy homeostasis or require the diversion of amino acids to other metabolic pathways (Chapters 14 and 17).

Newly synthesized proteins pose a significant burden on the chaperone machinery of the cell, which assists nascent chains to attain their proper three-dimensional structure. The reserve capacity of the protein-folding machinery in the various compartments is relatively limited, as attested to by the dire consequences of (*cis*) mutations affecting the ability of abundantly expressed proteins to fold (Carrell and Lomas 1997). Therefore, eIF2(αP)-mediated attenuation of protein synthesis protects chaperone networks from client protein overload. It may be important in this regard that eIF2(αP) targets the initiation phase of protein synthesis and does not attenuate elongation. It is now appreciated that the ribo-

some nascent chain complex is associated with cellular chaperones that function as components of a coupled translation and folding machine (Bukau 2005). Attenuated processivity of this machine would tend to prolong the engagement of chaperones with incomplete nascent chains, whereas attenuated initiation would allow chaperones to recycle efficiently. Similar considerations apply to the pool of ribosomes and translation factors; by attenuating the initiation phase of translation, eIF2(αP) signaling facilitates the reprogramming of translation around the changing complement of mRNAs in stressed cells.

Although ternary complexes of eIF2 + GTP + charged $tRNA_i^{met}$ are required for translation initiation at all AUG codons (Chapter 4), the effects of limiting their availability (by eIF2α phosphorylation) has surprisingly gene-specific consequences. The mRNA encoding the yeast transcription factor Gcn4p and its metazoan counterpart, ATF4, are both subject to paradoxical translational up-regulation when levels of ternary complexes decline (Dever et al. 1992; Harding et al. 2000b). Translation initiation at the protein coding AUG of these unusual mRNAs is normally repressed by the presence of several conserved inhibitory upstream open reading frames. However, stress conditions that lead to eIF2α phosphorylation and declining ternary complex formation derepress ATF4 and GCN4 translation by a mechanism of regulated translation reinitiation (Lu et al. 2004a; Vattem and Wek 2004; Chapter 9).

Translational up-regulation of these transcription factor(s) activates a gene expression program whose targets adapt the cell to the specific stresses that promote eIF2α phosphorylation. In yeast, mutations that abolish eIF2(αP) signaling by inhibiting kinase activity or preventing eIF2α phosphorylation *in cis* have consequences similar to those affecting Gcn4p, suggesting that this linear signaling pathway evolved predominantly to control transcription rather than protein synthesis (Dever 2002). The situation in mammals is less clear. The phenotypic overlap of ATF4 deletion and various mutations that inactivate signaling upstream in the pathway are incomplete (Harding et al. 2003); however, ATF4 is not the only effector of regulated gene expression by the eIF2(αP) signaling pathway (Jiang et al. 2003; Deng et al. 2004). Therefore, the relative contributions of translational attenuation and transcriptional activation to the downstream consequences of signaling by eIF2(αP) remain to be defined. It is important to note, in this regard, that despite considerable effort, no other mRNA has been clearly demonstrated to be subjected to eIF2(αP)-regulated translation reinitiation, and the mechanisms linking eIF2(αP) signaling to the ATF4-independent gene activation program of mammals remain obscure.

Regulating Cellular Levels of Phosphorylated eIF2α

The eIF2(αP)-dependent signaling nexus is regulated by kinases and phosphatases. There are four known eIF2α kinases in vertebrates: GCN2 responds predominantly to amino acid deprivation, PERK responds to the imbalance between the load of endoplasmic reticulum (ER) client proteins and chaperones (so-called ER stress), and HRI and PKR are activated, respectively, by unbalanced synthesis of heme and globin in the erythroid lineage or the presence of double-stranded RNA in virally infected cells (Chapter 20).

Despite a diversity in upstream activating events, these four kinases have similar catalytic domains and share a single known effector, eIF2(αP) (Chapter 12). Formal evidence for this last point is provided by the shared phenotype of mutations in yeast that eliminate GCN2, or block the ability of eIF2α to be phosphorylated by the kinase (replacement of eIF2α's serine 51 by an alanine, $eIF2\alpha^{S51A}$) (Dever et al. 1992). The situation in mammals is complicated by the presence of more than one kinase. Nonetheless, the overlap in phenotype between homozygosity for $eIF2\alpha^{S51A}$ and loss of PERK (the dominant eIF2α kinase under basal conditions, in many cells) suggests that in mammals, too, these kinases have a single cellular target (Harding et al. 2001; Scheuner et al. 2001; Zhang et al. 2002a). Furthermore, the $eIF2\alpha^{S51A}$ mutation abolishes all measurable consequences of PERK activation (Lu et al. 2004a). Thus, claims to the contrary notwithstanding (Cullinan et al. 2003), we believe that most if not all signaling by this family of kinases is mediated by a single substrate, eIF2α.

