Pancreatic eukaryotic initiation factor-2α kinase (PEK) homologues in humans, *Drosophila melanogaster* and *Caenorhabditis elegans* that mediate translational control in response to endoplasmic reticulum stress

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In response to different cellular stresses, a family of protein kinases regulates translation by phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF-2α). Recently, we identified a new family member, pancreatic eIF-2α kinase (PEK) from rat pancreas. PEK, also referred to as RNA-dependent protein kinase (PKR)-like endoplasmic reticulum (ER) kinase (PERK) is a transmembrane protein implicated in translational control in response to stresses that impair protein folding in the ER. In this study, we identified and characterized PEK homologues from humans, *Drosophila melanogaster* and *Caenorhabditis elegans*. Expression of human PEK mRNA was found in over 50 different tissues examined, with highest levels in secretory tissues. In mammalian cells subjected to ER stress, we found that elevated eIF-2α phosphorylation was coincident with increased PEK autophosphorylation and eIF-2α kinase activity. Activation of PEK was abolished by deletion of PEK N-terminal sequences located in the ER lumen. To address the role of *C. elegans* PEK in translational control, we expressed this kinase in yeast and found that it inhibits growth by hyperphosphorylation of eIF-2α and inhibition of eIF-2B. Furthermore, we found that vaccinia virus K3L protein, an inhibitor of the eIF-2α kinase PKR involved in an anti-viral defence pathway, also reduced PEK activity. These results suggest that decreased translation initiation by PEK during ER stress may provide the cell with an opportunity to remedy the folding problem prior to introducing newly synthesized proteins into the secretory pathway.

Key words: eIF-2α, ER stress, protein kinase, protein synthesis, translation.

INTRODUCTION

In response to cellular stress, a family of related protein kinases regulates translation initiation by phosphorylation of eukaryotic initiation factor-2 (eIF-2) [1,2]. Composed of three subunits, eIF-2 associates with Met-tRNA$_i^{Met}$ and GTP and facilitates ribosomal selection of the start codon [3]. During this initiation process, GTP complexed with eIF-2 is hydrolysed to GDP. Phosphorylation of the α subunit of eIF-2 (eIF-2α) at Ser-51 reduces the function of the guanine-exchange factor, eIF-2B, that recycles eIF-2-GDP to the GTP-bound form required for subsequent rounds of translation initiation. Two well-characterized eIF-2α kinases were found to inhibit general translation in mammalian cells in response to different stress conditions. RNA-dependent protein kinase, PKR, is important for an anti-viral defence pathway mediated by interferon and is thought to control cell proliferation and apoptosis [4–8], and the haem-regulated inhibitor kinase reduces protein synthesis in erythroid tissues in response to hemin deficiency [9]. In the yeast *Saccharomyces cerevisiae*, the eIF-2α kinase GCN2 (general control non-derepressible-2) facilitates gene-specific translation in response to starvation for one of several different amino acids [1,10–12]. While the catalytic domains of each of these eIF-2α kinases share sequence and structural features, their flanking regulatory sequences are distinct, enabling each kinase to respond to different stress signals.

Recently, we identified a new member of this kinase family from rat pancreatic tissue, designated pancreatic eIF-2α kinase, or PEK [13]. As the name implies, PEK mRNA was found to be expressed at the highest levels in pancreas, although lower amounts were also detected in other rat organs. PEK immunoprecipitated from pancreas or islet cells was found to phosphorylate eIF-2α specifically on Ser-51 and addition of recombinant PEK to reticulocyte lysates resulted in a dose-dependent inhibition of translation [13]. Given that the expression of PEK mRNA is elevated in pancreas, a cell type containing an expanded endoplasmic reticulum (ER) network and specialized for protein secretion, it is inviting to consider a role for PEK in translational control in response to ER stress. Indeed, Harding et al. [14] reported that the mouse homologue of PEK, designated PERK (PKR-like ER kinase), is glycosylated and associated with the ER. The N-terminal sequences of mouse PERK are thought to be located in the lumen of the ER, with the kinase domain residing in the cytoplasm. Autophosphorylation activity of this transmembrane kinase was observed to be elevated in response to ER stress, brought about by the addition of tunicamycin, an inhibitor of protein glycosylation, or thapsigargin, which leads to release of calcium from the ER. These

Abbreviations used: eIF-2, eukaryotic initiation factor-2; eIF-2α, α subunit of eIF-2; ER, endoplasmic reticulum; PEK, pancreatic eIF-2α kinase; PKR, RNA-dependent protein kinase; PERK, PKR-like ER kinase; GCN2, general control non-derepressible-2; DmPEK, *Drosophila melanogaster* PEK homologue; CePEK, *Caenorhabditis elegans* PEK homologue; HsPEK, human PEK homologue; EST, expressed sequence tag; 5′-RACE, rapid amplification of cDNA 5′-ends; ORF, open reading frame; CMV, cytomegalovirus; GRP, glucose-regulated protein.

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The nucleotide sequences of human, *Drosophila melanogaster* and *Caenorhabditis elegans* pancreatic eukaryotic initiation factor-2α kinases have been submitted to GenBank under the corresponding accession numbers AF193339, AF193340 and AF193341.

observations suggest that this mouse homologue of PEK impairs protein synthesis in response to ER stress. Together, these reports suggest that PEK functions to reduce translation initiation in response to ER stress.

This study began with the initial characterization of a human cDNA, later observed to be the human homologue of PEK (HsPEK). HsPEK mRNA was found to be expressed in almost 50 different tissues, with highest levels in secretory tissues, including salivary gland, stomach and pituitary gland, as well as pancreas. We show that the eIF-2α kinase activity of PEK immunoprecipitated from cultured mammalian cells is elevated in response to ER stress. Deletion of the N-terminal sequences of PEK thought to be located in the lumen of the ER greatly reduced the catalytic activity of this eIF-2α kinase. In addition to characterizing HsPEK, we also analysed cDNA clones encoding PEK homologues from *Caenorhabditis elegans* (CePEK) and *Drosophila melanogaster* (DmPEK) and here we describe their sequence relationships. We show that recombinant CePEK phosphorylates eIF-2α at the Ser-51 regulatory site. CePEK expressed in yeast was found to inhibit growth. This reduced-growth phenotype was alleviated by substituting alanine for Ser-51 in eIF-2α, or by mutating eIF-2B so that the guanine-exchange factor was insensitive to inhibition by phosphorylated eIF-2α. We also found that expression of vaccinia virus K3L protein, an inhibitor of PKR, suppressed the growth defect associated with PEK. Together, these results indicate that PEK is expressed at elevated levels in a variety of mammalian secretory tissues, in addition to pancreas, and that this eIF-2α kinase facilitates translational control in response to ER stress in diverse metazoic organisms.

MATERIALS AND METHODS

Cloning of HsPEK cDNA

We searched for novel mammalian eIF-2α kinases in the GenBank database by using the sequences conserved among eIF-2α kinases and the presence of an insert that separates subdomains IV and V as a query, and the BLAST and BLAST2 programs with the default settings. An expressed sequence tag (EST) with the accession number F07171, corresponding to a clone from a human infant brain cDNA library, was identified in this search and obtained subsequently from the Lawrence Livermore National Laboratory (Livermore, CA, U.S.A.). The sequence of the 2.1-kb cDNA was determined by the dideoxy method.

To identify the 5′ portion of this cDNA sequence, several rounds of rapid amplification of cDNA 5′-ends (5′-RACE) were carried out using Marathon human brain and pancreas cDNA libraries (Clontech) and nested oligonucleotide primers. DNA products obtained from the nested PCR reactions were cloned into the pCR2.1 vector (Invitrogen) and amplified in *Escherichia coli*. Plasmid inserts were analysed by dideoxy sequencing of both strands. During the course of our isolation of the cDNA clones, several human EST sequences contributed to GenBank, including AA669109, AA419404, AA419589 and T18601, were found to be identical to portions of the HsPEK cDNA. Further extension of the 5′ portion of the cDNA could not be obtained directly, presumably because of the presence of GC-rich sequences in the 5′ region of the mRNA.

