PERK (eIF2 α kinase) is required to activate the stress-activated MAPKs and induce the expression of immediate-early genes upon disruption of ER calcium homeostasis

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The eIF2 α (eukaryotic initiation factor-2 α) kinase PERK (double-stranded RNA-activated protein kinase-like ER kinase) is essential for the normal function of highly secretory cells in the pancreas and skeletal system, as well as the UPR (unfolded protein response) in mammalian cells. To delineate the regulatory machinery underlying PERK-dependent stress-responses, gene profiling was employed to assess global changes in gene expression in PERK-deficient MEFs (mouse embryonic fibroblasts). Several IE (immediate-early) genes, including *c-myc*, *c-jun*, *egr-1* (early growth response factor-1), and *fra-1* (fos-related antigen-1), displayed PERK-dependent expression in MEFs upon disruption of calcium homeostasis by inhibiting the ER (endoplasmic reticulum) transmembrane SERCA (sarcoplasmic/ER Ca²⁺-ATPase) calcium pump. Induction of *c-myc* and *egr-1* by other reagents that elicit the UPR, however, showed variable dependence upon PERK. Induction of *c-myc* expression by thapsigargin was shown to be linked to key signalling enzymes including PLC (phospholipase C), PI3K (phosphatidylinositol 3-kinase) and p38

MAPK (mitogen-activated protein kinase). Analysis of the phosphorylated status of major components in MAPK signalling pathways indicated that thapsigargin and DTT (dithiothreitol) but not tunicamycin could trigger the PERK-dependent activation of JNK (c-Jun N-terminal kinase) and p38 MAPK. However, activation of JNK and p38 MAPK by non-ER stress stimuli including UV irradiation, anisomycin, and TNF- α (tumour necrosis factor- α) was found to be independent of PERK. PERK plays a particularly important role in mediating the global cellular response to ER stress that is elicited by the depletion of calcium from the ER. We suggest that this specificity of PERK function in the UPR is an extension of the normal physiological function of PERK to act as a calcium sensor in the ER.

Key words: eukaryotic initiation factor-2 α (eIF2 α), endoplasmic reticulum (ER), mitogen associated protein kinase (MAPK), calcium homeostasis, stress response, immediate-early (IE) genes.

INTRODUCTION

The ER (endoplasmic reticulum) depends upon a specialized environment of high Ca²⁺ concentration to fold, modify and assemble newly synthesized proteins destined for secretion or insertion into one of several cellular membranes. Perturbation of the ER environment results in the inability of the ER to properly fold proteins and stimulates the UPR (unfolded protein response). Various pathophysiological conditions and pharmacological reagents that alter the Ca²⁺ levels, oxidizing environment, or glycosylation machinery in the ER can trigger the UPR [1–3]. Reagents that perturb Ca²⁺ homeostasis in the ER are particularly potent elicitors of the UPR. The dominant folding protein, BiP/GRP78 (binding protein/glucose-regulated 78 kDa protein) plays an important role in the storage of ER Ca²⁺ with a stoichiometry of 1–2 moles of Ca²⁺/mole of BiP/GRP78 [4]. BiP/GRP78 also acts as the principal regulator of three ER-resident transmembrane proteins, PERK (double-stranded RNA-activated protein kinase-like ER kinase) IRE-1 (inositol requiring-1) and ATF6 (activating transcription factor 6) which are primary sensors and transducers of the UPR [5–9]. A decrease in ER Ca²⁺ results in disassociation of BiP/GRP78 from PERK and IRE1 resulting in their activation

[10,11], whereas, dissociation of BiP/GRP78 from ATF6 leads to its translocation to the Golgi, a prerequisite for its activation [12,13]. The ER is the major storage organelle in the cell for Ca²⁺ and mobilization of ER Ca²⁺ has a potent impact on a variety of calcium-sensitive signal transduction pathways in the cytoplasm and nucleus [14–16]. Thus major perturbation of ER calcium homeostasis can have manifold effects upon stress-related signalling pathways in addition to eliciting the UPR.