Phosphorylation of eIF2α is highly dynamic and regulated. In addition to the aforementioned kinases, there are two known phosphatases that dephosphorylate eIF2(αP). A constitutive complex, consisting of an eIF2α-specific regulatory subunit (CReP) and the catalytic subunit of protein phosphatase I (PP1c), contributes to the high basal eIF2(αP)-directed phosphatase activity of mammalian cells (Jousse et al. 2003). In stressed cells, the eIF2α phosphorylation-dependent gene expression program activates a second related phosphatase regulatory subunit encoded by the *GADD34* gene (Novoa et al. 2001; Brush et al. 2003). *GADD34*, too, forms a complex with PP1c and significantly increases the eIF2(αP)-directed phosphatase activity of cells, ensuring recovery of protein synthesis during the later phases of stress responses that activate the eIF2α kinases (Kojima et al. 2003; Novoa et al. 2003). We do not know whether, between them, *GADD34* and CReP account for all eIF2(αP) dephosphorylation, nor do we know whether the four known kinases account for all the eIF2α-directed kinase activity of cells.

Given the diversity in upstream activators, all of which are channeled to a single downstream event, we propose that the consequences of eIF2α phosphorylation, in metazoans, be referred to as the integrated stress response (ISR, Fig. 1). As we shall see, the ISR, with its translational and transcriptional components, forms an important strand of several stress-responsive signal transduction pathways. Much of what we know about the role of eIF2α phosphorylation in health and disease has been
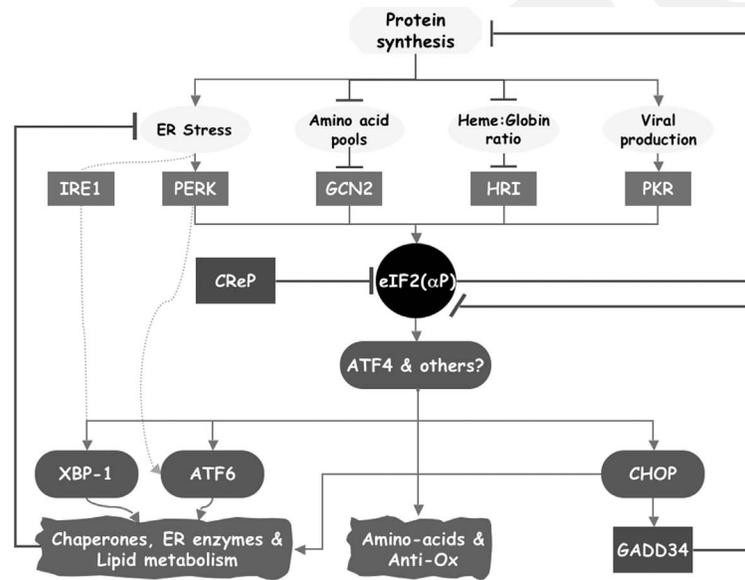

Figure 1. Schematic overview of the eIF2α-phosphorylation-dependent integrated stress response (ISR). The four eIF2α kinases—PERK, GCN2, HRI, and PKR—are modulated by distinct stress signals, which in turn are influenced by rates of protein synthesis. eIF2α phosphorylation and attenuated protein synthesis are especially important in limiting the levels of ER stress. Expression of ATF4 and possibly other transcription factors is increased by eIF2α phosphorylation, and these activate downstream genes. The latter include the genes encoding the transcription factors ATF6 and XBP-1, which are also controlled by parallel ER stress signals (*dashed arrows*) and genes downstream from that which encode chaperones, ER enzymes, enzymes involved in lipid metabolism, amino acid transporters, and enzymes involved in amino acid metabolism and anti-oxidative stress response genes. Also induced is the gene encoding *CHOP*, a transcription factor that activates *GADD34*, which encodes an eIF2(αP)-specific regulatory subunit of a phosphatase that inhibits signaling in the ISR. CReP is a constitutive regulatory subunit of a phosphatase that dephosphorylates eIF2(αP). (*Arrows*) Activating signals; (*blunted lines*) inhibitory signals.

derived by analysis of mutations that affect this pathway. Next, we consider these mutations from the perspective of their effects on defined physiological processes.

PATHOPHYSIOLOGICAL MECHANISMS: eIF2α PHOSPHORYLATION AND PROTEIN FOLDING

PERK and HRI Couple Protein Synthesis to the Rate of Posttranslational Protein Metabolism

Evidence for the role of eIF2α phosphorylation in preventing protein misfolding is provided by the analysis of mutations in PERK and HRI. PERK is an ER-localized type-I *trans*-membrane protein whose lumenal domain senses the balance between unfolded/misfolded lumenal client proteins and the host of chaperones in the organelle. An imbalance, also referred to as ER stress, leads to PERK activation that occurs by oligomerization in the plane of the membrane, *trans*-autophosphorylation, and a marked enhancement in kinase activity and in the kinase's affinity for its substrate, which take place on the cytosolic face of the ER membrane (Bertolotti et al. 2000; Marciniak et al. 2006). The resultant increase in eIF2(αP) levels attenuates protein synthesis and diminishes the load of newly synthesized unfolded ER client proteins. PERK thus matches the rate of client protein synthesis on the cytoplasmic side of the ER to the folding environment on the lumenal side (Harding et al. 1999; Sood et al. 2000).

In the absence of PERK, cells are unable to modulate protein synthesis in response to protein misfolding in the ER lumen, and *PERK*$^{-/-}$ cells experience higher levels of ER stress when subjected to manipulations that adversely affect the folding environment in the ER (Harding et al. 2000a). PERK deletion in mice and homozygosity for loss-of-function mutations in humans (the Wolcott Rallison syndrome) are associated with profound dysfunction in a number of tissues constituted of mainly secretory cells. Thus, the mutant mice and humans develop a syndromatic form of diabetes mellitus with onset in infancy, exocrine pancreatic dysfunction, and a severe bone defect (Delepine et al. 2000; Harding et al. 2001; Zhang et al. 2002a). As expected, hypersensitivity to conditions that promote protein misfolding in the ER is also observed in the eIF2α^{S51A} mutation that abolishes responsiveness to PERK (Scheuner et al. 2001).

