P58\textsuperscript{IPK}, a Novel Endoplasmic Reticulum Stress-inducible Protein and Potential Negative Regulator of eIF2\(\alpha\) Signaling*

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The unfolded protein response, which is activated in response to the loss of endoplasmic reticulum (ER) Ca\(^{2+}\) homeostasis and/or the accumulation of misfolded, unassembled, or aggregated proteins in the ER lumen, involves both transcriptional and translational regulation. In the current studies we sought to identify novel ER stress-induced genes by conducting microarray analysis on tunicamycin-treated cells. We identified P58\textsuperscript{IPK}, an inhibitor of the interferon-induced double-stranded RNA-activated protein kinase, as induced during ER stress. Additional studies suggested that P58\textsuperscript{IPK} played a role in down-regulating the activity of the pancreatic eIF2 kinase/eukaryotic initiation factor 2\(\alpha\) (eIF2\(\alpha\)) like ER kinase/activation transcription factor (ATF) 4 pathway. Modulation of P58\textsuperscript{IPK} levels altered the phosphorylation status of eIF2\(\alpha\), and thereby affected expression of its downstream targets, ATF4 and Gadd153. Overexpression of P58\textsuperscript{IPK} inhibited eIF2\(\alpha\) phosphorylation and reduced ATF4 and Gadd153 protein accumulation, whereas silencing of P58\textsuperscript{IPK} expression enhanced pancreatic eIF2\(\alpha\)-like ER kinase and eIF2\(\alpha\) phosphorylation and increased ATF4 and Gadd153 accumulation. These findings implicate P58\textsuperscript{IPK} as an important component of a negative feedback loop used by the cell to inhibit eIF2\(\alpha\) signaling, and thus attenuate the unfolded protein response.

The endoplasmic reticulum (ER)\(^3\) is an important organelle in which newly synthesized secretory and membrane-associated proteins are correctly folded and assembled. Perturbations in the ER environment result in a condition known as ER stress, which can threaten cell survival. ER stress can be induced in cells by a variety of treatments including agents known to affect calcium homeostasis, inhibitors of glycosylation, and/or the accumulation of misfolded, unassembled, or aggregated proteins in the ER lumen.

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§ The abbreviations used are: ER, endoplasmic reticulum; ATF, activation transcription factor; CHOP, CCAAT enhancer-binding protein homologous protein; eIF2\(\alpha\), a subunit of eukaryotic initiation factor-2; GRP78, glucose-regulated protein 78; PEK, pancreatic eIF2 kinase; PERK, PKR-like ER kinase; dsRNA, double stranded RNA; PRR, double-stranded RNA-dependent protein kinase; UPR, unfolded protein response; XBP-1, X-box binding protein-1; MEF, mouse embryo fibroblasts; RT, reverse transcriptase; DAPI, 4,6-diamidino-2-phenylindole; siRNA, small interference RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly(ADP-ribose) polymerase.

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PERK, like IRE1, is a transmembrane protein, comprised of a cytoplasmic domain possessing kinase function and a stress-sensing luminal domain (20). PERK and IRE1 are activated through a similar mechanism involving oligomerization and autophosphorylation (1, 2, 30). Although PERK-mediated eIF2a phosphorylation leads to a general suppression of translation, it promotes the preferential translation of certain mRNAs. Most notable among these is the transcription factor ATF4 (18).

Genome-wide expression analysis using DNA microarrays has revealed that activation of the UPR in yeast results in the up-regulation of more than 350 genes. These include genes involved in various aspects of the secretory pathway, such as protein folding, ER to Golgi vesicular transport, and ER-associated protein degradation (22). Although many of the molecular details of the UPR have been conserved in yeast and mammalian systems, the scope of UPR outputs in the mammalian cell is more complex and diverse. Accordingly, it is likely to involve a greater number of proteins and gene expression changes than seen in yeast.

The present study utilized DNA microarray analysis to search for novel genes induced by ER stress in mouse embryonic fibroblasts (MEFs). We report here the identification of P58IPK, an inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (PKR), as a gene whose expression is up-regulated in response to ER stress. Additional studies provide evidence that ATF6 contributes to the induction of P58IPK and P58IPK plays a role in regulating the activity of the PERK/eIF2a/ATF4 pathway.