In mammalian cells, the UPR is characterized by transcriptional activation of chaperone and protein-folding genes, regulation of growth arrest and apoptosis genes, and repression of global protein synthesis [17]. Among the four ER-resident transmembrane proteins, IRE1 α , IRE1 β , and ATF6 play a dominant role in mediating transcriptional regulation, whereas PERK is purported to be responsible for repressing global protein synthesis via phosphorylation of the α subunit of eIF2 α (eukaryotic initiation factor 2 α) [18,19]. Phosphorylation of eIF2 α , on the other hand, can also indirectly control gene transcription by positively regulating the translation of transcription factors as has been shown for mammalian ATF4 and yeast GCN (general control of non-derepressible) 4 [20,21]. The translational derepression of ATF4 upon increased phosphorylation of eIF2 α by PERK in turn leads to

Abbreviations used: ATF4, activating transcription factor 4; BiP/GRP78, binding protein/glucose-regulated 78 kDa protein; CHOP, CCAAT/enhancer-binding protein-homologous protein; CPA, cyclopiazonic acid; DAG, diacylglycerol; DTT, dithiothreitol; *egr-1*, early growth response factor-1; eIF2 α , eukaryotic initiation factor-2 α ; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; *fra-1*, fos-related antigen-1; GADD153, growth arrest and DNA damage-inducible protein; GCN, general control of non-derepressible; IE, immediate-early; IP3, inositol 1,4,5-trisphosphate; IRE-1, inositol requiring-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; PERK, pancreatic eIF-2 α kinase; PERK, double-stranded RNA-activated protein kinase-like ER kinase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; RT-PCR, reverse transcription-PCR; SERCA, sarcoplasmic/ER Ca²⁺-ATPase; UPR, unfolded protein response.

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the up-regulation of *CHOP/GADD153* (CCAAT/enhancer-binding protein-homologous protein/growth arrest and DNA damage-inducible protein) [20] and *ATF3* [22] expression. Thus by activating ER-resident protein kinases, cells respond to ER stress via intracellular signalling pathways to regulate gene expression. However, the relevant signalling pathways remain to be elucidated.

Signal transduction from the ER to the cell nucleus could be mediated by similar signal cascades associated with plasma membrane-initiated cell signalling. ER stress, for example, is coupled to the activation of stress-activated protein kinases [23,24]. These kinases, including JNK (c-Jun N-terminal kinase) and p38 MAPK (mitogen-activated protein kinase) are known to be activated through a cascade of kinase activities preferentially triggered by physical stresses and inflammatory cytokines, which distinguish them from the ERK (extracellular signal-regulated kinase) pathway [25,26]. JNK and p38 MAPK are activated by a diverse array of ER stress-inducing agents such as thapsigargin, tunicamycin, and DTT (dithiothreitol), which cause depletion of ER Ca^{2+} , inhibition of N-linked glycosylation of proteins, or impairment of disulphide bond formation, respectively [23,27–30]. In addition, several IE (immediate-early) genes, including *c-myc* and *egr-1*, have been shown to be transcriptionally activated as a consequence of ER-stress [31–35]. In as much as the ER-resident transmembrane proteins play a major role in ER adaptation, their activities may also be required for the broader cellular adaptations to ER stress mediated by activation of JNK and p38 MAPK pathways and the IE genes which all impact cell proliferation and viability.

Recently we and others discovered that PERK, an ER transmembrane kinase, is required for the normal growth and viability of the endocrine and exocrine pancreas [36,37] leading us to propose that PERK may regulate signal transduction pathways underlying growth control. To investigate this question further we have established MEFs (mouse embryonic fibroblasts) from *Perk*^{-/-} mice [38] and have identified genes that exhibit an aberrant response to ER stress. We show in the present study that the normal response of *c-myc* and *egr-1* expression to the loss of Ca^{2+} homeostasis in the ER requires PERK.

Moreover, PERK is required for the activation of JNK and p38 MAPK induced by the loss of ER Ca^{2+} homeostasis, but PERK is not strictly required for the activation of JNK and p38 MAPK when ER stress is elicited by others reagents that induce the UPR. Thus PERK plays a particularly important role in mediating the global cellular response to ER stress that is elicited by the depletion of Ca^{2+} from the ER. We suggest that this specificity of PERK function in the UPR is an extension of the normal physiological function of PERK to act as a Ca^{2+} sensor in the ER.

EXPERIMENTAL

Cell culture

MEFs were isolated from *Perk*^{+/+} and *Perk*^{-/-} embryos at days 14–15 of gestation [38]. The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% (v/v) FBS (foetal bovine serum) at 37°C in a humidified 5% CO_2 atmosphere.

cDNA microarray analysis

The total RNA used for microarray analysis was isolated using Atlas Pure Total RNA Labelling System (Clontech Laboratories, Inc.) according to the manufacturer's instructions. The isolated RNA was further treated with DNase I (Ambion, Inc.) for DNA decontamination. The *Perk*^{+/+} and *Perk*^{-/-} MEFs at passage 2 were

grown to 90% confluence and treated with or without 1 μM thapsigargin for 1 h before the RNA was isolated. Total RNAs for standard RT-PCR (reverse transcription-PCR) analysis were prepared by TRI reagent (Sigma).