To obtain the 5′ end of the cDNA, a pBelo BACII library was screened for the *HsPEK* gene. A 0.6-kb DNA probe encoding residues 95–306 of the N-terminal portion of HsPEK was radiolabelled with ^{32}P by random-primed labelling and used in a hybridization screen of the BAC (bacterial artifical chromosome)-based library (Genome Systems). The isolated clones, designated 19031 and 19032, were sequenced directly to determine the 5′ portion of the HsPEK open reading frame (ORF).

Analysis of HsPEK mRNA levels by Northern blot and RNA master blot

Multiple-tissue and endocrine-system Northern blots containing 2 μg per lane of poly(A)$^+$ RNA from different human tissues were purchased from Clontech. An RNA master blot containing poly(A)$^+$ RNA from 50 different human tissues was also purchased from Clontech. The poly(A)$^+$ RNA samples on the master blot were normalized for equal sample amounts by probing for eight different 'housekeeping' genes. The master blot also included human genomic DNA and *E. coli* DNA among other negative-control samples. All three poly(A)$^+$ RNA human blots were analysed for PEK mRNA levels using a similar protocol. A 1.7-kb cDNA fragment encoding the kinase domain of HsPEK was radiolabelled with ^{32}P using a Random Primed Labelling Kit (Boehringer Mannheim) and used as a probe in the Northern- and master-blot analyses. Prehybridization and hybridization steps were carried out using ExpressHyb Hybridization Solution (Clontech) for 3 h at 68 °C. The filters were washed with a solution containing $0.1 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1 % SDS at 50 °C. Alternatively, a 260-bp cDNA probe encoding a portion of human ubiquitin was radiolabelled and used to probe the same three RNA blots. The blots were exposed to X-ray film using an intensifying screen at −80 °C. The relative levels of HsPEK and ubiquitin mRNAs in the different tissues were determined by measuring the band intensities in the Northern blots or the spot intensities in the RNA master blot in the autoradiograms using a Bio-Rad GS-670 imaging densitometer. Measurements were carried out with autoradiograms of different-length exposures to ensure the linearity of the measurements.

Bacterial expression of PEK

Recombinant PEKs were expressed in *E. coli* using the T7 promoter included in the pET vectors (Novagen). To express CePEK in *E. coli*, we obtained EST clones yk39f10 and yk427c7 from Dr Yuji Kohara (National Institute of Genetics, Mishima, Japan). These EST clones were used as templates in PCR reactions to generate a 2.0-kb DNA fragment encoding CePEK residues 480–1077 and a 3.3-kb fragment containing residues 26–1077. Both amplified DNA fragments were engineered to include an *Nde*I restriction site at their 5′ ends and a *Sal*I site at their 3′ ends. Following cleavage with *Nde*I and *Sal*I, the amplified CePEK DNA fragments were inserted between these sites in p242, a modified version of pET15b. Plasmid p513 expresses CePEK-Δ1-479 fused to an N-terminal polyhistidine sequence from an inducible T7 promoter and p552 encodes a fusion protein containing CePEK residues 26–1077. To express recombinant HsPEK for antibody production, we excised a 2.6-kb *Eco*RV–*Xho*I DNA fragment from EST clone T18601 (provided by Dr Graeme I. Bell, University of Chicago, Chicago, IL, U.S.A.). This DNA fragment encodes residues 588–1115 of HsPEK and the *Eco*RV restriction site is included in the HsPEK sequences, whereas the *Xho*I site at the 3′ end is of vector origin. The HsPEK DNA fragment was inserted between the *Eco*RV and *Xho*I sites of pET30c, generating p459, which encoded kinase sequences fused to an N-terminal polyhistidine tag.

E. coli strain BL21 (DE3) (F$^-$ *ompT* r_B^- m_B^- containing lysogen DE3) transformed with plasmids p459, p513 or p552 was

grown at either 30 or 37 °C in Luria–Bertani medium supplemented with 50 μg/ml kanamycin or 100 μg/ml ampicillin until mid-logarithmic phase at which point 1 mM isopropyl β-D-thiogalactoside was added to the culture and incubated for an additional 3 h. Cells were collected by centrifugation, washed in a solution of 20 mM Tris/HCl (pH 7.9) and 500 mM NaCl and then resuspended in solution A (20 mM Tris/HCl, pH 7.9, 500 mM NaCl and 10 % glycerol) and protease inhibitors (100 μM PMSF, 0.15 μM aprotinin, 1 μM leupeptin and 1 μM pepstatin) with 10 mM imidazole, followed by cell lysis using a French press. Lysates were clarified by centrifugation at 39000 ***g*** and applied on to a column containing nickel-chelation resin (Qiagen, Hilden, Germany) and polyhistidine-tagged PEK protein was eluted with solution A containing elevated concentrations of imidazole. CePEK was assayed for phosphorylation *in vitro* of eIF-2α and recombinant HsPEK was used for antibody production in rabbits. HsPEK was combined with Freund's complete adjuvant and injected intradermally, followed by multiple rounds of subcutaneous injection using HsPEK and Freund's incomplete adjuvant.

Transfection of HsPEK cDNA into cultured 293T cells

HsPEK cDNAs were inserted downstream of the CMV (cytomegalovirus) promoter in plasmid pcDNA3 (Invitrogen). Plasmid p578 encoded wild-type HsPEK in the *Xho*I site of pcDNA3, p579 encoded HsPEK-K621M and p580 contained PEK-Δ2-582. 293T human embryonic kidney cells grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum in 100-mm dishes were transfected with 2.5 μg of plasmid DNA encoding different versions of HsPEK or vector alone using 18 μl of Lipofectamine (Life Sciences). After transfection (40 h), cells were exposed to thapsigargin for 1 h, harvested and lysed in RIPA buffer supplemented with protease inhibitors.

eIF-2α kinase assay *in vitro*

Cultured NIH 3T3 murine fibroblast cells were grown in the absence of ER stress or exposed to 1 μM thapsigargin or 5 μM A23187 for 1 h, harvested and lysed in RIPA buffer (50 mM Tris/HCl, pH 7.9, 150 mM NaCl, 1.0 % Nonidet P40, 0.5 % sodium deoxycholate and 0.1 % SDS) in the presence of protease inhibitors. Cell lysates were precleared by the addition of Protein A–Sepharose and centrifugation at 4 °C. Anti-PEK polyclonal antibody was added to the lysate, and PEK was immunoprecipitated with Protein A–Sepharose. The PEK immunocomplex was rinsed twice with RIPA buffer supplemented with protease inhibitors, followed by two washes with kinase buffer (20 mM Tris/HCl, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$ and 5 mM β-mercaptoethanol) supplemented with protease inhibitors. Immunoprecipitated PEK was incubated in a 25-μl solution of kinase buffer, protease inhibitors, recombinant eIF-2α and 10 μCi of [γ-^{32}P]ATP in a final concentration of 10 μM ATP at 30 °C for 2 and 4 min as indicated. Reactions were terminated with the addition of an equal volume of 2 × SDS sample buffer, followed by heating at 95 °C. Phosphorylated proteins were analysed by electrophoresis on an SDS/polyacrylamide (12.5 %) gel, followed by autoradiography. The relative levels of substrate phosphorylation were determined by measuring the band intensities in the autoradiograms using a Bio-Rad GS-670 imaging densitometer. Measurements were carried out with autoradiograms of different-length exposures to ensure the linearity of the measurements.

Phosphorylation of eIF-2α by recombinant proteins containing the kinase domain of CePEK was carried out as described previously [15]. The reaction volume of 20 μl included 1 μg of yeast eIF-2α, 10 μCi of [γ-^{32}P]ATP in a final concentration of 60 μM ATP and CePEK (25 or 50 ng) or 1 μg of yeast cell lysate expressing PKR. CePEK and PKR reaction mixtures were incubated for 2 and 20 min, respectively, at 30 °C. Phosphorylated proteins were analysed by electrophoresis on an SDS/polyacrylamide (12.5 %) gel, followed by autoradiography. In these assays, phosphorylation of eIF-2α by each of these kinases was dependent upon the presence of Ser-51. However, in kinase reactions containing higher concentrations of CePEK, such as 400 ng/20 μl, and which were incubated for 10 min or longer, we detected significant amounts of phosphorylation of mutant eIF-2α-S51A substrate.