MATERIALS AND METHODS

Cell Culture, Treatments, Plasmid Construction, and Transfection—MEFs, human embryonic kidney fibroblasts (HEK-293), and human cervical carcinoma HeLa cells were cultured in Dulbecco's modified essential medium (Invitrogen). All media were supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units of penicillin/ml, and 100 µg of streptomycin (Invitrogen) per ml, and were maintained in a humidified atmosphere containing 5% CO₂. Recombinant DNA techniques were performed by standard procedures. Plasmid pCGN-atf4 was kindly provided by Dr. R. Prywes (Columbia University) and used to construct pCGN-p50atf6 by inserting a PCR fragment spanning the HI sites of the pCGN vector. HEK-293 and HeLa cells were grown to 80% confluency in 100-mm plates and transfected using Polyfect reagent (Qiagen, Valencia, CA, USA). Six µg of pCGN-p50atf6, pCDNA3-atf4 (kindly provided by Dr. J. Leiden, University of Chicago), pCDNA1-p58IPK (kindly provided by Dr. M. G. Katze, University of Washington, pCDNA-perk (kindly provided by Dr. D. Ron, New York University School of Medicine), or an empty vector were transfected per plate. Tunicamycin and thapsigargin were from Sigma.

Western Blot Analysis—Whole cell lysate protein aliquots (20–30 µg) were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Schleicher & Schuell) by standard techniques. Blots were hybridized with the following antibodies: monoclonal anti-P58IPK (gift from Dr. M. G. Katze, University of Washington), polyclonal anti-Gadd153 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), polyclonal anti-phospho-eIF2a (Cell Signaling Technology, Beverly, MA), polyclonal anti-phospho-eIF2a (Cell Signaling Technology, Beverly, MA), polyclonal anti-phospho-PERK (Cell Signaling Technology), polyclonal anti-PKR (Cell Signaling Technology), polyclonal anti-Perkins (Cell Signaling Technology), monoclonal anti-cleaved PARP (Cell Signaling Technology), monoclonal anti-cleaved caspase-3 (Cell Signaling Technology), monoclonal anti-Grave (9E10) (Santa Cruz Biotechnology Inc.), and monoclonal anti-GAPDH (Abcam Ltd., Cambridge, UK). Secondary horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Amersham Biosciences. Protein bands were detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

RT-PCR and Northern Blot Analysis—To analyze RNA expression by reverse transcription-PCR (RT-PCR), total RNA from each sample was treated with DNAse I and used for RT-PCR with SuperScript One-Step RT-PCR with the Platinum Tag system (Invitrogen). The primers for gene-specific RT-PCR analysis were as follows: for P58IPK, GAGGTTTGTTGGGATGCAG (5‘) and GCTTCTCACTGGATCTACATG (3‘); for ATF4, AGGGATCTGGCTGGATGCTCCGTG (5‘) and AGTGATATCCTCCTCTACCGCC (3‘); for ATF6, ATCGATTTCAACCTGACACCC (5‘) and CTGTCGTCTTACAGCAGACATATC (3‘); for Gadd153, CGTACCAGTGGTCTTCTGTTG (5‘) and CTGTCGTCTTACTGTTGAGATG (3‘); GAPDH, ACATCAAGAAGGTTGGAACGG (5‘) and CTGTCGTCTTACTGTTGAGATG (3‘). Equal aliquots of cell lysates were electrophoresed through 2% agarose gels. For Northern blot analysis, 4-µg aliquots of total RNA (harvested using Nucleospin RNA kit (Clontech, Palo Alto, CA)) were run on agarose-formaldehyde gels and transferred onto GeneScreen Plus membranes (PerkinElmer Life Sciences). cDNAs corresponding to atf4, gadd153, and gsp78 (a generous gift from Amy S. Lee), were labeled by the random primer method and used to detect corresponding mRNAs on Northern blots. An end-labeled 24-µp oligonucleotide complementary to 18 S rRNA (ACGGATCTCGTGTTCTGGACAC) was used as a probe to verify RNA integrity and loading differences.

cDNA Array Analysis—Total RNA was extracted from all samples using a Nucleospin RNA kit (Clontech, Palo Alto, CA). Atlas Human 1.2 K filters (www.Clontech) each containing 1174 genes were used. Total RNA (5 µg) was reverse transcribed and labeled with [α-32P]dATP using the Clontech cDNA array labeling kit. Hybridizations and washes were performed as recommended by the manufacturer. The cDNA array membranes were visualized for analysis by using a PhosphoImager (Amersham Biosciences), and were quantitated as described (31).