A conceptually similar scenario exists with regard to HRI. This eIF2α kinase is expressed prominently in erythroid precursors, where it is repressed by free heme. When heme is limiting, HRI phosphorylates eIF2α, thereby attenuating protein synthesis, which in erythroid precursors

amounts mostly to inhibition of globin-chain production. This translational repression maintains a balance between heme and globin production to ensure that the newly synthesized globin chains are incorporated into hemoglobin molecules. As long as heme biosynthesis is maintained, HRI is dispensable. However, when heme biosynthesis becomes limiting (commonly a consequence of nutritional iron deficiency), HRI is rendered essential. In its absence, unchecked protein synthesis leads to the accumulation of unliganded globin chains that misfold and are converted to dangerous proteotoxins. What would otherwise be a mild red cell production defect in wild-type animals is converted to a life-threatening anemia due to destruction of red cell precursors in the bone marrow of $HRI^{-/-}$ mice (Chen 2000; Han et al. 2001).

Recently, HRI has been implicated in a different context in which translational control protects cells from proteotoxicity. Arsenite, an abundant environmental toxin that leads to protein misfolding in the cytoplasm, was noted to promote eIF2α phosphorylation through the activation of HRI. It is likely, although unproven, that the hypersensitivity of $HRI^{-/-}$ cells to arsenite is due, at least in part, to their inability to attenuate the load of newly synthesized proteins in response to the threat of toxin-induced misfolding (McEwen et al. 2005).

These dramatic phenotypes of loss-of-function mutations in upstream activators of the ISR point to the critical role of eIF2α phosphorylation in controlling protein synthesis under specific stressful circumstances. It is tempting to speculate that modulation of protein synthesis may have a broad role in protecting cells from physiological and environmental use-dependent attrition; i.e., that eIF2α phosphorylation counteracts processes that contribute to aging. The insulin-producing β cells of the pancreas is one scenario where this process is believed to have an important role in pathophysiology.

Obesity in humans and mice leads to insulin resistance, which is counteracted by increased production of pro-insulin in the ER of the pancreatic β cells. This adaptation is promoted by a dramatic glucose-dependent translation up-regulation of insulin and other secreted proteins in the β cell (Skelly et al. 1996). PERK-dependent eIF2α phosphorylation protects the β cell from the consequences of the elevated client protein load and, at the expense of imposing transient limitation of insulin biosynthesis, ensures the long-term survival of the tissue (Fig. 2). The ultimate failure of this protective mechanism is believed to contribute to the onset of diabetes mellitus in the obese, a theory that is supported by the observation that minor defects in the ISR accelerate the onset of glucose intolerance (Scheuner et al. 2005).
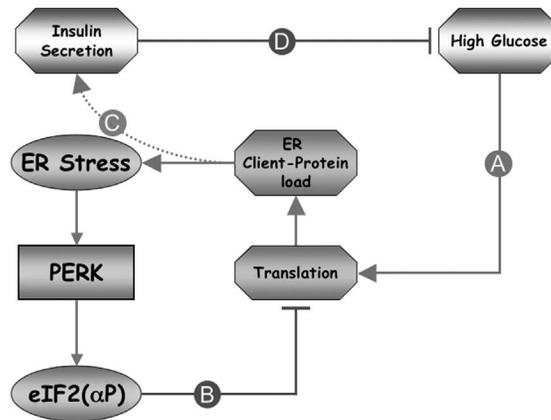
Figure 2. eIF2α phosphorylation in the pancreatic β cell. High-serum glucose (*A*) stimulates translation in the β cell, increasing ER client protein load and promoting a physiological ER stress. The latter is modulated by PERK-mediated eIF2α phosphorylation (*B*), which protects the β cell from ER stress at the expense of insulin synthesis (*C*). However, this compromise is highly advantageous to β cells, which are rapidly destroyed in the absence of PERK. Insulin resistance, which decreases signaling in *D,* places extra stress on the β cell by increased signaling in *A*. The survival of the cell depends, in part, on the adequacy of the protective PERK-dependent mechanism described here.

Unfolded Protein Load and eIF2(αP) Dephosphorylation

The important role of eIF2(αP)-mediated translational control in checking the risk of chaperone overload and misfolding is also supported by the consequences of pharmacological and genetic manipulation of eIF2(αP)-directed phosphatase activity. A screen for chemical compounds that protect cultured cells from the lethal effects of tunicamycin (an agent that perturbs the folding environment in the ER by blocking glycosylation) led to the identification of salubrinal, an in vivo inhibitor of CReP and GADD34 (Boyce et al. 2005). Furthermore, CReP knockdown by RNA interference (RNAi) (Jousse et al. 2003) and deletion of GADD34 (Marciniak et al. 2004) are both protective against experimental models of ER stress. These findings are consistent with a simple model whereby diminished phosphatase activity sustains eIF2(αP) levels and attenuates newly synthesized unfolded protein load and with it levels of ER stress.