The Clontech Atlas Mouse Nylon Arrays 1.2 and 1.2 II containing 2350 genes in total were employed in this study. The ³²P-labelled probe was prepared from 5–8 μg of total RNA and hybridization was performed according to Clontech protocols. The hybridized array was exposed to a Kodak phosphor-image screen for 2–4 days and scanned and visualized using a Storm 860 PhosphorImager (Molecular Dynamics) at 100 μm resolution. The intensity of hybridization signals was quantified using Clontech AtlasImage 2.01 software. The fold change of each gene (cDNA) between two samples, for example mutant versus wild-type or treated versus untreated, was calculated after normalization to the sum of the signal values over background of all genes on the array. Two separate sets of treatment and RNA isolation were used for the assay. In total, four compared values of an individual gene were obtained by cross-comparison between these two sets of samples. The differentially expressed genes in the MEFs were selected only when they consistently showed at least a 2 fold change from multiple calculations.

RT-PCR analysis

The mRNA levels of genes of interest were analysed by one-step RT-PCR using Promega Access RT-PCR System. In a standard 20 μl reaction volume, 0.1 μg of total RNA was added for the analysis and 3–5 μl of final reaction mixture was electrophoresed on a 1.5% agarose gel. The DNA fragment was visualized by ethidium bromide staining. The primer sequences (Clontech 7832-1) were designed by Clontech to be used for the RT-PCR analysis of genes on the Atlas Array. The aliquots were removed from each reaction after 13–40 PCR cycles (when visible products first appeared and before they reached a saturation plateau). The cDNA intensities were quantified by ImageQuant 5.1 software (Molecular Dynamics) and compared between samples. The expression of *egr-1* and *c-myc* was also evaluated by real-time quantitative PCR (TaqMan, Applied Biosystems). The total RNA template for reverse transcription was purified from treated cells using the RNeasy mini kit (Qiagen). cDNA was reverse-transcribed using Moloney-murine leukaemia virus polymerase and random hexamer priming (both from Promega). Real-time quantitative PCR reactions contained 0.5–1.0 μg of input cDNA and 2 μM of the appropriate primer pairs. SYBR Green detection (Eurogentec) was used to quantify the amplification of *egr-1* and *c-myc*. The amplification of tubulin in each cDNA sample was used to normalize the data for the test genes.

Kinase inhibitors analysis

The majority of kinase inhibitors were obtained from Calbiochem. MEFs were pretreated with inhibitors for 30 min before thapsigargin was added to the culture. The concentration used for various kinase inhibitors was: PD98059 (ERK kinase inhibitor), 20 μM ; U73122 [PLC (phospholipase C) inhibitors], 10 μM ; KT5720 (protein kinase A inhibitor), 500 nM; LY294002 [PI3K (phosphatidylinositol 3-kinase) inhibitor], 20 μM ; SB203580 (p38 MAPK inhibitor), 20 μM . The less active PLC inhibitor U73343 was obtained from Sigma and was also used at 20 μM .

Stress treatment and immunoblot analysis

MEFs were grown on 35 mm plates to 90% confluence and switched to fresh media containing 0.5% FBS for 18–24 h. Cells were then treated with a variety of stress-inducing agents,

including thapsigargin (1 μ M), DTT (10 mM), tunicamycin (10 μ g/ml), UV light (200 J/m²), anisomycin (10 μ g/ml), and TNF- α (tumour necrosis factor- α) (10 ng/ml). At the indicated time after treatment, cells were washed with PBS and lysed by the addition of 80 μ l of cold RIPA buffer [1% NP 40 (Nonidet P40), 0.5% sodium deoxycholate, 0.1% SDS, 1 $\times$ PBS, pH 8.0] containing 1 $\times$ protease and phosphatase inhibitor cocktails (Sigma). Cells were scraped from the plate and incubated on ice for 10 min. Total cell lysates were obtained after centrifugation at 14000 g for 10 min at 4°C and the protein concentration was determined by Bio-Rad protein assay reagent. Proteins (15–20 μ g) were separated by SDS 4–15% gradient PAGE and transferred to PVDF membrane. Immunoblotting was performed as previously described [37]. Primary antibodies used are mouse anti-eIF2 α (kindly provided by Scot Kimball, Pennsylvania State University, PA, U.S.A.), 1:2000; rabbit anti-phospho-eIF2 α (Biosource International), 1:1000; rabbit anti-JNK, 1:1000; rabbit anti-(p38 MAPK), 1:1000; rabbit anti-(phospho-p38 MAPK), 1:1000; rabbit anti-ERK, 1:1000; rabbit anti-phospho-ERK, 1:1000 (all from Cell Signaling Technology); mouse anti-phospho-JNK, 1:200 (sc-6254, Santa Cruz Biotechnology). Secondary antibodies used are horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (1:5000, Jackson Immuno Research). The immuno-reactive signal was detected by ECL[®] Plus reagents (Amersham-Pharmacia Biotech) and visualized by a Storm 860 PhosphorImager. Quantification of the signals was conducted using ImageQuant 5.1 software.