Immunoblot analysis of PEK and eIF-2α

Lysates prepared from *E. coli* or cultured mammalian cells were separated by electrophoresis on an SDS/polyacrylamide gel and transferred on to nitrocellulose filters. Filters were blocked in a TBS-T solution containing 20 mM Tris/HCl (pH 7.6), 150 mM NaCl, 0.1 % Tween 20 and 4 % non-fat dry milk. To measure HsPEK protein levels, we incubated the filters in a TBS-T solution containing rabbit polyclonal antibody prepared against HsPEK. Filters were washed in TBS-T and PEK–antibody complex was detected using horseradish peroxidase-labelled secondary antibody and chemiluminescent substrate. Because our polyclonal antibody prepared against HsPEK did not recognize CePEK, we used rabbit polyclonal antibody that recognized the polyhistidine tag (Pierce) of the recombinant CePEK. The polyhistidine-specific antibody was conjugated directly to horseradish peroxidase and recognized using chemiluminescent substrate.

Immunoblots measuring eIF-2α phosphorylation were carried out with lysates prepared from yeast or cultured mammalian cells separated by SDS/PAGE. Yeast lysates were prepared as described in [16] and mammalian cell lysates were prepared as summarized above for immunoprecipitation kinase assays with the addition of 50 mM NaF and 40 mM β-glycerophosphate. Phosphorylated eIF-2α was visualized using affinity-purified antibody that specifically recognized eIF-2α phosphorylation at Ser-51, which was provided kindly by Dr Gary Krause (Wayne State University, Detroit, MI, U.S.A.) [17]. Total eIF-2α in yeast lysates was detected by immunoblot using rabbit polyclonal antibody prepared against a polyhistidine-tagged version of yeast eIF-2α expressed and purified from *E. coli*. To carry out immunoblots containing mammalian lysates, we utilized monoclonal antibody that recognized both phosphorylated and non-phosphorylated forms of eIF-2α [18], provided generously by Dr Scot Kimball (Pennsylvania State University, College of Medicine, Hershey, PA, U.S.A.), followed by anti-mouse secondary antibody that was conjugated to horseradish peroxidase.

Expression of CePEK in yeast

To express CePEK in *S. cerevisiae*, PCR was used to amplify the CePEK coding sequences from residues 26–1077 using EST clone yk427c7 as the DNA template. Engineered into the 5′ end of the CePEK PCR product were an *Xma*CI and an *Nde*I restriction site, which included an initiation codon followed by six histidine codons. In addition, a *Sal*I site was included at the 3′ end of the PCR product. The CePEK DNA was inserted between the *Xma*CI and *Sal*I sites of pEMBLyex4 [19], resulting in plasmid p551. Plasmid p551 encoding CePEK, p434 including human PKR in pEMBLyex4 or vector pEMBLyex4 alone were introduced into an isogenic set of yeast strains including H1816 (*MAT*a *ura3-52 leu2-3 leu2-112 gcn2*Δ *sui2*Δ *trp1*Δ-63 [*GCN4-*

lacZ, *TRP1*] at *trp1*Δ-63, p1097[*SUI2 LEU2*]), H1817 (*MAT*a *ura3-52 leu2-3 leu2-112 gcn2*Δ *sui2*Δ *trp1*Δ-63 [*GCN4-lacZ*, *TRP1*] at *trp1*Δ-63, p1098[*SUI2-S51A LEU2*]), H1894 (*MAT*a *ura3-52 leu2-3 leu2-112 gcn2*Δ *trp1*Δ-63) [20] and GP3299 (*MAT*a *ura3-52 leu2-3 leu2-112 gcn2*Δ *gcd2*Δ::hisG, pAV1033[*GCD2-K627T*, *TRP1*]) containing the *GCD2-K627T* mutant allele [21]. Plasmid-containing strains were selected for by uracil prototrophy. Strain H1894 containing p551 expressing CePEK was transformed with p348 encoding the vaccinia virus K3L protein expressed from the *ADH1* promoter, p349 containing the *K3L* gene inserted into pYCDE2 [22] in the opposite orientation, such that the K3L antisense RNA was expressed from the *ADH1* promoter, or pYCDE2 vector alone. Yeast cells were grown to saturation in synthetic medium [23] supplemented with 2 % glucose, 2 mM leucine, 0.5 mM isoleucine and 0.5 mM valine (SD medium), diluted to either $A_{600} = 0.05$ or $A_{600} = 0.005$ and 8 μl of the culture was spotted on to agar plates containing SD medium or synthetic medium containing 10 % galactose, 2 % raffinose and the required amino acids (SGal medium). Agar plates were incubated for 4 days at 30 °C and photographed.

RESULTS

Identification of the human PEK homologue

This study originated with a search for novel eIF-2α kinases by using the BLAST program and a query containing sequences conserved among the eIF-2α kinase family. We initially identified an EST clone F07171 as a candidate in this search. Sequence analysis of the 2.1-kb insert in this EST clone revealed sequences highly similar to subdomains V–XI of the eIF-2α kinase catalytic domain, followed by 3′-untranslated sequences 1010 nucleotides in length that ended in a poly-A tract. Utilizing 5′-RACE, we obtained a larger portion of this cDNA with a combined length of 4.2 kb. Concurrently with this project, a screen for new threonine kinases in pancreatic tissue using anti-phosphothreonine antibodies and a cDNA expression library derived from rat pancreatic islets isolated a full-length cDNA that was found to encode a new eIF-2α kinase designated PEK [13]. Comparisons between rat PEK and the newly isolated human cDNA using the BLAST2 program revealed 87 % sequence identity over their entire length, indicating that we had isolated the human homologue of PEK (Figure 1). Despite repeated attempts using the 5′-RACE method, we failed to obtain the 5′ portion of HsPEK, which in the alignment with the rat homologue would encode an additional 65 N-terminal residues.

To obtain the 5′ portion of the HsPEK coding region, we first isolated BAC genomic clones that contained the *HsPEK* gene. By direct sequencing of the BAC isolates using HsPEK-specific oligonucleotide primers, we found a putative N-terminal coding region that closely matched that of rat PEK (Figure 1). Combining the cDNA and gene sequences, the HsPEK ORF is 1115 residues in length with a predicted molecular mass of 125000 Da. The kinase domain of HsPEK is 525 residues in length and like its rat counterpart contains a 223-residue sequence inserted between subdomains IV and V (Figure 1). This insert is characteristic of eIF-2α kinases and ranges in size between 35 residues in length in PKR to 550 residues in PfPk4 from *Plasmodium falciparum* [24,25]. HsPEK contains a 590-residue N-terminal region juxtaposed to its kinase domain. Within these sequences there is a predicted transmembrane region that is thought to be important for PEK localization in the ER (Figure 1). The *HsPEK* gene was mapped using the sequence-tagged site marker WI-6863 and was designated Hs.102506, located on chromosome 2 in the 111-114 cM genetic-map position. Recently, the sequence of HsPEK cDNA was published by Shi et al. [26] and was found to be identical to the HsPEK cDNA that we had isolated.

Levels of HsPEK are elevated in pancreas and other selected secretory tissues

Similar amounts of poly(A)$^+$ RNA extracted from different human tissues were analysed by Northern blotting for HsPEK transcript levels. A radiolabelled DNA probe encoding the kinase domain of HsPEK was found to hybridize to a transcript of about 5.5-kb in length (Figure 2, top panel). Whereas the HsPEK mRNA was detected in each of the eight tissues analysed, pancreas was found to have the highest level of transcript, with about six times that found in heart. Following this analysis, ubiquitin mRNA levels were measured in the blot as a standard to ensure that similar amounts of poly(A)$^+$ RNA were present in each lane (Figure 2, bottom panel).