The transcription factor CHOP has an especially interesting role in linking eIF2α phosphorylation and ER stress. The *CHOP* gene is strongly

induced by ER stress, as part of the PERK→eIF2(αP)→ATF4-dependent gene expression program mentioned above (Fig. 1) (Harding et al. 2000b). *CHOP* deletion has been noted to protect against the lethal affects of ER stress (Zinszner et al. 1998), whereas CHOP overexpression sensitizes cells (McCullough et al. 2001). GADD34 is a direct transcriptional target of CHOP, such that eIF2(αP)-directed phosphatase activity is diminished in stressed $CHOP^{-/-}$ cells. As a consequence, mutant cells synthesize fewer proteins and, compared with the wild type, confront their chaperones with a diminished load of unfolded client proteins. Mutant cells are thus partially protected from the lethal consequences of perturbations to the protein-folding environment in the ER (Marciniak et al. 2004).

This protection extends beyond experimental pharmacological inducers of protein misfolding to other paradigms with disease relevance. The dominant C96Y mutation in mouse insulin 2 (the so-called Akita mutation) causes the protein to misfold, and the ensuing compromised protein-folding environment in the ER greatly shortens β-cell life span, leading to the development of diabetes mellitus in the affected mice. A $Ddit3^{-/-}$ ($CHOP^{-/-}$) genotype is partially protective against β-cell death and delays the onset of diabetes mellitus in $Ins2^{C96Y/+}$ (Akita) mice (Oyadomari et al. 2002).

CHOP deletion has also been noted to protect dopaminergic neurons of mice against 6-hydroxydopamine injection (Silva et al. 2005). This compound is believed to mimic endogenous and environmental agents that contribute to dopaminergic cell death, the underlying cellular substrate for the development of Parkinson's disease. Interestingly, exposure to 6-hydroxydopamine causes ER stress in cultured dopaminergic neurons (Ryu et al. 2002; Holtz and O'Malley 2003), suggesting that the effect of CHOP deletion might be mediated through the ability of sustained elevation of eIF2(αP) levels to protect against ER stress.

In both models, CHOP is induced by the ISR, which is a PERK→eIF2(αP)→ATF4-dependent strand of an evolutionarily conserved response to protein misfolding in the ER known as the unfolded protein response (UPR). The UPR has two other well-characterized strands which also contribute to the activation of genes that enhance the ability of the ER to cope with unfolded and misfolded client proteins: One mediated by the ER-localized signal transducer IRE1 and its downstream effector, the transcription factor XBP-1, activation of which involves a non-canonical mRNA splicing event (Patil and Walter 2001; also see Chapter 10). The other is mediated by the release of the transcription factor ATF6 from its ER tether in stressed cells (for review, see Mori

2000). This three-stranded UPR protects cells from ER stress by attenuating client protein load and by augmenting ER capacity (Harding et al. 2002; Kaufman 2002).

CHOP constitutes a relatively downstream component of the ISR/UPR that contributes to *GADD34* activation, which, in turn, counteracts the effects of PERK in the UPR and serves as a negative feedback loop that maintains protein synthesis. This eIF2(αP)$\rightarrow$ATF4$\rightarrow$CHOP$\rightarrow$GADD34–|eIF2(αP) negative feedback loop presumably evolved in response to the inherent reciprocal relationship between protein synthesis and chaperone reserve. High levels of eIF2(αP) favor chaperone reserve over protein synthesis and, under some circumstances, survival of the individual cell. However, this adaptation tends to compromise the ability of the stressed cell to fulfill its physiological mission (e.g., synthesize insulin, in the example described above) and, over time, might even compromise the cell's ability to synthesize proteins required for its own survival.

Metazoans need to balance these contradictory influences and set the thresholds for activating the various components of the ISR/UPR at their appropriate level. The data fit a model whereby thresholds for activation that proved adaptive under the selective pressures that existed when the system evolved might not be appropriate for all circumstances. According to this theory, the protection against agents that perturb folding in the ER afforded by CHOP deletion or GADD34 inhibition (by salubrinal) likely reflects a failure of homeostasis operating at the level of client protein synthesis (Fig. 3). However, it is not possible to exclude an alternative hypothesis whereby CHOP and GADD34 evolved primarily to kill cells experiencing insurmountable levels of ER stress. The killing of severely damaged ER stressed cells could also have a homeostatic role, as removal of the damaged cells would provide space for regeneration; it is interesting in this regard that CHOP and GADD34 are relatively late additions, being found in vertebrates, but not in simpler metazoans that do not engage in tissue regeneration. This second hypothesis, too, fits a model of failure of homeostasis, but one that is based primarily on the control of cell death rather than protein synthesis.