RESULTS

PERK is required for the thapsigargin-induced expression of multiple IE genes

To determine the global regulatory effects of PERK on gene expression, we have employed cDNA microarray analysis to examine differences in the expression of 2350 mouse mRNAs in *Perk*^{-/-} versus *Perk*^{+/+} MEFs. We identified 31 genes with a difference of greater than 2 fold expression between *Perk*^{+/+} and *Perk*^{-/-} MEFs under normal growth conditions, and 27 genes were differentially expressed upon treatment with thapsigargin to elicit the UPR. Several mitogenesis-related IE genes, including those encoding *c-myc*, *c-jun*, *egr-1*, *fra-1*, *TIS21* (NGF inducible protein) and tristetraproline, displayed PERK-dependent expression in MEFs upon thapsigargin treatment (Table 1). Except for *c-jun* whose expression was down-regulated in *Perk*^{-/-} MEFs, the expression of all other IE genes was differentially up-regulated in *Perk*^{+/+} MEFs. RT-PCR analysis of the mRNA levels of selected IE genes such as *egr-1* and *c-myc* confirmed that their expression was induced by thapsigargin in a PERK-dependent manner (Figure 1A). The expression of *CHOP*, previously shown to be positively regulated by PERK in response to thapsigargin [20,39], was significantly reduced in *Perk*^{-/-} MEFs by both microarray and RT-PCR analysis (results not shown).

Table 1 Differentially expressed IE genes in thapsigargin-treated MEFs

GenBank [®] accession number	Gene/protein name	Ratio MEF ^{+/+} /MEF ^{-/-}
M20157	<i>egr-1</i>	7.05
X01023	<i>c-myc</i>	2.72
M64292	<i>TIS21</i>	2.65
AF017128	<i>fra-1</i>	2.50
M57422	Tristetraproline	2.40
J04115	<i>c-jun</i>	2.33

Induction of IE gene expression is usually transient and thus disruption of PERK function may only change their temporal expression rather than ablating their expression. To address this issue, we analysed the mRNA levels of *c-myc* and *egr-1* at various time points after thapsigargin treatment. Standard RT-PCR analysis revealed that whereas *c-myc* mRNA levels in *Perk*^{+/+} MEFs continued to rise during 2 h of thapsigargin treatment, their levels in *Perk*^{-/-} MEFs showed no increase but declined after 1 h of treatment (Figure 1B). Thus PERK is required for thapsigargin-induced increase of *c-myc* expression. During the first 30 min after the addition of thapsigargin, *egr-1* expression increased in both *Perk*^{+/+} and *Perk*^{-/-} cells. In contrast, wild-type MEF *egr-1* mRNA levels continued to increase from 30 min to 2 h, though *egr-1* mRNA levels in *Perk*^{-/-} MEFs dramatically declined (Figure 1B). Therefore PERK is required to sustain the induction and/or stability of the *egr-1* mRNA.

We also examined the response of the IE genes to CPA (cyclopiazonic acid), another SERCA (sarcolemmal/ER Ca²⁺-ATPase) inhibitor, as compared with the response to thapsigargin using a more sensitive real-time PCR method for quantifying the RNA levels. In the presence of 10 μ M CPA, *c-myc* mRNA levels increase at least 2 fold within 1 h and decline slightly, at 2 h in *Perk*^{+/+} cells while levels in *Perk*^{-/-} remain for the most part, unchanged over the course of the treatment (Figure 1C). We also observe an approximately 2 fold PERK-dependent increase in *egr-1* mRNA levels in response to CPA at 1 h; however, there is a significant reduction after 2 h of treatment. It has been shown that both CPA and thapsigargin are specific inhibitors for the SERCA pump in the ER membrane and initiate the release of calcium from the same intracellular stores. However, they do differ in their affinity for SERCA and elicit unique patterns of intracellular calcium transients, which could result in differences in the kinetics of responses to them [40,41]. The temporal differences we observe in IE mRNA up-regulation in response to CPA as compared with thapsigargin may reflect these subtle functional differences; however, overall it is clear that both SERCA inhibitors will up-regulate IE gene expression at the mRNA level in a PERK-dependent manner.