To address whether HsPEK mRNA is elevated in other tissues, we carried out a Northern-blot analysis using poly(A)$^+$ RNA extracted from 50 different human tissues and applied it to a filter using a dot-blot apparatus. We found HsPEK transcript in each of the adult and fetal human tissues, whereas no radiolabelled probe was found to hybridize to the control RNA samples prepared from yeast or *E. coli* (Figure 3A). We carried out a second hybridization analysis using the same blot and a radiolabelled ubiquitin DNA probe (Figure 3B). The relative levels of HsPEK and ubiquitin transcripts in the different tissues were quantified by measuring the spot intensities in the autoradiograms by densitometry. The ratios of HsPEK mRNA to ubiquitin transcripts for each tissue were determined and normalized to heart. In this dot-blot analysis we again measured the highest levels of HsPEK transcript in pancreas, with 4.7 times that measured in heart (Figure 3B, dot C1 versus D3). Other tissues with elevated HsPEK mRNA levels, and their relative amounts compared with heart, include salivary gland, 4.1; stomach, 3.4; pituitary gland, 3.2; adrenal gland, 2.5; thyroid, 2.3; and prostate, 2.3. Among major human fetal organs, HsPEK mRNA was highest in brain, heart and spleen.

To test further whether HsPEK 5.5-kb transcript levels are elevated in the aforementioned tissues, we carried out a Northern-blot analysis using poly(A)$^+$ RNA prepared from selected human endocrine tissues that was separated by gel electrophoresis. The 5.5-kb HsPEK mRNA was detected in each of the tissues analysed, with highest levels detected in pancreas, stomach and thyroid (Figure 4).

Activation of PEK catalytic activity in response to ER stress

Previously, using bandshift analysis and orthophosphate metabolic labelling, it was shown that PEK autophosphorylation was enhanced in response to ER stress [14]. To address whether the eIF-2α kinase activity of PEK is induced in response to ER stress, we cultured NIH 3T3 mouse fibroblast cells in the presence or absence of ER stress. ER stress was elicited by treating the cells with thapsigargin or A23187. Cells were collected, lysed and PEK was immunoprecipitated using our rabbit polyclonal antibody prepared against HsPEK. PEK in the immunocomplex was incubated with [γ-^{32}P]ATP and recombinant eIF-2α. Radiolabelled proteins were separated by SDS/PAGE, followed by autoradiography (Figure 5). In the presence of either A23187 or thapsigargin, phosphorylation of eIF-2α substrate was increased 3- and 4-fold, respectively, compared with the reactions containing PEK prepared from cells not subjected to ER stress.

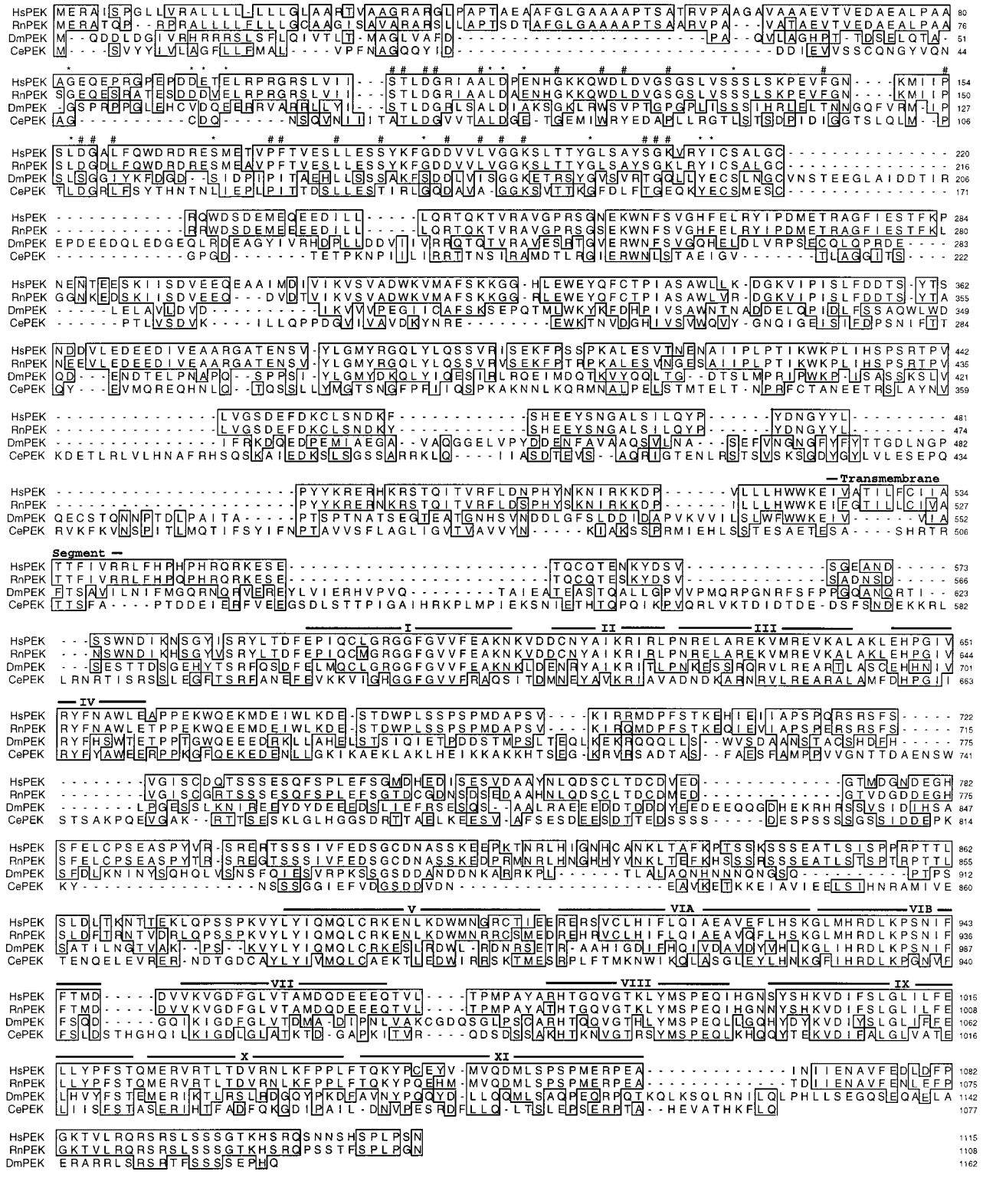


Figure 1 Comparison of PEK from humans with homologous sequences from rat, *D. melanogaster* and *C. elegans*

Sequence alignments were compiled in part using the BLAST2 program. Amino acid residues that are identical between the PEK homologues are boxed, and gaps in the alignment are represented by dashes. The predicted transmembrane segment [13] and kinase subdomains [55] are indicated by bars above the alignment. Positions of residues are listed on the right. # and * above the PEK sequences represent residues that are identical between *C. elegans* IRE1 aligned with two or more PEK homologues; # shows that this residue is also conserved in an alignment with human IRE1.

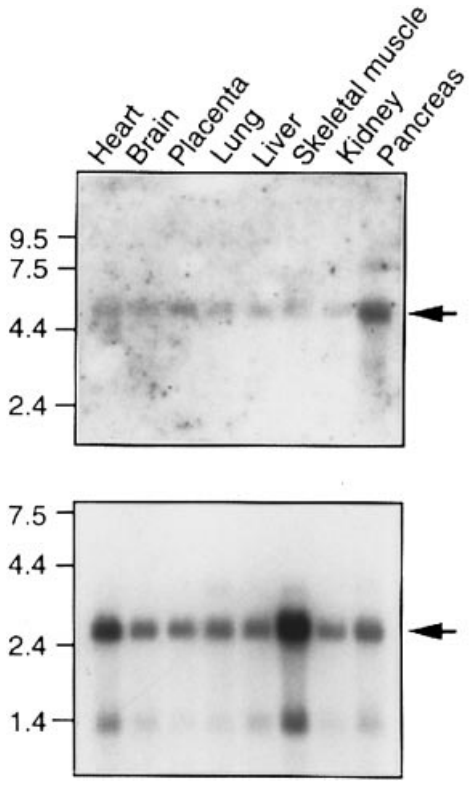


Figure 2 Expression of HsPEK mRNA in several different human tissues

A Northern-blot analysis was carried out using 2 μg of poly(A)$^+$ RNA prepared from each of the indicated human tissues. (Top panel) A radiolabelled HsPEK cDNA probe was incubated with filter-bound RNA and, following washing steps using high-stringency conditions, the filter blot was exposed to autoradiography. The arrow indicates the 5.5-kb HsPEK mRNA. (Bottom panel) A similar hybridization analysis was carried out using the same blot filter and a radiolabelled probe encoding human ubiquitin. Sizes of RNA standards (kb) are shown on the left. Relative levels of HsPEK mRNA, normalized for ubiquitin transcript levels, were measured by densitometry. HsPEK transcript levels in pancreas were six or more times than those measured in the other tissues.