Besides being intuitively appealing, the model, whereby the eIF2(αP)$\rightarrow$ATF4$\rightarrow$CHOP$\rightarrow$GADD34–|eIF2(αP) loop provides advantages to the organism that are independent of cell death, is supported by at least two observations: (1) the impaired survival of GADD34 mutant cells exposed to high doses of thapsigargin (an agent that strongly activates PERK and promotes a sustained shutdown of protein synthesis in the mu-
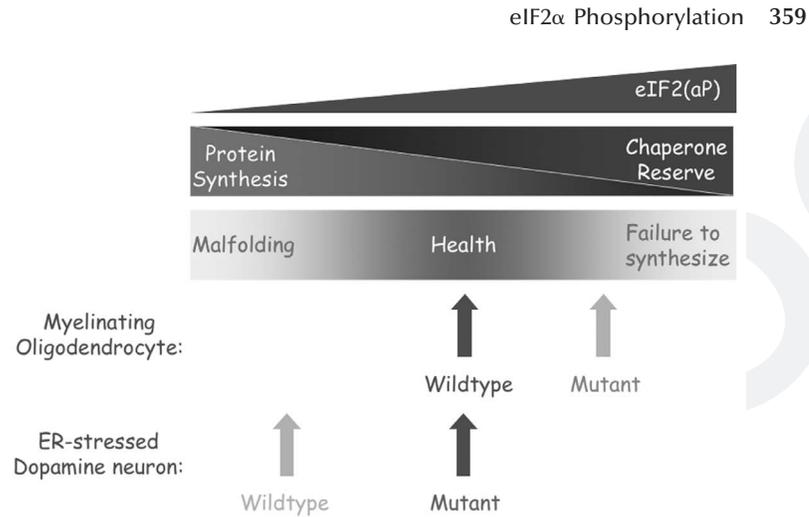
Figure 3. Failure of homeostasis in the ISR. CHOP-mediated GADD34 expression affects the balance between protein synthesis and chaperone reserve (see Fig. 1). The set point for this component of the ISR maintains homeostasis of the wild type under conditions that prevailed during selection of the phenotype. In the hypothetical example considered here, the set point allows the oligodendrocyte to cope with the physiologically stressful process of myelination. The wild-type cell strikes an appropriate balance between too much eIF2α phosphorylation, which risks failure to synthesize enough myelin constituents, and too little eIF2α phosphorylation, which risks protein misfolding in the ER. However, in certain unusual situations, such as the 6-hydroxydopamine-stressed dopamineric neuron, the set point of the wild type is maladaptive, favoring survival of the mutant. This reflects a failure of homeostasis.

tant cells [Novoa et al. 2003]) and (2) the impaired survival of CHOP mutant mice with a confounding second mutation in proteolipid protein (PLP); specifically, a mutation that causes the protein to misfold in the ER of oligodendrocytes and induces a model of Pelizaeus-Merzbacher leukodystrophy. The reduced survival of the *PLP^mut*; CHOP knockout mice is associated with more, not less, oligodendrocyte cell death (Southwood et al. 2002), arguing that the ability of CHOP to promote protein synthesis rather than cell death accounts for its survival advantage in that context.

The observations made on mice and cells with mutations in ISR components suggest that the pathway could be manipulated pharmacologically to beneficial ends. Specifically, inhibitors of eIF2α dephosphorylation might protect cells against ER stress. However, the safety of such manipulation will need to be considered carefully. In the next section, we discuss further complexity introduced into the ISR by its downstream gene expression program.

PATHOPHYSIOLOGICAL MECHANISMS: eIF2α PHOSPHORYLATION AND GENE EXPRESSION

The well-recognized consequences (i.e., scoreable phenotypes) of eIF2α phosphorylation in yeast are attributed to activation of a gene expression program mediated by the translational up-regulation of the transcription factor Gcn4p. The upstream eIF2α kinase, Gcn2p, is activated by uncharged tRNAs and thus responds to decreased availability of amino acids. The gene expression program activated by Gcn4p promotes the import and synthesis of multiple amino acids and is thus referred to as the general (amino acid) control (GC) response (Chapter 9). Amino acid transporters and biosynthetic enzymes are also among the genes prominently up-regulated by the mammalian ISR, but this pathway has diversified to include target genes of other classes, as described below.

Integrated Stress Response and Oxidative Stress

Impaired signaling in the ISR by mutations at any level in the pathway is associated with enhanced accumulation of reactive oxygen species (Scheuner et al. 2001; Harding et al. 2003). These observations reveal that the mammalian ISR has evolved a significant role in combating oxidative stress. This is played out partially at the level of enhanced import of cysteine, a precursor of the cell's major redox buffers, but also includes up-regulation of genes directly involved in redox homeostasis. This aspect of the ISR is especially important during ER stress and likely reflects the role of the ER as a producer of H_2O_2 and a consumer of reduced glutathione (for review, see Tu and Weissman 2004), the consequences of which are counteracted by the gene expression program induced by the ISR (Harding et al. 2003).

The challenge to redox homeostasis posed by ER activity is intrinsic to the biochemistry of the major ER oxidase, ERO1 (Tu and Weissman 2004). Interestingly, *ERO1a*, encoding one of the two mammalian isoforms of this enzyme, is activated by CHOP. This connection can be rationalized as an attempt by stressed cells to up-regulate their capacity to process ER client proteins, whose folding depends on disulfide bond formation. However, in the context of severe ER stress, the up-regulation of *ERO1a* poses a significant challenge to the ability of the cell to maintain a reduced cytoplasm, accounting for the tendency of CHOP to promote oxidative stress (McCullough et al. 2001; Ikeyama et al. 2003; Marciniak et al. 2004).

ISR and Conventional UPR Target Genes

The induction of ERO1α is but one example of the role of ISR in enhancing the capacity of the ER to process client proteins. To achieve this goal, it also collaborates with other strands of the UPR to activate genes that enhance the cell's capacity to cope with ER client protein load. This is mediated in part by transcriptional activation of key UPR regulators such as XBP-1 and ATF6, transcription factors that transduce ER stress signals to the nucleus (Calfon et al. 2002; Harding et al. 2003). It is unclear how PERK-dependent eIF2(αP)-mediated ER stress signaling transcriptionally up-regulates *XBP-1* and *ATF6α*, as these genes are induced normally in *ATF4*$^{-/-}$ cells (Harding et al. 2003).

