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Perk Is Essential for Translational Regulation and Cell Survival during the Unfolded Protein Response

Heather P. Harding, Yuhong Zhang, Anne Bertolotti, Huiqing Zeng, and David Ron*
Skirball Institute of Biomolecular Medicine
Departments of Medicine and Cell Biology and
The Kaplan Cancer Center
New York University School of Medicine
New York, New York 10016

Summary

Malfolded proteins in the endoplasmic reticulum (ER) inhibit translation initiation. This response is believed to be mediated by increased phosphorylation of eukaryotic initiation factor 2α (eIF2α) and is hypothesized to reduce the work load imposed on the folding machinery during stress. Here we report that mutating the gene encoding the ER stress–activated eIF2α kinase PERK abolishes the phosphorylation of eIF2α in response to accumulation of malfolded proteins in the ER resulting in abnormally elevated protein synthesis and higher levels of ER stress. Mutant cells are markedly impaired in their ability to survive ER stress and inhibition of protein synthesis by cycloheximide treatment during ER stress ameliorates this impairment. PERK thus plays a major role in the ability of cells to adapt to ER stress.

Introduction

To attain their proper three-dimensional structure, secreted, membrane-bound, and resident proteins of the exocytic compartment must fold in the lumen of the endoplasmic reticulum (ER). This process is often perturbed when cells are deprived of essential nutrients or exposed to toxins or as a consequence of mutations in the synthesized proteins themselves (Lee, 1992). Conditions that impair protein folding in the ER have a significant adverse effect on cellular survival. However, little is known about the mechanisms involved. At least two functionally distinct signaling pathways have evolved to help maintain a proper folding environment in the ER. The first well-characterized component of the unfolded protein response (UPR) mediates the transcriptional induction of genes encoding ER chaperones, disulfide exchange proteins, and prolyl-peptidyl isomerases (Kozutsumi et al., 1988; Gething and Sambrook, 1992). In yeast, signaling in this pathway is initiated by the product of the *IRE1* gene, an ER resident transmembrane stress receptor that responds to a luminal signal correlating with the accumulation of malfolded proteins (Cox et al., 1993; Mori et al., 1993).

A second arm of the UPR consists of a profound attenuation of translation initiation (reviewed in Brostrom and Brostrom, 1998). This correlates with enhanced phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) (Prostko et al., 1993). Phosphorylated eIF2α (eIF2α-P) binds the guanine nucleotide exchange factor eIF2B with high affinity interfering with the assembly of a 43S translation initiation complex (Clemens, 1996). It is believed that reduced protein synthesis rates during ER stress serve to reduce the load of substrates presented to the folding machinery in the ER lumen. However, the homeostatic role of this response has not been tested experimentally.

Recently, an ER resident transmembrane protein kinase known as PERK (or PEK; Shi et al., 1998) has been shown to be activated by ER stress (Harding et al., 1999). PERK phosphorylates eIF2α in vitro and, when overexpressed in cells, attenuates protein synthesis (Shi et al., 1998; Harding et al., 1999). PERK is highly likely to participate in coupling ER stress to translation inhibition because its luminal domain is related in sequence to that of IRE1, suggesting that these two arms of the UPR are activated by the same signal(s).

Here we report on the phenotypic consequences of an induced recessive mutation in mouse *Perk*. Our data prove that *Perk* is required for both the phosphorylation of eIF2α and the attenuation of translation that occurs in response to ER stress. Furthermore, reduced survival of $Perk^{-/-}$ cells, when exposed to agents that cause ER stress, suggests that in this physiological context reduced translation is an adaptive response.

Results

The *Perk* gene was targeted in mouse embryonic stem cells. The mutation replaces the transmembrane domain by insertion of a *Neo*-expressing gene (Figure 1A). Homozygosity for the mutant allele was attained with high frequency by culturing $Perk^{+/-}$ ES cells in the presence of high concentrations of the selection agent, G418 (Figure 1B). Cells carrying the homozygous mutation expressed 10-fold less *Perk* mRNA than the wild-type cells (Figure 1C). The predicted mutant gene product is either a secreted or truncated protein that could not be properly targeted to the ER membrane. Western blot analysis of detergent lysates from $Perk^{-/-}$ cells reveals the absence of PERK protein, whereas the expression of IRE1α is unaffected in these cells (Figure 1D).

When exposed to agents that induce ER stress, wild-type cells exhibit a profound attenuation in global translation rates. This is reflected in the reduced incorporation of [^{35}S]methionine/cysteine into cellular proteins following a short labeling pulse in the presence of ER stress–inducing toxins (Figure 2A). By contrast, $Perk^{-/-}$ cells do not reduce translation rates when exposed to either thapsigargin, tunicamycin, or dithiothreitol, agents that have been shown to activate PERK in wild-type cells (Harding et al., 1999). Rather, when exposed to agents that cause ER stress, the mutant cells reproducibly exhibited an increase in content of ^{35}S-labeled cellular proteins. This small increase may be due to a decrease in secretion that occurs during ER stress. Arsenite, a stress-causing agent that induces phosphorylation of eIF2α and attenuates translation (Brostrom

*To whom correspondence should be addressed (e-mail: ron@saturn.med.nyu.edu).