DAPI Staining—DAPI staining was performed as described previously (24). In brief, prior to staining, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and then washed with phosphate-buffered saline. DAPI was added to the fixed cells for 30 min to which they were examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei. Percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted x 100. A minimum of 400 cells were counted for each treatment.

Construction of Small Interference RNA (siRNA) Duplexes and Transfection—Twenty-one nucleotide double-stranded RNAs were transcribed in vitro using the Silentce™ siRNA construction kit according to the manufacturer instructions (Ambion Inc.). The targeting sequence of human P58IPK (accession number U28424), corresponding to nucleotide positions 137–157 (coding region), was AATTACTTGCGGACCACTCC (sense) and CTGTCGTCTTACTGTTGAGATG (antisense). Twenty-one nucleotide double-stranded RNAs were transfected into 6-well plates on the day before transfection at a concentration of 10⁵ cells per well. Cells were transfected with Oligofectamine reagent according to the manufacturer's instructions (Invitrogen). A mixture of 1.25 µg of P58IPK siRNA and 25 µg of 1 µm siRNA duplex. In a separate tube, 12 µl of Opti-MEM 1 was incubated with 3 µl of Oligofectamine for 5 min at room temperature. The two mixtures were combined, gently mixed, and incubated for another 20 min at room temperature. The entire mixture was added to the cells in 0.8 ml of 10% fetal bovine serum containing Dulbecco's modified Eagle's medium without antibiotics.

Cells were assayed at different time intervals after transfection.

[35S]Methionine Metabolic Labeling—Cells were seeded into 6-well plates at a density of 10⁶ cells per well. Twenty-four h following P58IPK siRNA transfection, cells were placed for 30 min in methionine-free minimal essential medium (BIOSOURCE, Camarillo, CA), and labeled by addition of [35S]methionine (20 µCi/ml, 1000 Ci/mmol; Amersham Biosciences) to the culture medium for 2 h. Cells were washed twice with ice-cold phosphate-buffered saline and collected in lysis buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 50 mM NaCl, 1% Triton X-100), 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 µl/mg leupeptin, 5 µg/ml apropinin). The protein concentration was measured using the Bio-Rad protein DC assay kit. The [35S]methionine incorporation was measured by cold trichloroacetic acid precipitation and analyzed by SDS-PAGE. For trichloroacetic acid precipitation, equal amounts of protein were added to 0.5 ml of 0.1 M HCl in 10% trichloroacetic acid containing 0.2% sodium azide and placed on ice. Ice-cold 20% trichloroacetic acid (0.5 ml) was mixed with 20 µl of 1 µm siRNA duplex. In a separate tube, 12 µl of Opti-MEM 1 was incubated with 3 µl of Oligofectamine for 5 min at room temperature. The two mixtures were combined, gently mixed, and incubated for another 20 min at room temperature. The entire mixture was added to the cells in 0.8 ml of 10% fetal bovine serum containing Dulbecco's modified Eagle's medium without antibiotics.

Cells were assayed at different time intervals after transfection.
Induction of P58IPK expression is indicated by the (Fig. 3A). Hence, we

Materials and Methods., as described under “Materials and Methods.” Induction of P58IPK expression is indicated by the arrow. B, HEK-293 and MEF cells were treated with either 2 μg/ml tunicamycin or 1 μm thapsigargin for the times shown, whereupon p58IPK and gapdh mRNA levels following exposure to ER stress agents were detected by RT-PCR as indicated under “Materials and Methods.” Assessment of gapdh levels served to control for RNA integrity and loading. C, immunoblot analysis of P58IPK and GAPDH protein levels in HEK-293 and MEF cells treated with tunicamycin (2 μg/ml) for the times indicated.

RESULTS

Identification of P58IPK as an ER Stress-Induced Gene—We sought to investigate the UPR stress response in mammalian cells by assessing changes in gene expression profiles after ER stress using the Atlas cDNA Gene Array (Clontech). This array contains cDNAs for 1174 genes involved in apoptosis, cell cycle control, stress responses, transcription, and signaling. Total RNA was isolated from MEFs that were either left untreated or treated for 6 h with 2 μg/ml tunicamycin, an agent that causes ER stress by inhibiting protein N-glycosylation. Analysis of the resulting signals on cDNA arrays (carried out as described under “Materials and Methods”) revealed 19 candidate genes whose expression was increased in response to tunicamycin treatment. Among these were a number of genes previously shown to play a role in the UPR response, including grp78, gadd153, and erp72. One novel gene whose expression was significantly elevated in MEFs following ER stress was p58IPK, an established inhibitor of the eIF2α kinase PKR, a kinase related to the ER-specific kinase, PERK (Fig. 1A). Hence, we further investigated the regulation of p58IPK by ER stress and examined its potential role during the UPR response.