It is apparent from the above that the prosurvival effects of the ISR are attributed to regulation of both protein synthesis and gene expression. The latter conclusion has received direct support from an experimental system that allows conditional activation of PERK by an artificial ligand, independently of the induction of ER stress. Transient, preemptive activation of PERK (and induction of the ISR) markedly protected HT22 cells from subsequent challenges that involve oxidative stress, although the latter were applied *after* the translational effects of PERK activation had worn off (Lu et al. 2004b). Further support for the protective role of attenuated eIF2 activity (the predicted consequence of eIF2α phosphorylation) was provided by the results of a somatic cell genetic screen in which interference with eIF2α expression protected HT22 cells from oxidative stress (Tan et al. 2001).

ATF4 activation is unlikely to account for all of these protective effects, as the contribution of ATF4 to the survival of ER-stressed cells can be substituted by supplementing the medium with nonessential amino acids and reducing substances, whereas that of the upstream kinase PERK cannot be substituted by this simple expedient (Harding et al. 2003). It is therefore likely that eIF2α phosphorylation activates other, yet to be identified, transcription factors, as exemplified recently by the case of NF-κB (Jiang et al. 2003; Deng et al. 2004). Establishing the relative contribution of the ISR's translational and transcriptional arms to its cytoprotective actions will therefore require a more-detailed understanding of the mediators of the transcriptional program and their genetic manipulation.

The prosurvival effects of the ISR may have an important role in cancer biology. The survival of cancer cells in vivo requires that they negotiate the ischemic environment of tumors. The attendant oxygen and nutrient deprivation challenge the protein-folding environment in the ER

and induce a protective UPR of which PERK activation and the ISR are prominent features (Koumenis et al. 2002). Remarkably, transformed cells lacking either PERK or its downstream effector, ATF4, are markedly impaired in their ability to survive as tumors in vivo (Bi et al. 2005). These observations suggest that inhibition of the ISR may have a role in cancer therapy.

The discussion, so far, has centered on the role of ISR in cell survival, specifically in the context of misfolded protein stress. However, there are reasons to believe that ISR signaling, induced by physiological levels of ER stress, also contributes to cell function. This aspect is especially interesting with regard to the intersection of the ISR with calcium signaling in excitatory cells.

The ER is a major repository of intracellular calcium, and its stores are tapped in a variety of excitation-coupled phenomena (e.g., secretion and muscle contraction). The most important outcome of this is to raise cytosolic calcium levels and activate cytosolic effector mechanisms; inevitably, however, such signaling is associated with a measure of ER calcium store depletion. Severe calcium store depletion is a powerful activator of PERK (Harding et al. 1999), presumably a consequence of the role of ER calcium in chaperone function. There are reasons to believe that ER calcium excursions in the physiological range might also activate PERK, as physiological agonists that deplete ER calcium stores have been noted to activate the ISR (Kimball and Jefferson 1990; Alcazar et al. 1997). More recently, analysis of $PERK^{-/-}$ mice has revealed a broad defect in excitation-coupled secretion in the pancreas and contraction in smooth muscle cells (Huang et al. 2006). This suggests that physiological levels of excitatory activity lead to PERK activation and ISR signaling, which are, in turn, required for the proper function of the excitatory apparatus.

PATHOPHYSIOLOGICAL MECHANISMS: eIF2α PHOSPHORYLATION AND NUTRITION

Availability of amino acids is the primary regulator of eIF2α phosphorylation in yeast. In mammals, too, nutrient availability regulates eIF2α phosphorylation, but the connection has acquired a surprising measure of complexity, and eIF2α phosphorylation has emerged as an important signaling node in integrating nutritional and metabolic cues. Below, we consider several examples with potential pathophysiological implications.

GCN2 and the Adaptation to a Diet with Imbalanced Amino Acid Content

Remarkably, $GCN2^{-/-}$ mice and worms exhibit no obvious impairment when maintained on a normal diet. Even when placed on a diet deficient in an essential amino acid, adult $GCN2^{-/-}$ mice fare no worse than their wild-type littermates (D. Ron and H.P. Harding, unpubl.), although recent evidence suggests that the mutant animals are unable to conserve muscle mass under these circumstances (Anthony et al. 2004). Furthermore, when pregnant dams are placed on an amino-acid-deficient diet, their developing $GCN2^{-/-}$ pups are at a disadvantage compared to the wild type, but this too is not an especially strong phenotype (Zhang et al. 2002b), all suggesting that mammals have redundant physiological pathways for dealing with amino acid deprivation.

It has long been known that omnivorous animals such as rats will avoid otherwise palatable and nutritious foods that are deficient in merely one essential amino acid. The aversion to imbalanced foods is presumably highly adaptive as it directs the foraging animal to avoid dangerous micro-nutrient deficiency. It is also relatively easy to measure this response by comparing the consumption of two otherwise identical meals, one of which is deficient in a single amino acid. Bilateral lesions in the anterior piriform cortex abolish the bias against the imbalanced foods (Gietzen 1993). Interestingly, the consumption of an imbalanced meal leads to eIF2α phosphorylation in neurons in that nucleus (Gietzen et al. 2004), and such phosphorylation and the associated aversive response are missing in mice lacking GCN2 (Hao et al. 2005; Maurin et al. 2005).