Minimal phosphorylation of the mutant eIF-2α-S51A substrate was detected and no PEK activity was found in immunoprecipitations prepared using preimmunization rabbit serum. In parallel, we measured levels *in vivo* of eIF-2α phosphorylation using polyclonal antibody specific to eIF-2α phosphorylated at Ser-51 in an immunoblot assay. Coincident with this elevated PEK catalytic activity, we found that NIH 3T3 cells treated with either thapsigargin or A23187 had significantly elevated eIF-2α phosphorylation compared with cells not subjected to ER stress (Figure 5C). Similar levels of eIF-2α protein were present in the lysate preparations as judged by immunoblot using monoclonal antibody that recognized both phosphorylated and non-phosphorylated forms of this translation-initiation factor. To address whether activation of PEK is elevated in response to ER stress in other cell types, we immunoprecipitated PEK from AR42J cells, a rat exocrine pancreas tumour line [27] treated in the presence or absence of A23187. Again we found that the eIF-2α kinase activity of PEK from AR42J cells was increased significantly in response to ER stress (results not shown).

N-terminus of PEK facilitates phosphorylation of eIF-2α

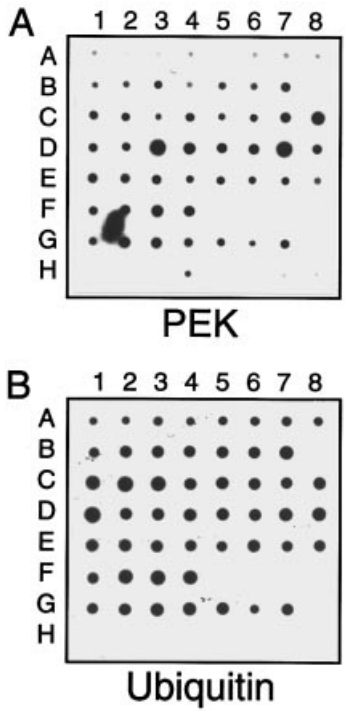
To understand better the regulation of PEK activity in response to ER stress, we expressed wild-type and mutant forms of PEK in 293T human embryonic kidney cells using the CMV promoter in plasmid pcDNA3 (Invitrogen). The 293T cells are readily transfected and express the large T antigen, enhancing replication of the simian virus 40-origin-based pcDNA3. Following the transfection procedure (40 h), cells were subjected to thapsigargin treatment for 1 h, lysed and levels *in vivo* of eIF-2α phosphorylation were measured using polyclonal antibody specific to the phosphorylated initiation factor (Figure 6). In the vector-transfected cells there was elevated phosphorylation of eIF-2α in response to ER stress. By comparison, over-expression of wild-type PEK led to high levels of eIF-2α phosphorylation that were independent of thapsigargin exposure (Figure 6D).

Consistent with the findings of Harding et al. [14], our immunoblot analysis of PEK levels in cells transfected with vector alone showed higher-molecular-mass forms of PEK in response to ER stress (Figure 6A). It was found that this retarded migration was alleviated by protein phosphatase treatment [14], suggesting that PEK autophosphorylation at multiple residues is coincident with activation of this eIF-2α kinase. In cells over-expressing wild-type PEK, multiple higher-molecular-mass forms of the eIF-2α kinase were observed by immunoblot using lysates prepared in the absence of thapsigargin treatment. Upon ER stress, over-expressed wild-type PEK migrated uniformly as a higher-molecular-mass band (Figure 6B). Endogenous PEK from vector-transfected cells was also immunoprecipitated using our polyclonal antibody prepared against this kinase and incubated with [γ-^{32}P]ATP and eIF-2α substrate. We found a modest increase in PEK and eIF-2α phosphorylation in response to ER stress. The catalytic activity of over-expressed PEK in the immunoprecipitation assay were substantially elevated in the presence or absence of ER stress. Harding et al. [14] also found that over-expressed PEK led to higher levels of autokinase activity independent of ER stress.

We also transfected expression plasmids encoding the kinase-defective PEK-K621M and PEK-Δ2-582, containing only the kinase catalytic domain, into the 293T cells. Both mutant versions of PEK were impaired for phosphorylation of eIF-2α in the immunoprecipitation assay (Figure 6C). Immunoblot analysis indicated that there were similar levels of transfected wild-type and mutant versions of PEK, with PEK-Δ2-582 migrating as a single band with a molecular mass of 66000 Da, consistent with that predicted from the DNA sequence (Figure 6A). Similarly, PEK-K621M migrated as a single species with a molecular mass similar to endogenous PEK from vector-transfected cells grown in the absence of ER stress. We conclude that the N-terminus of PEK is required for catalytic activity. Additionally, these results support further the idea that the migration shift of PEK is the result of autophosphorylation coincident with induced PEK catalytic activity in response to ER stress. Phosphorylation of eIF-2α in 293T cells expressing PEK-K621M was increased in response to ER stress, suggesting that this version of PEK does not function significantly as a dominant-negative mutant (Figure 6D). In the case of PEK-Δ2-582, there were modest levels of phosphorylation *in vivo* of eIF-2α independent of ER stress. We interpret this observation to suggest that PEK-Δ2-582 retains some low-level eIF-2α kinase activity that is not subject to regulation by ER stress. Alternatively, PEK-Δ2-582 may phosphorylate endogenous PEK, contributing to elevated eIF-2α phosphorylation in the absence of ER stress.

PEK homologues in *D. melanogaster* and *C. elegans*

Comparisons between HsPEK and sequences in the GenBank database using the BLAST program indicated that this eIF-2α



C

	1	2	3	4	5	6	7	8
A	whole brain 1.2	amygdala 0.8	caudate nucleus 0.4	cere-bellum 0.4	cerebral cortex 0.8	frontal lobe 0.7	hippo-campus 0.7	medulla oblongata 0.6
B	occipital lobe 0.6	putamen 0.7	sub-stantia nigra 1.5	temporal lobe 0.6	thalamus 1.2	sub-thalamic nucleus 0.6	spinal cord 1.0	
C	heart **1.0**	aorta 0.8	skeletal muscle 0.4	colon 1.6	bladder 0.9	uterus 1.5	prostate 2.3	stomach 3.4
D	testis 0.9	ovary 1.4	pancreas 4.7	pituitary gland 3.2	adrenal gland 2.5	thyroid gland 2.3	salivary gland 4.1	mammary gland 1.1
E	kidney 2.1	liver 1.5	small intestine 2.0	spleen 1.6	thymus 1.6	periph-eral leukocyte 1.1	lymph node 1.2	bone marrow 0.9
F	appendix 2.3	lung 1.4	trachea 1.9	placenta 1.7				
G	fetal brain 2.5	fetal heart 2.1	fetal kidney 1.6	fetal liver 0.9	fetal spleen 2.1	fetal thymus 0.9	fetal lung 1.9	
H	yeast total RNA	yeast tRNA	*E. coli* rRNA	*E. coli* DNA	Poly r(A)	human cot1 DNA	human DNA	human DNA

Figure 3 RNA dot-blot showing expression of HsPEK transcript in all human tissues characterized, with highest levels in secretory cell types

(**A**) An RNA dot-blot containing poly(A)$^+$ RNA prepared from 50 different human tissues was applied to a filter in an 8×8 grid. The filter was incubated with a radiolabelled HsPEK cDNA probe, and after high-stringency washing the filter was exposed to autoradiography. (**B**) To normalize the amounts of poly(A)$^+$ RNA in each dot, we carried out a similar hybridization analysis using a radiolabelled ubiquitin cDNA probe. The resulting autoradiograph is illustrated. (**C**) Key listing the human tissue used for each RNA preparation, and nucleic acid controls. Included are the relative levels of HsPEK transcripts, normalized for ubiquitin mRNA levels, determined for each tissue sample. Levels of HsPEK mRNA are presented relative to heart (grid reference C1).

kinase was also represented in *C. elegans* and *D. melanogaster*. The *PEK* gene in *C. elegans* was included in two overlapping cosmids designated F08B12 and F46C3, with accession numbers Z68104 and Z66563, respectively. To delineate the exon sequences of the *CePEK* gene, we identified an EST cDNA clone yK427c7 in GenBank that contained sequences corresponding to the predicted N-terminal portion of CePEK. We determined the CePEK sequence in the yK427c7 clone and found that it encoded an ORF that was 1051 residues in length, extending from the 5′ end of the insert. However, based on alignments between this ORF and the *CePEK* gene and mammalian PEK homologues, it appeared that the extreme N-terminal portion of the eIF-2α kinase was missing. Using the *CePEK* gene sequence to extend this ORF, we found a presumptive initiation codon 25 positions upstream of the sequences aligned with the 5′ end of the cDNA ORF. These sequences were included in the first exon of the *CePEK* gene as predicted by the program Genefinder. Together, the CePEK ORF is 1077 residues in length, with a predicted molecular mass of 120000 Da (Figure 1). Comparisons between the *C. elegans* and human homologues using the BLAST2 program revealed 25% sequence identity extending over nearly 1000 residues with a sum probability of random correspondence of $1e^{-71}$.