Various ER-stress stimuli result in differential PERK-dependent expression of *c-myc* and *egr-1*

c-myc can also be induced upon tunicamycin treatment in MEFs [35], suggesting that *c-myc* induction may be a common event under ER stress. To determine if the induction of *c-myc* is PERK-dependent in cells stressed with other reagents that elicit the UPR, we analysed the mRNA level of *c-myc* in wild-type and *Perk*^{-/-} MEFs treated with tunicamycin and DTT. We observed that *c-myc* mRNA levels were increased up to 2 fold 1 h after tunicamycin treatment in both *Perk*^{+/+} and *Perk*^{-/-} MEFs (Figure 2A), indicating that PERK is dispensable for the induction of *c-myc* expression by tunicamycin. The *c-myc* mRNA level continued to increase beyond the first hour of tunicamycin treatment in wild-type cells, whereas the *Perk*^{-/-} cells exhibited a reduction (Figure 2A). In cells treated with DTT, the level of *c-myc* mRNA was not increased until 2 h after treatment in wild-type cells (Figure 2B). By contrast, no significant change in *c-myc* mRNA levels was observed in *Perk*^{-/-} MEFs upon DTT treatment (Figure 2B), indicating that the induction of *c-myc* expression by DTT is PERK-dependent. Together, these results indicate that the PERK-dependent induction of *c-myc* expression is specific to certain but not all ER stress stimuli.

In parallel, we also analysed the expression of *egr-1* under the ER stresses elicited by tunicamycin and DTT. Tunicamycin could induce similar transient elevation of mRNAs in both *Perk*^{+/+} and

Figure 1 Differential expression of *c-myc* and *egr-1* in *Perk* mutant MEFs treated with thapsigargin

(A) Time-course analysis of mRNA levels of *c-myc* and *egr-1* during thapsigargin (Tg) (1 μM) treatment. Quantitative data are from three independent experiments and relative to the sample of wild-type cells without Tg treatment. (B) Real-time quantitative RT-PCR analysis of *Perk*^{+/+} and *Perk*^{-/-} MEFs treated with 1 μM Tg or 10 μM CPA. The relative levels of *c-myc* and *egr-1* expression were quantified using the Applied Biosystems Prism 7000 Sequence Detection Software and were normalized to tubulin expression.

Figure 2 The expression of *c-myc* and *egr-1* induced by tunicamycin and DTT

Wild-type^{+/+} and *Perk*^{-/-} MEFs were treated with 10 μg/ml tunicamycin (A) or 10 mM DTT (B). Total RNAs were isolated at the indicated time and mRNA levels of *c-myc* and *egr-1* were analysed by RT-PCR. Quantitative data are from three independent experiments and relative to the sample of wild-type cells without treatment.

Perk^{-/-} MEFs (Figure 2A), indicating that PERK is not necessary for the induction of *egr-1* expression by tunicamycin. Unlike tunicamycin, DTT did not cause a significant change in *egr-1* mRNA levels in *Perk*^{+/+} MEFs (Figure 2B). In *Perk*^{-/-} MEFs,

however, *egr-1* mRNA levels were greatly decreased at 1 h after DTT treatment (Figure 2B). These results indicate that although PERK is not essential for ER stress-induced expression of *egr-1*, it is required to maintain the steady state mRNA levels of *egr-1*.

Figure 3 Effects of various enzyme inhibitors on the expression of *c-myc* induced by thapsigargin

Wild-type MEFs were pretreated with enzyme inhibitors for 30 min and then incubated with 1 μ M of Tg for 1 h. (A) is a representative of RT-PCR analysis. (B) Quantitative data showing the average fold change in mRNA levels after Tg treatment obtained from three independent experiments. PD98059, ERK kinase inhibitor; U73122, PLC inhibitor; KT5720, protein kinase A inhibitor; LY294002, PI3K inhibitor; SB203580, p38 MAPK inhibitor.

Thapsigargin-induced *c-myc* expression is mediated through multiple signal transduction pathways

The induction of IE genes is correlated with a variety of signal transduction pathways [42]. To identify the specific signal transduction pathways underlying the PERK-dependent activation of *c-myc* by thapsigargin in MEFs, we employed an array of specific enzyme inhibitors to block major signalling pathways. We found that thapsigargin-induced expression of *c-myc* was significantly repressed by PLC inhibitor U73122, and partially blocked by the PI3K inhibitor LY294002 and the p38 MAPK inhibitor SB203580 (Figure 3). By contrast, the ERK kinase inhibitor PD98059 and the protein kinase A inhibitor KT5720 were unable to abrogate the thapsigargin-induced increase of *c-myc* expression. U73343, which is often used as a negative control for the PLC inhibitor U73122 had no effect on thapsigargin-induced expression of *c-myc* (results not shown). These results indicate that signal transduction pathways associated with PLC, PI3K and p38 MAPK activities are necessary for the thapsigargin-induced expression of *c-myc* in MEFs.