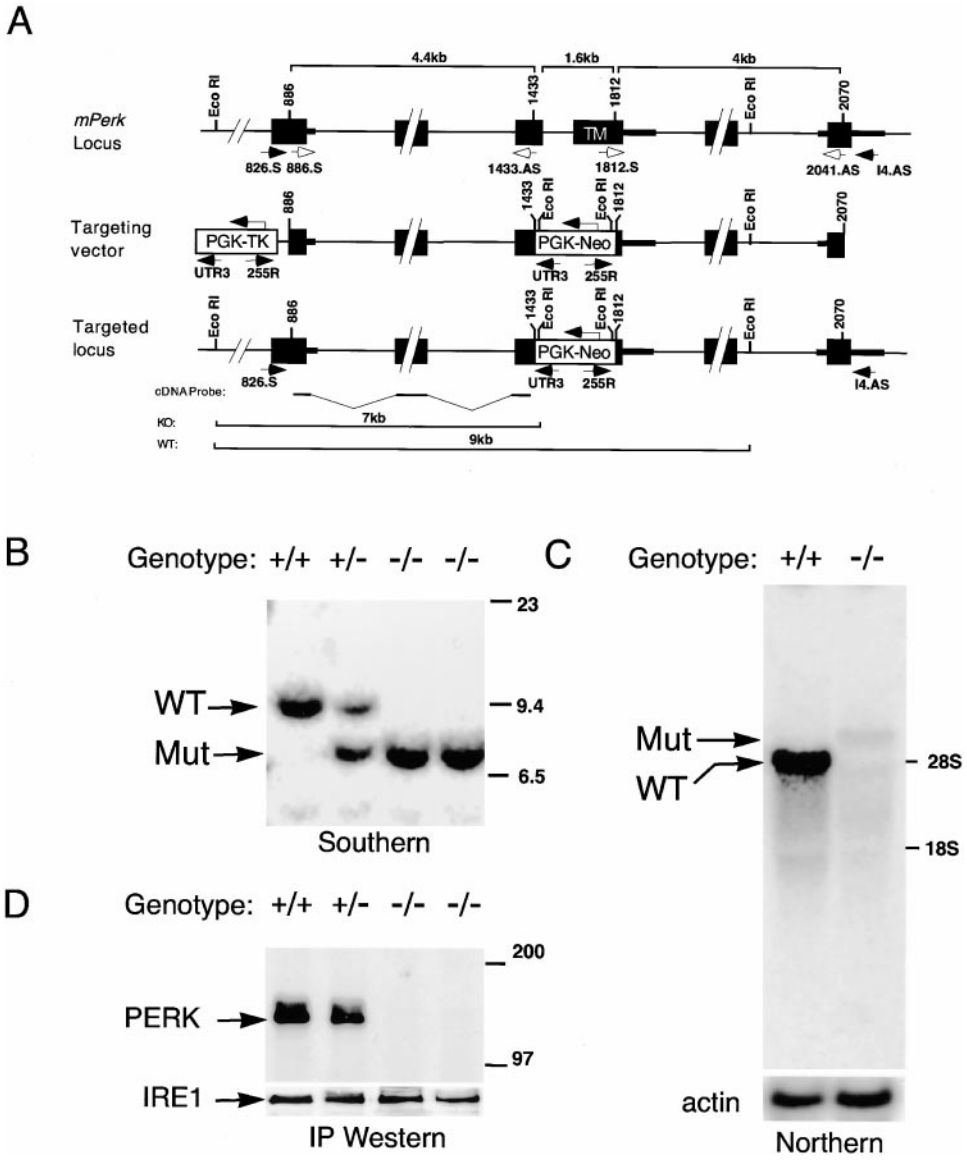

Figure 1. Structure of the Mutant *Perk* Allele

(A) Cartoon depicting the genomic structure of the region surrounding the transmembrane domain of *Perk*. The deletion encompasses residues 1433 and 1812 of the *Perk* cDNA and corresponds to the transmembrane domain of the protein.

(B) Southern blot of EcoR1-digested genomic DNA from cells with the indicated *Perk* genotype hybridized with a cDNA fragment corresponding to the genomic region indicated in the cartoon (upper panel).

(C) Northern blot of poly(A)$^+$ RNA (4 μg) isolated from wild-type and *Perk*$^{-/-}$ cells hybridized to the *Perk* (upper panel) and actin (lower panel) probes.

(D) Western blot of anti-PERK and anti-IRE1α reactive proteins immunoprecipitated from extracts of cells. The polyclonal antisera, directed against the C-terminal effector domains of PERK or IRE1α, were used in both the immunoprecipitation and subsequence Western blot. The position of the PERK and IRE1α proteins are indicated (lower panel).

and Brostrom, 1998), without activating PERK (Harding et al., 1999), inhibited translation and led to phosphorylation of eIF2α in both wild-type and *Perk*$^{-/-}$ cells, indicating that the effect of the mutation is specific for translational regulation by ER stress. The changes in protein synthesis rates were inversely related to the level of phosphorylation of eIF2α, with no increase in eIF2α phosphorylation in response to ER stress in the *Perk*$^{-/-}$ cells and an intermediate level of increase in *Perk*$^{+/-}$ cells, consistent with the reduced amount of PERK protein found in these cells (Figure 1D).