Several EST entries in the GenBank database included probable coding sequences for DmPEK. One EST cDNA clone, accession number AA439740, which contained sequences related to the N-terminus of PEK, was selected for further characterization. We determined the sequence of the entire insert of this cDNA clone, 4.2 kb in length, which included an 1162-residue ORF with a predicted molecular mass of 131000 Da (Figure 1). Sequences 5′ to this ORF contain an in-frame termination codon, indicating that the entire coding region was obtained. The 3′-untranslated region was 466 nucleotides in length and ended in poly-A sequences. The *D. melanogaster* ORF was 32% identical over the entire length of HsPEK, with a $1e^{-130}$ sum probability of random correspondence between the two sequences. We conclude that this ORF represents DmPEK.

A multi-sequence alignment between HsPEK and the rat, *D. melanogaster* and *C. elegans* counterparts revealed the greatest extent of identity between the kinase domains, with more divergence in the N-terminal regions (Figure 1). Within their kinase domains, each PEK homologue contained a large insert between subdomains IV and V, ranging in size from 208 residues in CePEK to 223 residues in the mammalian eIF-2α kinases. Following kinase subdomain XI, the C-terminal sequences of DmPEK and CePEK become quite dissimilar compared with HsPEK. There are also similarities between the PEK homologues dispersed over 570 residues N-terminal to the human kinase domain. The greatest sequence identities are concentrated between residues 100 and 350 of HsPEK (Figure 1). Interestingly, a portion of this N-terminal region of PEK, extending from about residues 100–220 of HsPEK, also shares sequence features with IRE1 (a mammalian homologue of inositol-requiring 1 from yeast), a transmembrane protein that functions in ER stress response pathway [28,29]. This is illustrated by the 29% sequence identity between this region of CePEK and *C. elegans* IRE1, predicted from the genome project, with a sum probability of random correspondence between the pairwise segments of $8e^{-5}$.

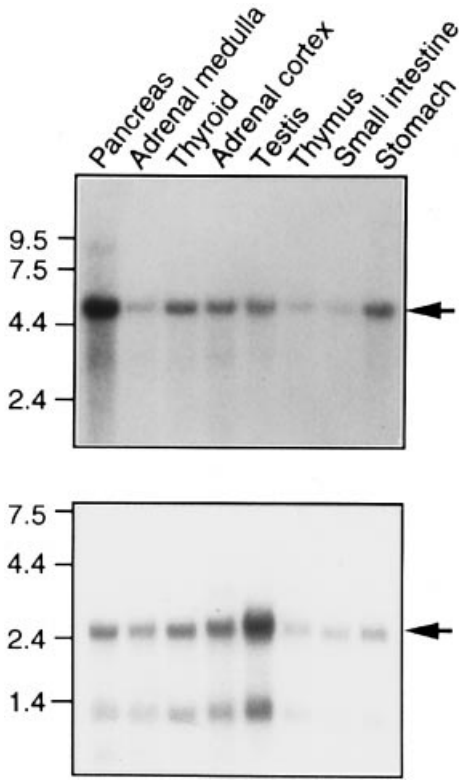


Figure 4 Expression of 5.5-kb HsPEK mRNA in endocrine tissues including pancreas

A Northern blot containing poly(A)$^+$ RNA purified from the indicated human tissues was hybridized with a ^{32}P-labelled DNA probe encoding the kinase domain of HsPEK. Following washes under high-stringency conditions, the HsPEK mRNA was visualized by autoradiography (top panel). The large arrow indicates a HsPEK transcript 5.5 kb in length. In the bottom panel is an autoradiogram derived from a similar hybridization analysis using the identical Northern blot and a radiolabelled probe encoding human ubiquitin. Sizes of RNA standards in kb are illustrated on the left. Relative levels of HsPEK mRNA, normalized for ubiquitin transcript levels, were measured by densitometry. Levels of HsPEK transcripts measured in each tissue relative to testis are as follows: pancreas, 7 times; stomach and thyroid, 3 times; and adrenal medulla, adrenal cortex, thymus and small intestine were similar to that measured in testis.

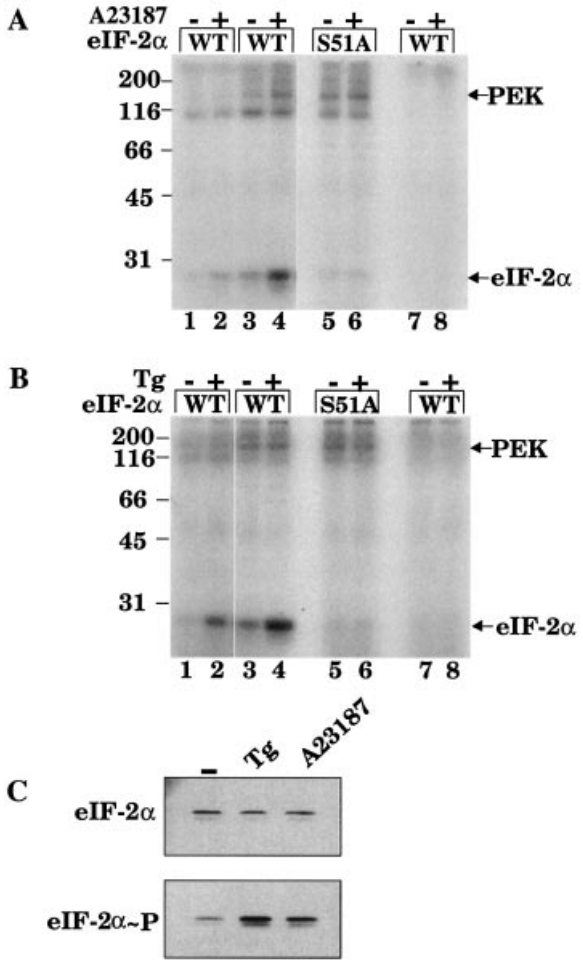


Figure 5 Activation of PEK catalytic activity in response to ER stress

Lysates were prepared from NIH 3T3 murine fibroblast cells that were subjected to ER stress by treatment with 5 μM A23187 or 1 μM thapsigargin (Tg) for 1 h. PEK was immunoprecipitated using rabbit polyclonal antibody specific to this eIF-2α kinase and incubated with [γ-^{32}P]ATP and recombinant eIF-2α. Radiolabelled proteins were separated by SDS/PAGE, followed by autoradiography (**A** and **B**). Kinase reactions represented in lanes 1–6 were prepared using polyclonal antibody specific to PEK, with lanes 1–4 containing wild-type eIF-2α substrate (WT) and lanes 5 and 6 including mutant eIF-2α-S51A (S51A). Reactions included in lanes 7 and 8 contain immunoprecipitants prepared using preimmune serum and wild-type eIF-2α substrate. Lanes 1 and 2 represent kinase reactions incubated for 2 min and lanes 3 and 4 were incubated for 4 min. Lysates prepared from cells grown in the presence of ER stress are indicated by $+$; those in the absence of stress by $-$. Phosphorylated PEK and eIF-2α are indicated by arrows. Molecular-mass markers are listed in kDa. (**C**) Lysates prepared from NIH 3T3 cells grown in the presence or absence of ER stress were analysed by immunoblot using polyclonal antibody that specifically recognized eIF-2α phosphorylated at Ser-51 (eIF-2$\alpha \sim$ P), or with monoclonal antibody that recognized total eIF-2α protein.