To validate the findings obtained by microarray analysis, p58IPK expression levels in MEFs and HEK-293 cells were examined using RT-PCR after treatment with either tunicamycin or thapsigargin (another ER stress agent that inhibits ER Ca2⁺-ATPase, thereby causing ER stress through a different mechanism). As shown, p58IPK mRNA levels increased in both

FIG. 1. Induction of P58IPK by ER stress-inducing agents. A, Atlas array analysis of gene expression profiles following treatment with tunicamycin. MEF cells were treated for 6 h with 2 μg/ml tunicamycin. Reverse transcribed, [α-32P]dATP-labeled cDNA probes synthesized from total cellular RNA present in either untreated (left) or tunicamycin-treated (right) populations were hybridized to Atlas Mouse 1.2 Array membranes, as described under “Materials and Methods.” Induction of P58IPK expression is indicated by the arrow. B, HEK-293 and MEF cells were treated with either 2 μg/ml tunicamycin or 1 μm thapsigargin for the times shown, whereupon p58IPK and gapdh mRNA levels following exposure to ER stress agents were detected by RT-PCR as indicated under “Materials and Methods.” Assessment of gapdh levels served to control for RNA integrity and loading. C, immunoblot analysis of P58IPK and GAPDH protein levels in HEK-293 and MEF cells treated with tunicamycin (2 μg/ml) for the times indicated.

FIG. 2. Overexpression of ATF4 induces p58IPK expression. A, HEK-293 cells were transfected with an empty vector or vectors expressing either atf4 or atf6. Cells were harvested 48 h after transfection and total RNA prepared for assessment of the expression levels of mRNAs encoding p58IPK and gapdh (to control for RNA integrity and loading) by RT-PCR. B, cells were transfected as described in the legend of panel A and total RNA was prepared to assess the expression levels of gadd153, atf4, atf6, and gapdh by RT-PCR.

cell lines in response to each ER stress agent in a time-dependent manner (Fig. 1B). Further characterization of the response was carried out through kinetic analysis of P58IPK protein expression by Western blot analysis. As depicted in Fig. 1C, P58IPK protein levels similarly increased following treatment with tunicamycin in both cell lines (Fig. 1C).

Because ATF4 and ATF6 are known to play a role in the transcriptional activation of ER stress-inducible genes, we examined their contribution to the induction of p58IPK expression. To investigate the potential role of ATF6, we transfected HEK-293 cells with a plasmid that expresses p50 ATF6 (the soluble form of ATF6, capable of translocating into the nucleus). Cells were harvested 48 h after transfection and p58IPK mRNA was examined using RT-PCR (Fig. 2). Overexpression of p50 ATF6 alone in HEK-293 cells resulted in up-regulation of the p58IPK mRNA, even in the absence of ER stress. In contrast, overexpression of full-length ATF4 alone did not alter p58IPK mRNA expression. To ensure that the ATF4 construct was functional in this assay, we also analyzed expression of gadd153, an established transcriptional target of both ATF4 and ATF6. gadd153 mRNA was induced similarly by ectopically overexpressed p50 ATF6 and ATF4, indicating that the ATF4 construct was functional. These observations indicate that p58IPK is a novel target of the ATF6, but not ATF4 pathway.