Treatment of wild-type cells with agents that cause ER stress resulted in dissociation of polysomes and accumulation of monosomes and ribosomal subunits, consistent with attenuation of an early step in mRNA translation (Prostko et al., 1993). In contrast, thapsigargin-treated *Perk*$^{-/-}$ cells had intact polyribosomes (Figure 2C), consistent with a defect in translational inhibition.

Activation of IRE1α during ER stress leads to decreased mobility of the protein on SDS-PAGE. This is likely due to IRE1 phosphorylation (Shamu et al., 1994;

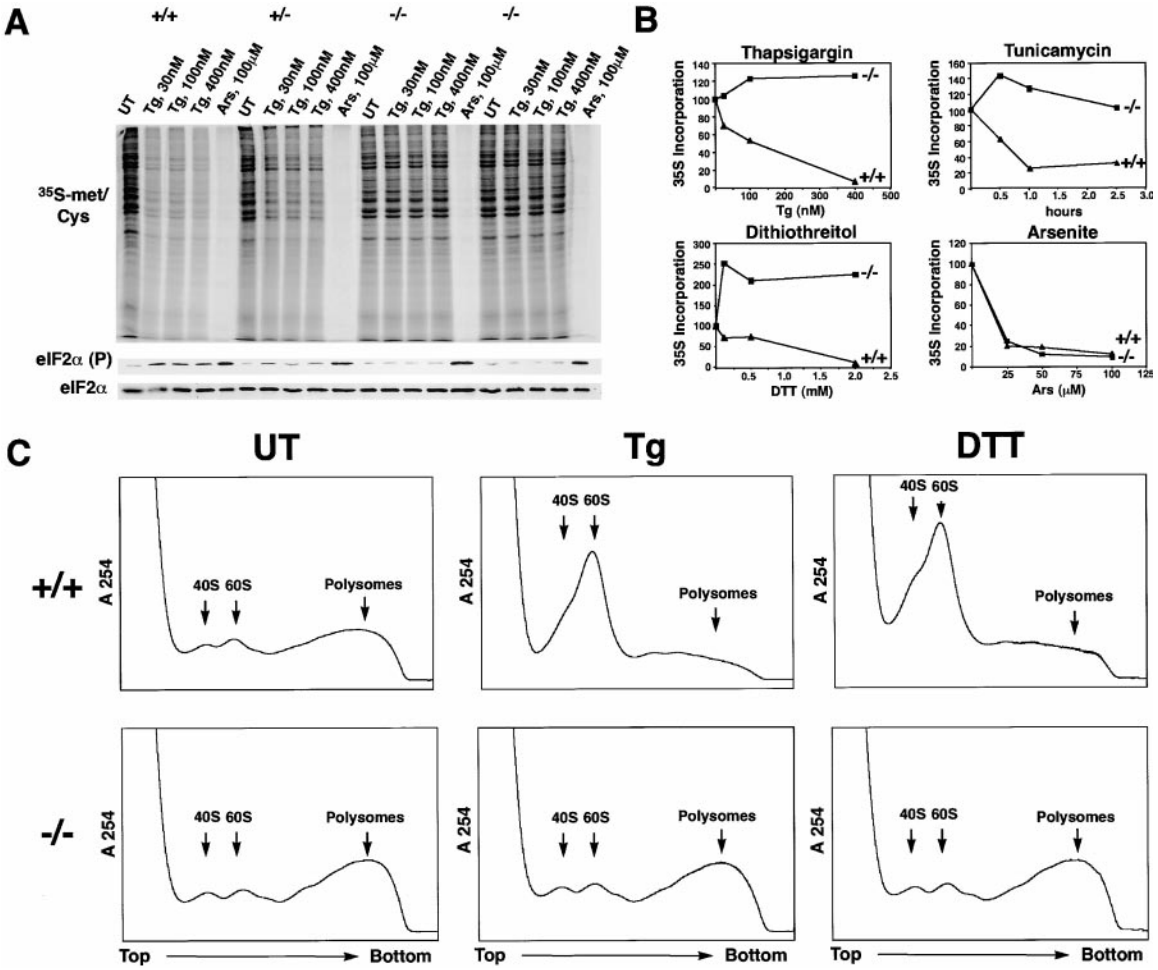

Figure 2. The *Perk* Mutation Prevents Attenuation of Protein Synthesis and Phosphorylation of eIF2α

(A) Protein synthesis rates measured by the incorporation of [^{35}S]methionine/cysteine into proteins during a 10 min pulse of labeling that followed 30 min of exposure to the indicated concentrations of thapsigargin. The labeled proteins in a whole-cell extract were resolved by SDS-PAGE and revealed by autoradiography (upper panel). eIF2α, in the same extracts, was revealed by Western blotting using a monospecific antiserum that recognizes only the phosphorylated form of the protein [eIF2α (P)] or a monoclonal antibody that reacts with all isoforms of eIF2α. The reproducibility of the results is emphasized by the two independently derived *Perk*$^{-/-}$ clones.