PERK is required for thapsigargin-induced activation of JNK and p38 MAPK but not ERK

ER stress induces the activation of p38 MAPK [24,28–30] and our finding that an inhibitor of p38 MAPK markedly diminished the induction of *c-myc* by thapsigargin, prompted us to investigate whether activation of p38 MAPK is PERK-dependent. The level of phosphorylated p38 MAPK was increased 2–3 fold in *Perk*^{+/+} but not in *Perk*^{-/-} MEFs after thapsigargin treatment (Figure 4), indicating that activation of the p38 MAPK signal pathway by thapsigargin is PERK-dependent.

In view of the cross-talk between different MAPK signalling pathways, we examined activation of ERK and JNK upon thapsigargin treatment. We did not observe an induction of ERK phos-

Figure 4 Effects of thapsigargin on the level of phosphorylated p38 MAPK, JNK and ERK

Wild-type and PERK mutant MEFs were treated with 1 μ M Tg for 30 min. Total and phosphorylated p38 MAPK, JNK, ERK and eIF2 α were measured by Western blot analysis of whole-cell extracts. Each sample is presented in triplicate obtained from three independent experiments.

phorylation upon thapsigargin treatment in either *Perk*^{+/+} or *Perk*^{-/-} MEFs. In contrast, phosphorylation of JNK was induced 2 fold in normal cells but not in *Perk*^{-/-} MEF cells (Figure 4).

PERK mediates the activation of JNK and p38 MAPK through distinct signalling pathways from those used by other non ER-stress stimuli

JNK and p38 MAPK are activated by a number of cellular stresses in addition to those that perturb the function of the ER. To determine whether the activation of JNK and p38 MAPK by other cellular stresses also requires PERK function, we treated *Perk*^{+/+} and *Perk*^{-/-} MEFs with UV light, anisomycin and TNF- α and measured the levels of phosphorylated JNK and p38 MAPK. We found that the activation of JNK and p38 MAPK by UV, anisomycin and TNF- α was very similar between *Perk*^{+/+} and *Perk*^{-/-} MEFs (Figure 5). All three of these stresses led to a predicted strong activation of both p38 and JNK. These results indicate that the components involved in JNK and p38 MAPK activation are not generally defective in *Perk*^{-/-} MEFs, but rather PERK is required for their activation in the context of specific ER stresses. Phosphorylation of eIF2 α was induced in cells treated with UV but not in cells treated with anisomycin or TNF- α (Figure 5A). The GCN2 eIF2 α kinase has been shown to be responsible for phosphorylation of eIF2 α upon UV induction [43,44].

Only specific ER stresses can trigger PERK-dependent activation of JNK and p38 MAPK

In addition to thapsigargin, several other ER stress-inducing agents such as DTT and tunicamycin can also activate JNK [23,27]. To determine whether the PERK-dependent activation of stress-activated MAPKs is a universal consequence of ER stresses, we measured the levels of phosphorylated JNK and p38 MAPK in both *Perk*^{+/+} and *Perk*^{-/-} MEFs treated with DTT or tunicamycin. DTT treatment significantly stimulated a 2 fold PERK-dependent phosphorylation of eIF2 α within the first 30 min (Figure 6A); however, only modest stimulation of JNK and p38 MAPK phosphorylation was observed in the *Perk*^{+/+} MEFs (1.4 fold for JNK and 1.6 fold for p38 MAPK). The relatively low level of JNK and p38 MAPK phosphorylation induced by DTT in wild-type cells was not observed until after 2 h despite a large induction in eIF2 α phosphorylation after only 30 min. No induction of JNK and p38 MAPK phosphorylation

Figure 5 Effects of UV light, anisomycin and TNF- α on the phosphorylation of p38 MAPK and JNK in MEFs

Wild-type and PERK mutant MEFs were treated with UV light (A), anisomycin (B) and TNF- α (C) for the indicated time. Total and phosphorylated p38 MAPK, JNK and eIF2 α were measured by Western blot analysis of whole-cell extracts. Samples treated with TNF- α are presented in duplicate obtained from independent experiments

was observed in *Perk*^{-/-} MEFs (Figure 6A). Compared with other pharmacological reagents that can induce the UPR, tunicamycin triggered a relatively low level of eIF2 α phosphorylation in *Perk*^{+/+} MEFs (Figure 6B). Moreover, tunicamycin induced a modest level of JNK and p38 MAPK phosphorylation in both *Perk*^{+/+} and *Perk*^{-/-} cells.