GCN2-dependent eIF2α phosphorylation is both a necessary and sufficient signal, as localized instillation of an inhibitor of a single tRNA synthetase into the anterior piriform cortex elicited an aversive response in animals fed a balanced diet (Hao et al. 2005). It is likely that a transient decline in serum levels of a specific amino acid is sufficient to trigger GCN2 activation in neurons of the piriform cortex and that this upstream event initiates the aversive response. Given the prominent role of eIF2α phosphorylation in GCN2 activity in yeast, it is likely that in mammals, too, it is the mediator of kinase action, although this remains to be proven. Nor it is known how GCN2 activation and eIF2α phosphorylation trigger the necessary neuronal activity; the short latency of the response suggests that the effects are directly translational, but it is impossible at present to exclude a contribution at other levels of gene expression. Despite these uncertainties, the role of GCN2 in the mammalian aversive response

to imbalanced diet resembles its ancestral role in adapting unicellular organisms to an amino-acid-deficient environment.

GCN2 also regulates other neuronal activities, as brain explants of $GCN2^{-/-}$ mice exhibit a complex defect in long-term potentiation, and the knockout mice have a prominent defect in memory (Costa-Mattioli et al. 2005). Unlike the response to an imbalanced diet, it is unclear how neuronal stimulation in long-term potentiation experiments or during normal memory formation activates GCN2. It is possible that ion fluxes during neuronal activity affect amino acid import and thereby activate GCN2 by the canonical mechanism of uncharged tRNAs, but this is merely a speculation that the authors of this chapter find attractive.

GCN2, Tryptophan Metabolism, and Immunomodulation

An especially interesting example of the evolution of the role of GCN2 as sensor of uncharged tRNAs is provided by its role in the immune response. The tryptophan degrading enzyme indolamine 2,3-dioxygenase (IDO) is activated in certain antigen-presenting cells and its activity has an important role in modulating adjacent T cells, to attenuate TH1 cytotoxic responses. IDO is believed to have a role in such important phenomena as tumor anergy or preventing fetal allograph rejection. The role of tryptophan metabolism in IDO signaling is well documented in vitro, where supplementation with the amino acid prevents IDO expression in dendritic cells from affecting T-cell action. This led Mellor and Munn (2004) to hypothesize that a localized environment of tryptophan depletion contributes to IDO's immunomodulatory role.

Given the ability of GCN2 to respond to depletion of even a single amino acid, it was natural to inquire whether IDO expression led to kinase activation and an ISR in the responding T cell. This proved to be the case. Furthermore, T cells explanted from $GCN2^{-/-}$ mice were profoundly deficient in their response to IDO-expressing cells in coculture experiments, suggesting that GCN2 signaling has an important role in mediating the effects of IDO in the responding T cell (Munn et al. 2005).

These intriguing observations are clearly consistent with Munn and Mellor's hypothesis, but they are also open to an alternative interpretation whereby IDO leads to the conversion of tryptophan or another precursor to metabolites that diffuse to the T cell and activate GCN2 (e.g., by inhibiting tryptophan uptake into the cells or the tryptophanyl tRNA synthetase). The reversal of IDO's effects by tryptophan supplementation would then be explained by competition at the level of IDO (if the precursor were other than tryptophan) or at the level of the metabolites'

target. Either way, this study suggests that canonical GCN2 signaling and the downstream ISR have been co-opted for immunomodulation in T cells.

ISR and Intermediary Metabolism

The development of diabetes mellitus, exocrine pancreatic insufficiency, and bone disease in mice and humans lacking PERK is readily explained by impaired function and survival of secretory cells (β cells, acinar cells, and osteoblasts) deprived of PERK's modulatory role on client protein synthesis and deprived of the pro-survival benefits of the ISR. The profound metabolic consequences likely reflect this basic problem of ER homeostasis. However, analysis of more subtle mutations in *PERK* and *eIF2α* suggests a more complex role for the ISR in regulating intermediary metabolism.

The first clue came from mice homozygous for the *eIF2α^{S51A}* mutation, which precludes regulated phosphorylation. The embryos died of hypoglycemia shortly after birth, due to their inability to maintain blood glucose levels following disruption of the feto-maternal circulation. This defect appeared to be multifactorial, as it correlated with lower levels of gluconeogenic enzymes and lower glycogen stores in the liver of the mutant mice (Scheuner et al. 2001). It is presently unclear whether this defect represents a failure of ISR signaling in the postnatal liver and, if so, what are the kinases involved in such signaling normally? The *eIF2α$^{S51A/S51A}$* mice also have a profound defect in pancreatic islet development, which may have contributed to the hypoglycemia by impaired glucagon production.

More recently, Kaufman and colleagues have evaluated the heterozygous *eIF2α$^{S51A/+}$* mice, and these too have metabolic defects, albeit more subtle: When placed on a high-fat diet, the *eIF2α$^{S51A/+}$* mice are more glucose-intolerant than wild-type mice. This phenotype is due in part to compromised insulin secretion, which is consistent with the role of the ISR in preserving β-cell function (Scheuner et al. 2005). However, the mutant mice have other metabolic abnormalities, such as increased body weight and hyperlipidemia, which are not easily attributed to defective β-cell function. Instead, these characteristics are consistent with a role for the ISR in regulating aspects of lipid metabolism.