(B) Graphic presentation of the effects of stress-causing agents on protein synthesis rates in cells with the indicated *Perk* genotypes. The level of protein synthesis was quantified from autoradiograms of gels like the one shown in (A). The level of ^{35}S incorporation into proteins in the untreated cells is set at 100%. Tunicamycin was used at a dose of 2.5 μg/ml, and the effects of the various doses of thapsigargin, dithiothreitol, and sodium arsenite were measured after 30 min of treatment (shown is a result of a typical experiment reproduced in three different pairs of ES clones of each genotype).

(C) Polysome profiles of cytoplasmic lysates of wild-type and mutant cells treated with thapsigargin (Tg; 200 nM for 60 min) or DDT (2 mM for 60 min). Absorbence at 254 nm (y axis, reporting on RNA concentration) is plotted against migration in the 10%–50% sucrose gradient (x axis). The position of the polysomes and ribosomal subunit peaks is indicated. The monosomal 80S peak is not resolved from the 60S peak in these gradients.

Welihinda and Kaufman, 1996). To compare the activity of IRE1 in wild-type and mutant cells, we first used tunicamycin, an agent that blocks glycosylation and predominantly affects the folding of newly synthesized proteins, inducing a gradual accumulation of malfolded proteins. IRE1α activation occurred earlier in tunicamycin-treated mutant cells (Figure 3A). The *Perk* mutation results in loss of control over protein synthesis during ER stress; therefore, we predicted that recovery after reversal of conditions that impair protein folding would be delayed in mutant cells because they would have accumulated more unfolded proteins than the wild type. DTT, a reversible inhibitor of protein folding in the ER, was used to fully activate IRE1α in wild-type and mutant cells, and the return of IRE1α to the basal state was studied following washout of the DTT. IRE1α activation subsided within 4–6 hr in the wild-type cells but persisted for over 16 hr in the *Perk*$^{-/-}$ cells (Figure 3B).

ER stress specifically activates the processing of the ER resident proapoptotic cysteine protease, caspase-12 (Nakagawa et al., 2000). This can be followed by monitoring the accumulation of the activated cleavage product of the caspase in immunoblots of whole-cell extracts. Caspase-12 activation is a late event in the response to accumulation of malfolded proteins in the ER and is presumed to be triggered by insupportable

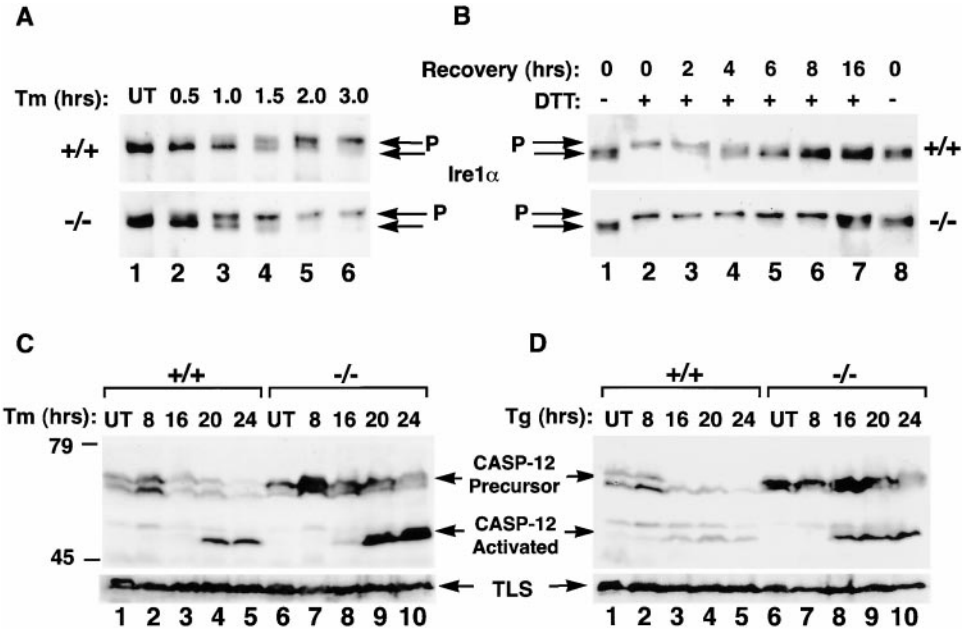

Figure 3. The *Perk* Mutation Is Associated with Heightened Activity of Parallel ER Stress Pathways

(A) Comparison of the activation status of endogenous IRE1α in cells with the indicated *Perk* genotypes at various times after treatment with tunicamycin (2 μg/ml). IRE1α activation is reflected in a shift in its mobility in this IP-Western blot with an antiserum directed against the C-terminal portion of the murine protein.

(B) Comparison of the rate of return of IRE1α to the basal state following its full activation by treatment of cells for 60 min with 2 mM DTT (lane 2) and washout of the agent for the indicated period of time ("recovery," lanes 3–7). Lanes 1 and 8 are samples from untreated cells and serve as a reference for the basal state of IRE1α. Shown is an IP-Western blot performed as in (A).