Overexpression of P58IPK Inhibits eIF2α Phosphorylation and ATF4 and Gadd153 Induction—To gain a better understanding of the biological role of P58IPK in the UPR, we tried to generate stable cell lines overexpressing P58IPK using both HEK-293 and HeLa cells. Despite our best efforts, no stable P58IPK-overexpressing clones could be obtained (data not shown). As an alternative strategy, we transiently transfected a plasmid expressing P58IPK into HeLa cells, where high transfection efficiencies could be achieved. ER stress is known to induce translational repression, which is mediated by phosphorylation of eIF2α. Phosphorylation of eIF2α leads to down-regulation of translation initiation through a well-characterized mechanism involving inhibition of eIF2β activity (32). P58IPK is a known inhibitor of the eIF2α kinase PKR, and it has been shown that overexpression of P58IPK can inhibit dsRNA-induced phosphorylation of eIF2α by PKR. Because ER stress leads to elevated P58IPK expression, we hypothesized that P58IPK might affect the eIF2α phosphorylation during the UPR. To address this possibility, HeLa cells were transiently transfected with a P58IPK expression vector or empty vector (Fig. 3A). Forty-eight hours post-transfection, cells were treated with tunicamycin, and protein lysates were analyzed for both eIF2α phosphorylation and total eIF2α protein levels.
ER Stress Induces P58IPK

**Fig. 3. Overexpression of P58IPK reduces eIF2α phosphorylation and expression of ATF4 and Gadd153.** A, 48 h after transfection with either an empty plasmid or a vector expressing p58IPK, HeLa cells were treated with 2 μg/ml tunicamycin for the indicated times and P58IPK and GAPDH protein levels were assessed by immunoblot. B, cells were transfected as described in panel A, whereupon they were treated with 1 μM thapsigargin (Tg) for 1 h and phospho-eIF2α and total eIF2α levels were assessed by immunoblotting. C, cells were treated as described in panel A, then treated with 2 μg/ml tunicamycin (Tn) for the times indicated. Expression of ATF4, Gadd153, and GAPDH proteins was assessed by immunoblot analysis. The values between the panels represent the -fold induction of ATF4 and Gadd153 levels compared with control levels after normalization to GAPDH levels, as determined by densitometric analysis. D, control and P58IPK-overexpressing HeLa cells were harvested at the indicated times, and PARP cleavage was assessed by Western blot analysis using a monoclonal antibody that recognizes cleaved PARP. E, HeLa cells that were treated with tunicamycin for the times indicated were fixed with 4% paraformaldehyde, stained with DAPI, and subjected to counting of apoptotic nuclei by fluorescence microscopy. Data represent the mean ± S.E.

Given that elevated P58IPK expression decreased the phosphorylation levels of eIF2α, we sought to analyze the levels of ATF4, a downstream target of this pathway, and Gadd153, whose expression is in turn regulated (at least in part) by ATF4. P58IPK overexpression profoundly inhibited ATF4 protein accumulation in tunicamycin-treated HeLa cells (Fig. 3C), consistent with previous reports showing that the production of ATF4 protein requires eIF2α phosphorylation (18). Gadd153 protein induction in the P58IPK overexpressed cells was ~70% of that seen in control cells after 4–6 h treatment with tunicamycin (Fig. 3C).

Previous reports have shown that P58IPK can protect cells from dsRNA or tumor necrosis factor-α-induced apoptosis. We were therefore interested in determining whether P58IPK could protect cells from ER stress-induced apoptosis. HeLa cells transiently transfected with either a p58IPK-containing expression vector or an empty vector were treated with tunicamycin for different time periods and then analyzed for apoptosis. A hallmark of apoptosis is cleavage of the nuclear 116-kDa PARP (poly(ADP-ribose) polymerase) protein to an 85-kDa inactive polypeptide. Inactivation of PARP through proteolytic cleavage facilitates chromosomal DNA fragmentation as part of the cellular apoptotic program (33). Our results show that the tunicamycin-induced PARP proteolysis was similar in vector and P58IPK-transfected HeLa cells (Fig. 3D). DAPI staining revealed a similar pattern of condensed and fragmented nuclei for both the control and P58IPK-transfected cells (Fig. 3E). Taken together, these results suggest that under the conditions utilized here, overexpression of P58IPK does not alter the apoptotic response to ER stress.

**Silencing of P58IPK by siRNA Induces Apoptosis—**Whereas elevated P58IPK expression failed to alter the cellular outcome following ER stress, it remained possible that a reduction in P58IPK could influence the response. We decided to employ the RNA interference technique to address this possibility. A small inhibitory double-stranded RNA homologous to a 21-nucleotide sequence unique to the human P58IPK was used to reduce P58IPK expression. As shown in Fig. 4A, transfection of HeLa or HEK-293 cells with P58IPK siRNA resulted in a reduction of P58IPK protein levels, although control transfections with siRNA specific for luciferase, carried out in parallel, showed no effect on P58IPK expression. These observations indicate that the P58IPK siRNA treatment specifically reduced the abundance of P58IPK protein.