PEK homologue from *C. elegans* phosphorylates eIF-2α at Ser-51 and controls translation in the yeast model system

To date, there has been no reported characterization of an eIF-2α kinase from *C. elegans*. We expressed two forms of CePEK in *E. coli*. In the first, CePEK sequences from residues 26–1077 were introduced into a pET expression vector, generating a fusion protein with N-terminal polyhistidine sequences. The second involved similar expression of CePEK residues 480–1077. Recombinant CePEK proteins were visualized by immunoblot analysis using polyclonal antibody specific to the polyhistidine tag. In close agreement with the molecular mass predicted for the CePEK ORF, we found that the extended version of CePEK and CePEK-Δ1-479 had molecular masses of 115000 and 72000 Da, respectively (Figure 7, top panel). Because the longer form of CePEK was insoluble in *E. coli*, we selected to purify the recombinant protein containing CePEK residues 480–1077 using nickel-chelation resin. Following a similar regimen described for the human homologue, we found that CePEK phosphorylated eIF-2α, whereas minimal phosphorylation of the mutant eIF-2α-S51A was detected (Figure 7, bottom panel). Similar phosphorylation specificity was observed for a control reaction containing human PKR. We conclude that CePEK expressed and purified from *E. coli* can phosphorylate eIF-2α at Ser-51 even in the absence of a large portion of its N-terminal sequences.

S. cerevisiae has proven to be an important model system for studying the role of eIF-2α kinases in the regulation of translation. Numerous studies reported that expression of PKR in yeast cells deleted for endogenous GCN2 can lead to slow growth due to hyperphosphorylation of eIF-2α [15,20,30]. To address whether CePEK can mediate translational control in yeast, we expressed the *C. elegans* kinase in strain H1816 (*gcn2*Δ)

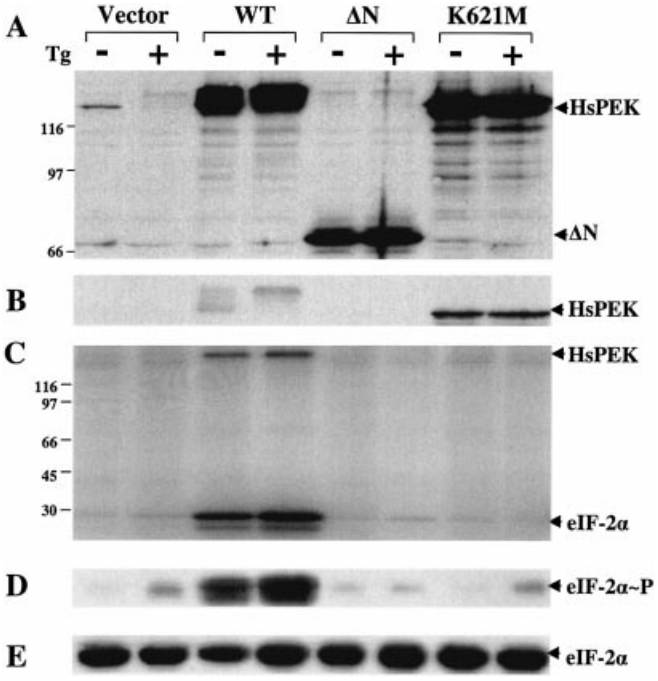


Figure 6 Requirement of the PEK ER luminal portion for kinase activity

Wild-type PEK (WT), PEK-K621M (K621M), PEK-Δ2-582 (ΔN) or vector alone were expressed in 293T human embryonic kidney cells using the CMV promoter. After transfection (40 h), cells were treated in the presence (+) or absence (−) of 1 μM thapsigargin (Tg) for 1 h. (**A**) PEK levels were measured by immunoblot using polyclonal antibody specific to this eIF-2α kinase. (**B**) To visualize the molecular masses of expressed wild-type PEK and PEK-K621M, the autoradiogram was re-exposed for a shorter length of time. (**C**) PEK was immunoprecipitated from 293T lysates, incubated with [γ-^{32}P]ATP and recombinant eIF-2α at 30 °C for 5 min. Radiolabelled proteins were separated by SDS/PAGE and visualized by autoradiography. Molecular-mass markers are indicated in kDa. Lysates were analysed by immunoblot using polyclonal antibody that specifically recognized eIF-2α phosphorylated at Ser-51 (eIF-2α ~ P; **D**), or with monoclonal antibody that recognized total eIF-2α protein (**E**).

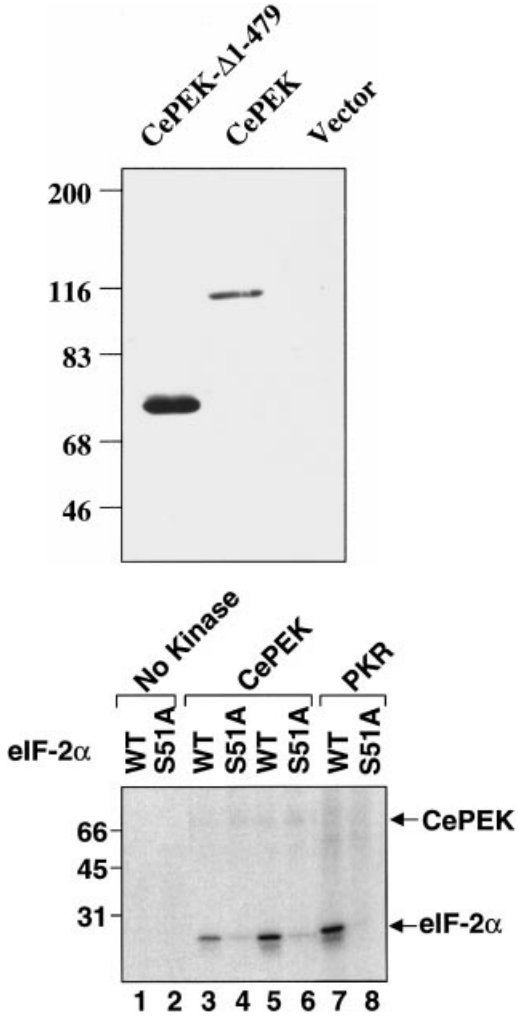


Figure 7 Phosphorylation of eIF-2α specifically at Ser-51 by CePEK

(Top panel) Immunoblot analysis of CePEK expressed in *E. coli*. CePEK residues 26–1077 (CePEK) or residues 480–1077 (CePEK-Δ1-479) were expressed as a polyhistidine fusion protein using the T7 promoter system in *E. coli*. Lysates were separated by electrophoresis in an SDS/polyacrylamide gel, and recombinant CePEK protein was visualized by immunoblotting using polyclonal antibody that recognized the polyhistidine tag. Vector represents a similarly prepared lysate from *E. coli* containing only the parent plasmid, p242 [12], a derivative of pET15b. (Bottom panel) Recombinant CePEK phosphorylates Ser-51 of eIF-2α. CePEK sequences 480–1077 were expressed and partially purified from *E. coli* in the form of a polyhistidine fusion protein. Recombinant CePEK was mixed with [γ-^{32}P]ATP and recombinant eIF-2α substrate containing wild-type Ser-51 (WT) or an alanine substituted for this phosphorylation site (S51A). In parallel, kinase reactions were carried out with 1 μg of total lysate prepared from yeast expressing human PKR, or with no added kinase. Following SDS/PAGE, radiolabelled proteins were visualized by autoradiography. The following amounts of the kinase preparations were added to a 20-μl reaction mixture: lanes 3 and 4, 25 ng of CePEK; lanes 5 and 6, 50 ng of CePEK; and lanes 7 and 8, 1 μg of total lysate prepared from yeast expressing human PKR. At the concentrations used in this experiment, both CePEK and PKR were found to phosphorylate wild-type eIF-2α, whereas minimal phosphorylation of the eIF-2α-S51A was observed. At elevated concentrations of CePEK enzyme, or extended incubation times, we found more significant phosphorylation of the mutant substrate. Sizes of protein standards in kDa are indicated on the left of both panels.

under the control of a galactose-inducible promoter. Yeast cells expressing CePEK, human PKR or vector alone were cultured in synthetic medium supplemented with glucose (SD medium) or galactose (SGal medium). Whereas no significant differences in growth were measured between yeast cells in glucose-containing medium, both the PKR- and CePEK-expressing strains displayed a striking slow-growth phenotype, indicative of reduced translation, when cultured in galactose medium (Figure 8, top panel). Coincident with this slow growth in galactose-inducing medium, we found levels of eIF-2α phosphorylation in H1816 cells expressing CePEK that were similar to that measured for the strain containing PKR (Figure 8, bottom panel).