(C and D) Activation of caspase-12 in mutant and wild-type cells, revealed by immunoblot of whole-cell lysates. Wild-type and mutant cells were exposed to thapsigargin (400 nM) or tunicamycin (2.5 μg/ml) for the indicated period of time. The inactive caspase-12 precursor form and active cleaved forms are indicated. The TLS signal on an immunoblot run in parallel controls for gel loading and protein integrity.

levels of ER stress (Nakagawa et al., 2000). When treated with tunicamycin or thapsigargin, *Perk*$^{-/-}$ cells accumulate much higher levels of activated caspase-12 than identically treated wild-type cells (Figures 3C and 3D). An earlier stress-induced increase in the inactive caspase-12 precursor was reproducibly noted in the wild-type cells (Figures 3C and 3D, lane 2). This was greatly magnified in the *Perk*$^{-/-}$ cells, which even had higher basal levels of caspase-12 precursor (lanes 6 and 7). Regardless of mechanism, these differences point to heightened activity of parallel ER stress pathways in *Perk*$^{-/-}$ cells.

The *Perk* mutation had a profound negative impact on the ability of cells to survive exposure to agents that cause ER stress. Wild-type and *Perk*$^{-/-}$ ES cells were plated at low density and exposed to tunicamycin or thapsigargin. After exposure, the toxin was removed, and the cells were allowed to resume growth in normal media. In this assay, outgrowth of clones reflects the ability of the cells to resist the impact of the toxin. *Perk*$^{-/-}$ cells were considerably more sensitive to the effects of the ER stress-inducing toxins than were wild-type ES cells (Figures 4A and 4B). The enhanced sensitivity of *Perk*$^{-/-}$ cells to agents that induce ER stress could be rescued in *trans* by introducing the *Perk* gene back into the cells (Figure 4C). This result provides evidence that enhanced sensitivity of the mutant cells is due to the lack of PERK protein rather than a gain of function associated with a product of the mutant allele. *Perk*$^{+/-}$ cells were not impaired in their ability to resist ER stress–inducing toxins (data not shown), indicating that the decreased phosphorylation of eIF2α observed in these cells (Figure 2A) was insufficient to elicit a phenotype in this assay. The death-promoting effects of arsenite, a toxin that does not induce ER stress but does activate a cellular response that includes strong inhibition of translation, was not influenced by the *Perk* genotypes (data not shown), indicating that the impact of the *Perk* mutation is specific to the ER stress pathway.

Enhanced sensitivity of the *Perk*$^{-/-}$ cells is also reflected in the FACS profiles of cellular DNA following exposure to ER stress. This revealed a marked increase in the fraction of *Perk*$^{-/-}$ ES cells with a hypodiploid genomic DNA content, indicative of programmed cell death (Figure 4D). Interestingly, treatment with cycloheximide effected a partial rescue of the *Perk*$^{-/-}$ phenotype. In wild-type and unstressed *Perk*$^{-/-}$ cells, cycloheximide treatment reduced colony formation. However, in ER-stressed *Perk*$^{-/-}$ cells, cycloheximide increases colony outgrowth (Figures 4A and 4B). This result suggests that inability to regulate protein synthesis plays a role in the reduced survival of ER-stressed *Perk*$^{-/-}$ cells. However, other modes by which *Perk* may affect survival of cells exposed to ER stress are not excluded.