Approximately 28 h after transfection with the P58IPK siRNA duplex, we observed that many cells showed reduced...
PhosphorImager.
P58IPK siRNA treatment markedly increased the level of eIF2 phosphorylation and inhibition of protein synthesis. A, 20 h after transfection of HEK-293 and HeLa cells with P58IPK siRNA or control siRNA (described in the legend of Fig. 4), whole cell protein extracts were subjected to immunoblot analysis to assess the levels of total eIF2α and phosphorylated eIF2α. B, whole cell extracts from HEK-293 and HeLa cells that were transfected with either the control siRNA duplex or the P58IPK siRNA duplex were subjected to pulse-labeling of total proteins using [35S]methionine (described under “Materials and Methods”). Proteins were resolved by 4–12% gradient SDS-PAGE and stained with Coomassie Blue R-250 (right panel), or visualized for analysis using a PhosphorImager.

ability to adhere to the plate and floated in the medium. To determine whether silencing P58IPK causes cell death, we examined the cell growth and viability of P58IPK siRNA-transfected cells. Cells transfected with the control siRNA grew very well. By contrast, cells transfected with P58IPK siRNA showed decreased growth compared with control transfected cells. Cells transfected with the control siRNA or P58IPK siRNA and were transfected with P58IPK siRNA and 20 h after transfection, lysates were prepared and subjected to Western blot analysis (Fig. 5B). As shown in the right panel of Fig. 5B, caspase-3 and PARP can be detected by Western blot analysis with an antibody that recognizes PARP only when it is phosphorylated on Thr980. HEK-293 cells transiently overexpressing P58IPK were used to generate a plasmid that expressed wild-type mouse PERK. Expression of PERK was confirmed by Western blot analysis with an antiserum reactive to the c-myc epitope tag expressed at the COOH terminus of PERK.

siRNA Silencing of P58IPK Induces PERK Phosphorylation—Having determined that P58IPK siRNA induces eIF2α phosphorylation we next wished to determine whether any of the known eIF2α kinases might be implicated in this process. P58IPK was originally discovered as an inhibitor of PKR, an interferon-induced, double-stranded RNA-activated kinase that is activated during virus infection (34). Activation of PKR by double-stranded RNA results in PKR dimerization and autophosphorylation at positions Thr446 and Thr451 in the activation loop (35). To determine whether PKR is activated by P58IPK siRNA, we examined its phosphorylation state. P58IPK siRNA did not markedly induce PKR phosphorylation, as determined by Western blotting with an antiserum reactive to phospho-Thr446 (Fig. 6A). It was interesting to note that total PKR protein levels were slightly increased by P58IPK siRNA. Although moderate, such increases in PKR expression were seen reproducibly.

PERK, a kinase found in the lumen of the ER, also phosphorylates eIF2α in response to various stimuli that induce ER stress. A very recent paper from Yan et al. (36) demonstrates that P58IPK is associated with the ER and represses PERK activity. Unfortunately, the low expression levels of endogenous PERK and the present unavailability of good anti-PERK antibodies did not allow a clear analysis of the effects of P58IPK siRNA on PERK phosphorylation. Thus, to gain insight into this potential regulation, we transiently transfected HEK-293 cells using a plasmid that expressed wild-type mouse PERK. PERK activation can occur through autophosphorylation of its cytoplasmic kinase domain, and can be monitored by immunoblotting with an antibody that recognizes PERK only when it is phosphorylated on Thr315/317. HEK-293 cells transiently overexpressing PERK were transiently transfected with the control siRNA or P58IPK siRNA and, 20 h after transfection, lysates were prepared and subjected to Western blot analysis (Fig. 6B). As described.
Silencing of P58IPK induces ATF4 and Gadd153. Western blot and Northern blot analyses of ATF4, Gadd153, and GRP78 expression in cells silenced with P58IPK siRNA. HEK-293 cells were transfected with P58IPK siRNA and 20 h later treated with 2 μg/ml tunicamycin for different time periods. A, immunoblot analysis of protein lysates from control transfected populations and from P58IPK siRNA-transfected cells to monitor ATF4, Gadd153, Grp78, and GAPDH protein levels. B, abundance of mRNAs encoding atf4, gadd153, grp78, and gpdh. Total RNA was prepared from transfected HEK-293 cells and analyzed by Northern blotting.

shown, P58IPK siRNA treatment markedly increased the level of PERK phosphorylation compared with control transfected cells.