At present, we are unable to offer a unified explanation for all these effects of altered signaling in the eIF2(αP) pathways, but interesting clues have surfaced recently. Hotamisligil and colleagues have documented higher levels of ER stress signaling in the adipose tissue and liver of mice placed on a high-fat diet (Ozcan et al. 2004). The mechanism for such activation remains unclear, but the phenomenon appears to be physio-

logically significant, as mutations affecting the IRE1→XBP-1 arm of the UPR profoundly reduce insulin sensitivity in mice fed a high-fat diet. PERK and IRE1 are activated by similar molecular mechanisms. Therefore, it is likely that mice on a high-fat diet (and obese people too) have enhanced PERK signaling in their liver and fat, and it follows that the $eIF2\alpha^{S51A/+}$ mice are impaired in conveying that signal.

The IRE1→XBP-1 arm of the UPR up-regulates phospholipid biosynthesis in yeast and mammals (for review, see Ron and Hampton 2004). The sterol-regulated transcription factors, SREBPs, are responsive to the lipid composition of the ER membrane. Furthermore, the machinery for their activation by regulated intramembrane proteolysis is shared by ATF6, a component of the UPR. We have recently discovered that the ISR inhibits SREBP target gene expression by attenuating SREBP activation (Harding et al. 2005). This inhibition could be mediated indirectly by an effect of the ISR on ER lipid composition or through cross-talk between the three arms of the UPR or, perhaps more directly, through the translational or transcriptional components of the ISR. Either way, the accumulating evidence for regulation of lipid metabolism by ER stress-mediated eIF2α phosphorylation suggests the need for further studies to clarify the role it has in the metabolic syndrome of obesity and diabetes mellitus.

PATHOPHYSIOLOGICAL MECHANISMS: TRANSLATIONAL REPRESSION CELL DEATH AND SURVIVAL

The discussion so far has emphasized the benefits of eIF2α phosphorylation and the downstream response, both to individual cells and to the organism as a whole. There are, however, a number of circumstances in which ISR activation correlated with more, not less, cell death. These will be considered next.

PKR, an eIF2α kinase activated by viral infection, is clearly important to vertebrate innate immunity. Evidence for this is provided by the observation that most animal cell viruses have evolved specific mechanisms for blocking eIF2α phosphorylation in their host cell (Chapter 20). It appears, however, that PKR provides its benefit to the organism by promoting the death of virally infected cells (Srivastava et al. 1998). Thus, in the context of viral infection, translational repression synergizes with other signals to promote apoptosis (Chapter 16).

Animal models of stroke are associated with conspicuous levels of eIF2α phosphorylation in the affected neurons (DeGracia et al. 1997), which correlate with PERK activation (Kumar et al. 2001). However, in these experimental systems, eIF2α phosphorylation correlates not with cell survival, but rather with cell death (DeGracia et al. 2002). This has led to

the speculation that impaired recovery of protein synthesis during the reperfusion phase of ischemic injury compromises neuronal survival (Paschen 2003). At the same time, however, hibernation, one of the most stress-resistant states of the central nervous system, is associated with very high levels of eIF2α phosphorylation (Frerichs et al. 1998). It therefore would appear that, like many regulated phenomena, the effects of eIF2α phosphorylation on nervous tissue survival are biphasic. A rare human genetic syndrome, childhood ataxia with cerebral hypomyelination (CACH, also known as vanishing white matter), is further instructive in this regard.

CACH is a severe disorder of the white matter associated with abnormalities in the myelin-producing oligodendrocytes. It is caused by loss-of-function mutations in any one of several subunits of eIF2B, the nucleotide exchange factor for eIF2 (Leegwater et al. 2001; Richardson et al. 2004). The CACH-associated mutations mimic the consequences of eIF2α phosphorylation and, to a first approximation, can be considered ISR-activating (Kantor et al. 2005). Furthermore, it is possible that eIF2α phosphorylation contributes directly to the pathophysiology of the CACH syndrome, as patients are reported to experience catastrophic deterioration in their condition following acute stressful conditions such as febrile illness or head trauma, which might compromise eIF2B function further through the induction of eIF2α phosphorylation.

The severe consequences of the CACH mutations point to the dangers of ISR hyperactivation, especially as it pertains to the myelin-producing oligodendrocyte. These cells seem to be perched in an especially precarious position as they are sensitive to both mild defects in mounting an ISR (Lin et al. 2005) and to excesses in eIF2α phosphorylation (Southwood et al. 2002) or to mutations that mimic its consequences (Fogli et al. 2004).

SUMMARY

The phosphorylation of eIF2α and signaling downstream represent an ancient and conserved adaptation to cell stress, which we refer to as the integrated stress response. The ISR influences the balance of precursors and macromolecular end products in protein, lipid, and carbohydrate metabolism, and in higher eukaryotes, this pathway has acquired a prominent role in regulating the protein-folding environment in the ER. Signaling in the ISR has biphasic and tissue-specific effects on cell survival under various stressful conditions. Because the pathway is regulated by specific kinases and phosphatases that are, in principle, amenable to pharmacological manipulation, the ISR is a potential drug target for the treatment of a variety of common disorders.

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