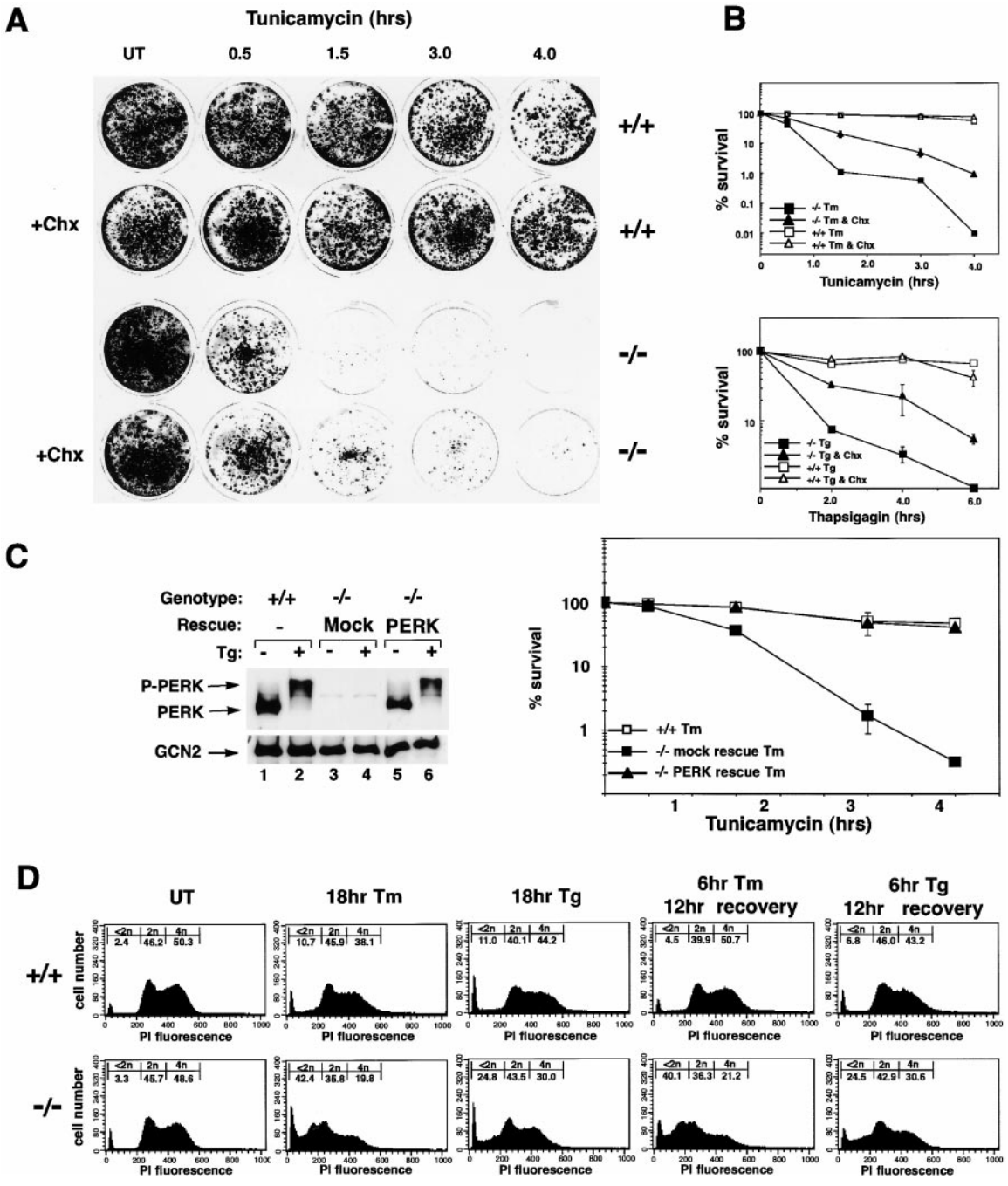

Figure 4. Reduced Survival of *Perk*$^{-/-}$ Cells Exposed to Agents that Cause ER Stress

(A) The ability of a brief pulse of tunicamycin treatment (2.5 μg/ml, for the indicated number of hours) to suppress the outgrowth of colonies scored 7 days later is compared between ES clones of wild-type and *Perk*$^{-/-}$ genotypes. Tunicamycin treatment was delivered in the absence or presence of 20 μg/ml cycloheximide [+CHX].

(B) Graphic display of the results shown in (A) and a similar experiment in which thapsigargin (200 nM) was used to induce ER stress. Shown are the mean ± SEM results of a typical experiment carried out in duplicate. Similar results were observed using two different clones of wild-type and *Perk*$^{-/-}$ ES cells in experiments reproduced three times.

(C) Survival of a representative pool of mock-rescued and PERK-rescued *Perk*$^{-/-}$ cells treated with tunicamycin as in (A) and (B). The immunoblot compares the levels of PERK protein in wild-type, *Perk*$^{-/-}$, and PERK-rescued *Perk*$^{-/-}$ ES cells. The GCN2 immunoblot controls for loading.

(D) FACS analysis of propidium iodide-stained genomic DNA content of ES cells with wild-type and mutant *Perk* genotypes following treatment with thapsigargin (Tg) or tunicamycin (Tm). The proportion of cells with hypodiploid (<2N), diploid (2N), and greater than diploid DNA content (4N) is indicated. Note the high proportion of treated *Perk*$^{-/-}$ cells with a hypodiploid content of DNA.

Discussion

Protein malfolding in the endoplasmic reticulum is a prominent feature of certain pathophysiological states associated with cellular dysfunction and death. These include ischemia, glucose deprivation, and mutations in abundantly expressed secreted proteins (Lee, 1992; Kaufman, 1999). Experiments presented here provide strong evidence that the ability to attenuate translation in response to ER stress plays an important role in mitigating the consequences of this insult to cellular homeostasis. These experiments also support an essential role for *Perk* in this adaptation and suggest that *Perk* deficiency may have a significant impact on physiological states associated with ER stress.

The mediators of cellular dysfunction during ER stress are not known. However, given that in wild-type cells less proteins are being synthesized than in *Perk*$^{-/-}$ cells, a deficit in production of proteins that normally fold in the ER is unlikely to contribute to cell death during ER stress. Rather, these results suggest that cellular dysfunction is brought about in some way by the accumulation of the malfolded proteins themselves, a consequence of their "proteotoxicity" (Hightower, 1991). This model is consistent with other experiments in which it has been demonstrated that overexpression of the ER chaperone BiP increases the survival of cells in response to ER stress (Morris et al., 1997). BiP promotes this effect apparently without increasing the maturation or secretion of proteins from the exocytic compartment (Dorner et al., 1992). According to this hypothesis, PERK is protective because it reduces the synthesis of malfolded proteins and BiP overexpression is protective by masking their toxicity.