P58IPK Silencing by siRNA Induces ATF4 and Gadd153—
Gadd153 is expressed at low or undetectable levels under normal growth conditions (37), but is highly induced following thapsigargin or tunicamycin treatments, and has been implicated in ER stress-induced apoptosis (38). Gadd153 transcription is induced by eIF2α phosphorylation through preferential translation of ATF4, as previously described (18). Because P58IPK siRNA increased the level of eIF2α phosphorylation, we predicted that it would likely induce ATF4 and Gadd153 expression. To determine the effect of P58IPK siRNA on ATF4 and Gadd153 expression, Northern and immunoblot analyses were performed. HEK-293 cells were transfected with control or P58IPK siRNA and 20 h later were treated with tunicamycin for different time intervals, whereupon total RNA and protein were prepared for analysis (Fig. 7, A and B). ATF4 protein was low in untreated cells, but increased in response to tunicamycin treatment. Transfection to decrease P58IPK levels in HEK-293 cells, did not significantly affect atf4 mRNA levels, but markedly increased ATF4 protein levels, even in untreated cells. This is consistent with earlier reports showing that ATF4 expression is regulated via its preferential translation during ER stress in an eIF2α-dependent manner (18). Gadd153, which was barely detectable in the control transfected cells, was likewise induced after P58IPK siRNA transfection. This was evident at both the mRNA and protein levels.

Transcriptional induction of gadd153 has been shown to closely parallel that of grp78 under many ER stress-triggering conditions and indeed like gadd153, grp78 mRNA levels were increased at 4 h following tunicamycin treatment. However, unlike gadd153, expression of grp78 was not increased by P58IPK siRNA. These findings suggest that silencing P58IPK specifically affects eIF2α stress signaling pathways and that the P58IPK protein plays a critical role in regulating ATF4 and Gadd153 protein levels.

The UPR, initiated in response to perturbations in the ER environment, is characterized by the activation of signaling pathways that transduce stress signals generated and sensed in the ER to other cellular compartments, thereby effecting changes in gene transcription and transiently suppressing translation. The biological objective of the UPR is to regain homeostasis in the ER by reducing demands on the organelle and increasing its capacity to carry out its protein folding and/or modifying functions. So far, three distinct pathways, IRE1/XBP-1, PERK/eIF2α/ATF4, and ATF6, have been shown to contribute to the transcriptional response, but there is increasing evidence for both cross-talk and redundancy in the pathways. For example, transcriptional up-regulation of XBP-1 through the ATF6 pathway provides more substrate for IRE1, thus potentiating IRE1 pathway signaling. In addition, some genes, such as gadd153, appear to be targets of more than one pathway (i.e. both ATF4 and ATF6 contribute to its transcription) (15, 17, 40).

In this report, we have identified P58IPK, an inhibitor of the interferon-induced protein kinase PKR, as a target for transcriptional up-regulation by the UPR. This finding is consistent with a previous report by Kaufman and colleagues (41) in which p58IPK was listed as one of 67 genes induced upon tunicamycin treatment in MEFs using a different cDNA array. However, the physiologic relevance of p58IPK induction was not addressed in that study, nor was its up-regulation verified by other methods in that report. In the current studies we have provided evidence suggesting that p58IPK induction is mediated via ATF6 and that P58IPK plays a role in down-regulating the activity of the PERK/eIF2α/ATF4 pathway. These findings identify a novel mechanism contributing to the regulation of the UPR and define a new avenue for cross-talk between ER stress-activated pathways.

That ATF6 contributes to the induction of p58IPK during the UPR is suggested by the finding that overexpression of its activated nuclear form (p50 ATF6) leads to elevated p58IPK expression. A 19-bp ER stress responsive element consisting of the consensus sequence CCAAT(N9)CCACG has been identified in the promoter region of the p58IPK gene (36). ER stress-responsive element sequences have been implicated in the transcriptional activation of downstream genes after treatment with agents that activate the mammalian UPR pathway (36), and ATF6 has been shown to interact with the ER stress-responsive element (16). Whereas definitive proof of its role in regulating p58IPK must await further investigation, a role for ATF6 in regulating p58IPK expression is consistent with studies of Okada and co-workers (42), in which microarray analysis revealed genes encoding molecular chaperones and folding enzymes as primary transcriptional targets of the ATF6 pathway. As a member of the DnaJ molecular chaperone family, P58IPK would fit into this category (34).