In summary, among the three reagents that induce the UPR, only disruption of ER calcium homeostasis by treatment of thapsigargin led to a strong induction of JNK and p38 MAPK phosphorylation that was PERK-dependent and highly correlated with eIF2 α phosphorylation. By contrast, DTT elicited a high level of PERK-dependent eIF2 α phosphorylation but this was associated with a delayed and weak induction of JNK and p38 MAPK phosphorylation, whereas tunicamycin stimulated a higher level of JNK and p38 MAPK phosphorylation but was independent of PERK and was not correlated with eIF2 α phosphorylation.

DISCUSSION

The UPR stimulates the expression of ER resident chaperones to adapt to increased demands for protein folding in the ER. Because

Figure 6 Effects of DTT and tunicamycin on the phosphorylation of p38 MAPK and JNK

Wild-type and PERK mutant MEFs were treated with 10 mM DTT (A) or 10 μ g/ml tunicamycin (B). Total or phosphorylated p38 MAPK, JNK and eIF2 α were measured by Western blot analysis of whole-cell extracts. Each sample is presented in duplicate obtained from independent experiments.

of the central role the ER plays in the function and viability of the cell, ER perturbations also impact upon a diverse array of other cellular functions. Underlying the general cellular response to ER stress are the stress-activated kinase pathways and the induction of IE genes, which can also be activated by non-ER mediated stresses. We found that the ER-resident transmembrane kinase PERK is required for either the induction or maintenance of the induced state of *c-myc* and *egr-1* gene expression under specific ER stresses. Of the ER stresses tested, each differentially impacts the expression of *c-myc* and *egr-1*. When ER calcium homeostasis is disrupted via inhibition of the ER SERCA calcium pump by thapsigargin, *c-myc* is dramatically induced only in *Perk*^{+/+} MEFs. Thapsigargin treatment has an early stimulatory effect on *egr-1* expression in both wild-type and *Perk*^{-/-} mutant MEFs. At later time points, however, *egr-1* mRNA levels continue to increase in wild-type cells but dramatically decline in *Perk*^{-/-} cells resulting in a ten fold difference at 2 h. Similar complexity in response to two other UPR inducing reagents, DTT and tunicamycin was observed. PERK is required for the activation of *c-myc* upon DTT treatment but not upon tunicamycin treatment. However, after two h of tunicamycin treatment under our experimental condition, the expression of *c-myc* declined substantially in *Perk*^{-/-} MEFs but continued to increase in *Perk*^{+/+} MEFs. A similar phenomenon was observed for the expression of *egr-1* in response to thapsigargin and DTT treatment; *egr-1* expression radically declined in *Perk*^{-/-} MEFs in contrast with wild-type cells in which *egr-1* continued to increase or remain at high levels. Thus for most ER stresses, PERK is required either for the initial activation of IE gene expression or for maintaining the activated state.

The IE genes such as *egr-1* and *c-myc* play crucial roles in regulating both cell proliferation and apoptosis [45–47]. CHOP/GADD153, a mediator of apoptosis, is also induced as part of

the UPR and its transcription is in part regulated by PERK via translational control of ATF-4, a CHOP transcriptional activator [20]. PERK is required for the developmental regulation of IGF-1 [48] and ER-stress induced translocation of the nuclear factor- κ B transcription factor to the nucleus [38]. Thus PERK regulates an array of factors that control growth and adaptation to stress. PERK activity is also modulated under non-stressed conditions, as a function of stimulus-coupled secretion that elicits calcium mobilization from the ER (B. C. McGrath and D. R. Cavener, unpublished work). Under normal physiological conditions that stimulate secretion and ER calcium mobilization, PERK may regulate factors which in turn modulate secretory capacity in concert with changes in developmental and physiological demand for secretory proteins. This function may be particularly important in the major secretory cells of the pancreas and skeletal system which exhibit gross dysfunctions in mice and humans deficient in PERK [37,48,49].

A plethora of extracellular and intracellular stimuli can activate the IE genes suggesting that PERK may be required for only specific pathways which mediate responses related to ER stress. We found that inhibition of PLC significantly represses *c-myc* induction by thapsigargin. PLC catalyses the cleavage of phosphatidylinositol 4,5-bisphosphate to generate the messengers DAG (diacylglycerol) and IP₃ (inositol 1,4,5-trisphosphate). IP₃, in turn, is required to activate the ER IP₃ receptor which releases Ca²⁺ from the ER. We suggest that the induction of *c-myc* expression by thapsigargin requires the concerted action of inhibiting the SERCA, which normally pumps cytoplasmic Ca²⁺ into the ER, and activation of the IP₃ receptor, which releases Ca²⁺ from the ER. Alternatively, *c-myc* induction may instead depend upon any one of several signalling and regulatory functions impacted by the generation of IP₃ and DAG messengers.