PERK's cytoplasmic effector domain is most closely related to that of the eIF2α kinase PKR (Harding et al., 1999). PKR promotes programmed cell death in response to double-stranded RNA and other conditions that activate its kinase (Der et al., 1997; Srivastava et al., 1998). However, our data indicate that *Perk* contributes to cell survival during ER stress. Therefore, from the perspective of survival, *Perk*'s role in ER stress is opposite to that of PKR during viral infection. This dichotomy may reflect differences in other, yet to be discovered effector functions of these two eIF2α kinases or differences in the physiological contexts in which PKR and PERK are activated.

The *Perk* mutation leads to increased activity of parallel ER stress pathways culminating in IRE1 phosphorylation and caspase-12 processing (Figure 3). IRE1 overexpression is a potent inducer of cell death (Wang et al., 1998); it is therefore possible that increased activity of IRE1α in ER-stressed *Perk*$^{-/-}$ cells may account for some of their reduced survival. The ability of IRE1 to couple stress-activated protein kinases (JNKs) to ER stress may be one component of the death pathway that is hyperactive in *Perk*$^{-/-}$ cells (Urano et al., 2000). Caspase-12 has recently been demonstrated to play an essential role in promoting programmed cell death during ER stress (Nakagawa et al., 2000). Therefore, increased production of activated caspase-12 in the mutant cells suggests that this proapoptotic protein might also play a role in the hypersensitivity of *Perk*$^{-/-}$ cells to ER stress.

The very low level of *Perk* mRNA in the mutant cells, together with the ability of physiological levels of PERK to rescue their hypersensitivity to ER stress when provided in *trans* (Figure 4C), argues against significant gain-of-function features for the mutant *Perk* allele. Nonetheless, it is formally possible that some of the effects of the *Perk*$^{-/-}$ genotype, reflected in attenuated eIF2α phosphorylation and persistence of protein synthesis during ER stress, may be due to interference with the activity of a related eIF2α kinase such as PKR and not solely the consequence of absent PERK activity. This caveat does not impact on the conclusions related to the role of translation inhibition in the adaptation to ER stress. However, here too we must exercise caution in interpreting the results. The salutary effects of cycloheximide on survival of *Perk*$^{-/-}$ cells exposed to ER stress (Figure 4A) and evidence for increased protein binding to BiP in ER-stressed *Perk*$^{-/-}$ cells (data not shown) are consistent with a direct role for translational attenuation in limiting the associated proteotoxicity. It is impossible, however, to exclude other effects of *Perk* that may promote cell survival during ER stress. These may include signaling downstream of PERK that operates independently of eIF2α phosphorylation or consequences of increased eIF2α phosphorylation that are not due to decreased synthesis of potentially toxic malfolded proteins. A well-characterized precedent for the latter mechanism exists in the case of the yeast eIF2α kinase GCN2. In addition to inhibiting translation rates globally, GCN2p activation by amino acid deprivation and phosphorylation of eIF2α preferentially promotes the synthesis of a transcription factor, GCN4p, that controls the expression of genes involved in amino acid biosynthesis (Hinnebusch, 1997). *Perk* too may participate in regulated gene expression through a similar mechanism operating in response to ER stress in mammalian cells.

Levels of ER stress comparable to those induced by the pharmacological agents utilized in this study are unlikely to be encountered by cells in natural situations. On the other hand, cells of long-lived organisms such as those in some nonrenewing tissues of mammalian species must endure the consequences of accumulation of malfolded proteins in the ER over relatively long periods of time. We speculate that *Perk*'s role in cellular physiology, presented here in relief using potent pharmacological agents, will be particularly important in the context of the ability of long-lived cells to survive low levels of chronic ER stress.

Experimental Procedures

ES Cell Culture and Gene Targeting

Parental W4 ES cells and derived mutant cell lines were maintained on feeders in ES media. The murine *Perk* gene was targeted with a positive–negative selection vector replacing 1.6 kb of DNA containing the transmembrane domain (residues 1434–1811 in the cDNA) with a *PGK-Neo* cassette in the reverse orientation. Both homology arms were obtained by long-range PCR on BAC DNA from a clone containing the *Perk* gene. The 5′ homology arm consists of the 4.4 kb genomic region lying 5′ of cDNA residue 1433. The 3′ homology arm consists of the 4 kb genomic region lying 3′ of cDNA residue 1812. Clones from two independently isolated heterozygous mutant lines were selected for conversion to homozygosity in 2 mg/ml G418. Eight *Perk*$^{-/-}$ lines were obtained. The lines were maintained on feeders and then passed two times without feeders on

gelatinized plates prior to immunoprecipitation, pulse labeling, colony outgrowth, and propidium iodide staining experiments. Blots of EcoRI-digested genomic DNA were hybridized with a probe corresponding to *Perk* cDNA bp 910–1386 (a region that is not deleted by the mutation). A Northern blot of poly(A)$^+$ mRNA was hybridized with the same *Perk* cDNA fragment and sequentially with an actin probe.