P58IPK was first recognized for its ability to inhibit the double-stranded RNA-activated protein kinase, PKR (34). PKR is part of the interferon-induced host defense against viral infection, and functions to repress translation initiation via phosphorylation of eIF2α (43, 44). The proposed inhibitory mechanism of P58IPK is as follows. Influenza virus activates the P58IPK pathway by promoting the dissociation of P58IPK from its own inhibitor, hsp40. The free P58IPK represses PKR-mediated eIF2α phosphorylation through direct protein-protein interaction, and thereby relieves the PKR-imposed block on mRNA translation (45). Importantly, unlike the situation seen here for ER stress, P58IPK expression is not altered by viral infection. Available evidence indicates that PKR is not involved in the ER stress response, as PKR knockout cells are unin-
paired in their ability to respond to ER stress by attenuating translation rates (20). Because PERK shares many properties with PKR, we investigated the possibility that P58IPK could modify this pathway. A very recent report by Yan and colleagues (36) provides evidence that P58IPK interacts with PERK and inhibits its activity. Our data showing that modulation of P58IPK levels alters the phosphorylation status of PERK and eIF2a is in agreement with this report. We found that overexpression of P58IPK inhibited eIF2a phosphorylation and reduced ATF4 protein accumulation, whereas silencing of P58IPK expression enhanced PERK and eIF2a phosphorylation and increased ATF4 accumulation. The current model for regulation of ATF4 protein levels proposes that, under normal conditions, ATF4 mRNA is expressed, but is translated poorly. However, under ER stress conditions, ATF4 protein is rapidly synthesized in a manner that is dependent on eIF2a phosphorylation. This then leads to enhanced expression of its target genes.

Previous studies implicate both ATF4 and ATF6 in the ER stress response. However, silencing of ATF6 to (relative to its effect on ATF4) likely reflects the contribution of P58IPK expression in cells with alternatively regulated ATF6. We propose that enhanced sensitivity to undergo apoptosis. Indeed, overexpression of ATF6 might contribute to the enhanced sensitivity to undergo apoptosis. Indeed, overexpression of ATF6 is transient in stressed cells. We propose that enhanced expression of P58IPK during the UPR serves as an important component of a negative feedback loop used by the cell to attenuate eIF2a signaling. This model is reminiscent of that proposed for Gadd34, another gene product induced during the ER stress response. Gadd34 functions in a manner that is dependent on eIF2a phosphorylation and reduces ATF4 protein accumulation, whereas silencing of P58IPK expression enhanced PERK and eIF2a phosphorylation and increased ATF4 accumulation. The current model for regulation of ATF4 protein levels proposes that, under normal conditions, ATF4 mRNA is expressed, but is translated poorly. However, under ER stress conditions, ATF4 protein is rapidly synthesized in a manner that is dependent on eIF2a phosphorylation. This then leads to enhanced expression of its target genes.

In summary, our findings are consistent with a model in which P58IPK functions to regulate PERK and eIF2a phosphorylation during normal physiologic conditions and under conditions of ER stress. Phosphorylation of PERK and eIF2a is required for translation attenuation, transcriptional induction, and cellular survival in response to ER stress. However, PERK and eIF2a phosphorylation and inhibition of protein translation is transient in stressed cells. We propose that enhanced expression of P58IPK during the UPR serves as an important component of a negative feedback loop used by the cell to attenuate eIF2a signaling. This model is reminiscent of that proposed for Gadd34, another gene product induced during the UPR, which has been shown to play an important feedback role in regulating eIF2a phosphorylation, through promotion of its dephosphorylation (29).

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In summary, our findings are consistent with a model in which P58IPK functions to regulate PERK and eIF2a phosphorylation during normal physiologic conditions and under conditions of ER stress. Phosphorylation of PERK and eIF2a is required for translation attenuation, transcriptional induction, and cellular survival in response to ER stress. However, PERK and eIF2a phosphorylation and inhibition of protein translation is transient in stressed cells. We propose that enhanced expression of P58IPK during the UPR serves as an important component of a negative feedback loop used by the cell to attenuate eIF2a signaling. This model is reminiscent of that proposed for Gadd34, another gene product induced during the UPR, which has been shown to play an important feedback role in regulating eIF2a phosphorylation, through promotion of its dephosphorylation (29).
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