In addition, we showed that thapsigargin-induced PERK-dependent expression of *c-myc* can be partially repressed by inhibitors of PI3K and p38 MAPK (Figure 3). PERK may be required to transduce signalling initiated by ER stress to multiple regulatory pathways which co-operate to regulate *c-myc* expression. Alternatively, PLC, PI3K and p38 MAPK may be functionally linked together in controlling *c-myc* expression. In support of the latter hypothesis, PLC- β has been shown to interact with both p38 MAPK and its upstream kinase, MAPK kinase 3 [50]. In addition, phosphorylation of p38 MAPK lies downstream of PI3K activation [51], suggesting a functional relationship exists between these proteins.

The stress-activated protein kinases JNK and p38 MAPK were previously shown to be induced by ER stress [23,24] and given our finding that *c-myc* induction by thapsigargin is partially dependent upon p38 MAPK activity, we investigated the potential role of PERK in regulating p38 MAPK and JNK. We found that PERK is indeed required for the activation of JNK and p38 MAPK upon treatment with thapsigargin, an inhibitor of the SERCA calcium pump. However, the activation of JNK and p38 MAPK by other ER stressors is independent or only partially dependent upon PERK. Moreover, the activation of JNK and p38 MAPK by extracellular and cytoplasmic stressors is independent of PERK. Thus PERK appears to play a restrictive role in regulating the activation of stress-activated kinases, JNK and p38 MAPK, upon disruption of ER Ca²⁺ homeostasis. For other ER stress-inducing reagents (e.g. DTT and tunicamycin) the activation of PERK and phosphorylation of eIF2 α is not required for the activation of JNK and p38 MAPK, with the possible exception of DTT which was shown to induce a modest and delayed phosphorylation of JNK and p38 MAPK only in *Perk*^{+/+} cells. Thus in general, activation of PERK and phosphorylation of eIF2 α is insufficient to activate JNK and p38 MAPK. We speculate that the release of

ER calcium stores upon thapsigargin treatment stimulates other calcium sensitive pathways, which act in concert with PERK to activate p38 and JNK. Other ER stressors, which directly disrupt protein folding or abrogate protein glycosylation, may lead to the activation of the stress-activated protein kinases by different pathways than those involved in perturbation of Ca²⁺ homeostasis.

The JNK and p38 MAPK pathways are particularly sensitive to disruption of intracellular Ca²⁺ signalling [51–56], and we propose that PERK plays an essential role to integrate ER-stress response and calcium-sensitive signalling pathways. In addition to its primary role in protein folding and quality control, the ER is also the major storage organelle for Ca²⁺. Mobilization of ER Ca²⁺ plays a fundamental role in regulating Ca²⁺ signalling cascades and exocytotic release of secretory granules [16]. Moreover, disruption of intracellular Ca²⁺ homeostasis or perturbation of Ca²⁺ signalling underlies a vast array of human diseases [14,15,57–59]. PERK may serve as an important cellular signalling mediator during the fluctuation of ER luminal calcium, which integrates the luminal function of protein folding and assembly with the dynamic physiological regulation of intracellular calcium.

It is shown that four ER transmembrane proteins – PERK, IRE1 α , IRE1 β and ATF6 – act in concert to mediate the UPR. The ER resident BiP/GRP78 negatively regulates each of these four proteins and consequently dissociation of BiP/GRP78 activates their function [10–13], suggesting that these four regulatory proteins could be activated simultaneously to activate the cascade of events comprising the UPR. One or more of these ER transmembrane proteins may functionally interact to mediate specific aspects of the UPR. For example, IRE1 has been shown to be required for the activation of JNK in MEFs upon treatment with thapsigargin and tunicamycin [23]. We show in the present study that thapsigargin, but not tunicamycin-induced activation of JNK is dependent on a functional PERK. Together, these findings suggest that PERK and IRE1 are both essential for the JNK activation upon disruption of ER Ca²⁺ homeostasis.

In conclusion, the PERK eIF2 α kinase is required to mediate the cellular responses to the disruption of ER Ca²⁺ homeostasis, and to a more limited extent, other ER stresses. We propose that these stress-related functions of PERK are an extension of the major role of PERK in regulating normal physiological processes mediated by Ca²⁺ mobilization and signalling. The direct downstream signalling components of PERK that mediate its ER-stress related functions remain to be discovered.

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