Immunoprecipitation and Western Blotting

ES cells of the indicated genotype were treated with 2.5 μg/ml tunicamycin, 400 nM thapsigargin, or 2 mM DTT for the indicated period of time, washed in ice-cold PBS-1 mM EDTA, and lysed in 400 μl/10 cm plate Triton buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM phenylmethysulfonyl fluoride, 4 μg/ml aprotonin, and 2 μg/ml pepstatin A). The lysates were cleared by centrifugation at 14,000 rpm, and 2.5 mg of extract from each sample was immunoprecipitated for 14 hr with 1 μl rabbit anti-PERK or IRE1α-specific antisera bound to 10 μl of protein A-Sepharose and the bound proteins washed four times for 5 min with Triton buffer. To detect caspase-12, cells were scraped into PBS containing 1 mM phenylmethysulfonyl fluoride, 4 μg/ml aprotonin, and 2 μg/ml pepstatin A, lysed in 2% SDS, and sonicated. Proteins were resolved by 7% (PERK and IRE1) or 10% (caspase-12) SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with primary antibody (see below), and the signal was revealed by chemiluminescence after reacting with HRP-conjugated protein A (1/5000), goat anti-mouse (1/3000), or goat anti-rat (1/3000), as required. The following primary antibodies were used: rabbit anti-PERK (1/10,000; Harding et al., 1999), rabbit anti-IRE1α (1/2000; Urano et al., 2000), rabbit anti-phospho eIF2α (1/400; DeGracia et al., 1997), monoclonal mouse anti-eIF2α monoclonal (Scorsone et al., 1987), monoclonal rat anti-caspase-12 (Nakagawa et al., 2000).

Metabolic Labeling

To measure translational inhibition, 50% confluent 60 mM dishes of cells of the indicated genotype were washed twice with PBS and placed in 1.5 ml of serum-free ES media containing 3 mg/L L-methionine and 4.8 mg/L L-cysteine. Cells were then pretreated for 0.5 hr with the indicated doses of DTT, thapsigargin, or sodium arsenite. Where indicated, tunicamycin (2.5 μg/ml) was added to the culture media 0.5, 1, or 2.5 hr before the labeling pulse. The cells were pulse labeled with 50 μCi of [^{35}S]Met/Cys express labeling mix (ICN) for 10 min, washed two times with ice-cold PBS containing unlabeled L-methionine (0.6 mg/ml) and L-cysteine (0.96 mg/ml), and lysed in 300 μl Triton buffer. The lysate was clarified in a tabletop centrifuge, and 20 μl was separated by SDS-PAGE. The gels were fixed, dried, and exposed for autoradiography.

Polysome Analysis

Cells at 50% confluence were treated for 1 hr with 2 mM DTT or 400 μM thapsigargin in serum-free medium. Cycloheximide (0.1 mg/ml) was added during the last 3 min of treatment. The plates were then washed twice with ice-cold PBS containing 0.1 mg/ml cycloheximide, and cells were collected by scraping into 1 ml of PBS. The cells from four 10 cm dishes were pooled and lysed in 500 μl polysome extraction buffer (15 mM Tris-Cl [pH 7.4], 15 mM $MgCl_2$, 0.3 M NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide, 1 mg/ml heparin, 1 mM phenylmethysulfonyl fluoride, 4 μg/ml aprotonin, and 2 μg/ml pepstatin A) and incubated on ice for 10 min. The lysates were cleared by centrifugation at 14,000 rpm and layered onto a 12 ml 10%–50% sucrose gradient prepared in the extraction buffer, and, after a 4 hr spin at 32,000 rpm in an SW40 rotor, the absorbence across the gradient at 254 nm was read.

Colony Outgrowth Assay

Cells were plated in ES media at a density of 8000 cells/well in 12-well dishes and grown for 16 hr. Duplicate wells were then treated with 2.5 μg/ml tunicamycin or 200 nM thapsigargin with or without 20 μg/ml cycloheximide for the indicated period of treatment. Colonies remaining on the plate 7 days later were revealed by crystal violet stain. Colony outgrowth was quantified using Imagequant software on images of the scanned stained dishes. The value obtained in the untreated plates was set as 100%. The *Perk* mutant cells were rescued by cotransfecting 35 cm plates with a PERK cDNA expression plasmid driven by a minimal HSV-TK promoter (or empty plasmid as a mock-rescue control) together with a *puromycin*^{r} marker using Lipofectamine 2000 (Life Technologies). It was necessary to use a weak promoter to drive expression of the transgene, because high level expression of PERK profoundly inhibits cell growth. Puromycin-resistant pools of rescued and mock-rescued cells were selected for their ability to resist 2 μg/ml tunicamycin for 9 hr (a dose found to kill all the *Perk*$^{-/-}$ cells). After 6 days, 300 surviving colonies were pooled from the pTK-PERK-transfected cells, and no surviving colonies were found in the mock-rescued plate. The pooled rescued cells were analyzed for PERK expression and survival at various doses of tunicamycin and thapsigargin as described above.

Propidium Iodide Staining

Cells, grown in 60 cm dishes, were treated with 2.5 μg/ml of tunicamycin or 200 nM thapsigargin for the indicated period of time. At harvest, the cells were washed twice in PBS and fixed in ice-cold ethanol for 2 hr at 4°C. The cells were then washed once with PBS and resuspended in a 1 ml solution containing propidium iodide 50 μg/ml and 100 μg/ml RNAse A in PBS, stained for 1 hr at 4°C, and then analyzed by FACScan.

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