Previously, it was observed that introduction of a mutant form of the eIF-2α gene, *SUI2*, which contains alanine for Ser-51, alleviates the slow-growth phenotype associated with expression of human PKR [20]. We similarly expressed CePEK in strain H1817 (*gcn2*Δ *SUI2-S51A*) that is isogenic to H1816, and found no detectable growth defect when these cells were grown in medium supplemented with galactose (Figure 8, top panel). As expected, no phosphorylation of eIF-2α was detected in H1817 expressing either CePEK or PKR (Figure 8, bottom panel). Furthermore, Pavitt et al. [21] noted that certain mutations in *GCD2*, encoding the δ subunit of eIF-2B, rendered the guanine-exchange factor less sensitive to inhibition by phosphorylated eIF-2α. We expressed either CePEK or human PKR in strain GP3299 (*gcn2*Δ *GCD2-K627T*) and found that the eIF-2Bδ mutation greatly relieved the slow-growth phenotype associated with the galactose-inducing conditions (Figure 8, top panel). Together, these results are consistent with the idea that CePEK

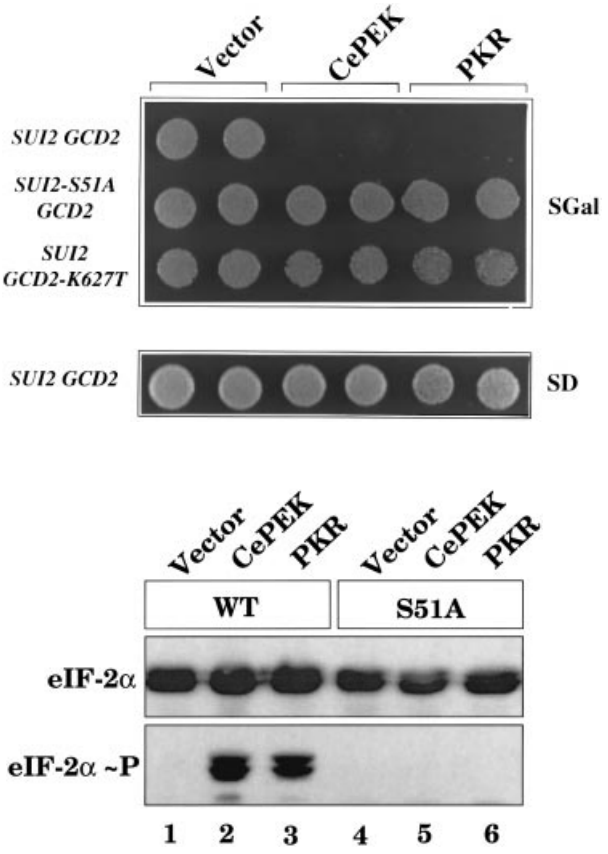


Figure 8 Expression of CePEK in yeast showing inhibition of growth due to hyperphosphorylation of eIF-2α

(Top panel) CePEK or human PKR were expressed using a galactose-inducible promoter in an isogenic set of yeast strains devoid of its endogenous eIF-2α kinase, GCN2. Yeast strains included H1816 (*gcn2*Δ *SUI2 GCD2*), H1817 (*gcn2*Δ *SUI2-S51A GCD2*) and GP3299 (*gcn2*Δ *SUI2 GCD2-K627T*). *SUI2* encodes eIF-2α and the mutant allele *SUI2-S51A* encodes a product that is blocked for phosphorylation by eIF-2α kinase [16,56]. *GCD2* encodes the δ subunit of eIF-2B and the *GCD2-K627T* mutant renders eIF-2B less sensitive to inhibition by phosphorylated eIF-2α [21]. Yeast cells were grown in culture and samples with a density of $A_{600} = 0.05$ were spotted on to agar plates containing synthetic medium supplemented with glucose (SD) or galactose (SGal). Agar plates were incubated for 4 days at 30 °C and photographed. While expression of CePEK and PKR in H1816 significantly reduced growth in the galactose-inducing medium, this growth defect was alleviated when CePEK was expressed in H1817 and GP3299 containing *SUI2-S51A* and *GCD2-K627T*, respectively. (Bottom panel) PEK, PKR or vector alone were expressed in strains H1816 containing wild-type eIF-2α (WT, lanes 1–3) and H1817 with mutant eIF-2α-S51A (S51A, lanes 4–6) using galactose-inducing medium. Lysates were prepared and analysed by immunoblot using polyclonal antibody that specifically recognized eIF-2α phosphorylated at Ser-51 (eIF-2α ~ P), or with monoclonal antibody that recognized total eIF-2α protein.

is an eIF-2α kinase that can regulate translation initiation through inhibition of eIF-2B.

Vaccinia virus K3L protein inhibits CePEK activity

Numerous viral proteins were identified that inhibit PKR and its associated anti-viral pathway [4,31,32]. One such protein from vaccinia virus, K3L, shares 28 % sequence identity with eIF-2α and is thought to function as a pseudosubstrate, blocking PKR activity by binding the kinase catalytic domain [33,34]. K3L protein was also found to inhibit haem-regulated inhibitor kinase and GCN2 [34,35]. These studies indicate that K3L is a specific inhibitor of eIF-2α kinases from yeast and mammals and suggest that these kinases share common structural features important

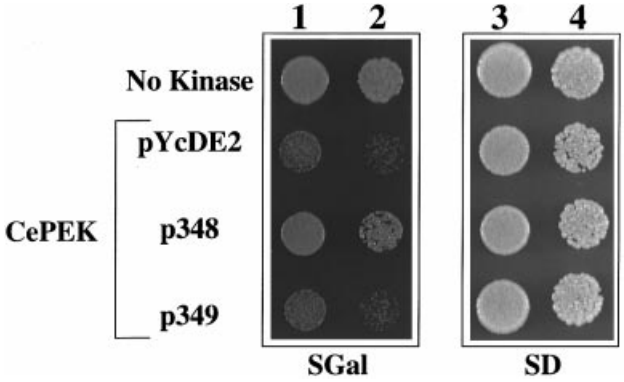


Figure 9 The expression of vaccinia virus K3L protein relieves the slow-growth phenotype associated with CePEK in yeast

The yeast strain H1894 (*gcn2*Δ *SUI2*) expressing CePEK from a galactose-inducible promoter was transformed with p348, encoding vaccinia K3L protein, p349 expressing the antisense K3L RNA or vector pYCDE2 alone. In parallel, we carried out a similar analysis using a version of H1894 expressing no CePEK and containing only vectors pEMBLyex4 and pYCDE2. Yeast cultures with a density of $A_{600} = 0.05$ (lanes 1 and 3) or $A_{600} = 0.005$ (lanes 2 and 4) were spotted on to agar plates containing synthetic medium supplemented with glucose (SD) or galactose (SGal). Agar plates were incubated for 4 days at 30 °C and photographed. Expression of CePEK in H1894 containing p349 or pYCDE2 reduced cell growth compared with the strain containing no CePEK. This growth inhibition by CePEK was relieved partially by introduction of K3L protein encoded in plasmid p348.

for recognition of their eIF-2α substrate. To determine whether K3L also inhibits PEK, we first expressed CePEK under the galactose-inducible promoter in H1894 (*gcn2*Δ), a strain isogenic to the H1816-based set described earlier. We then introduced into this H1894-derived strain plasmid p348, expressing K3L protein from the constitutive *ADH1* promoter, p349, containing the *K3L* gene in the opposite orientation to the *ADH1* promoter, or parent vector pYCDE2. The slow-growth phenotype associated with CePEK was relieved significantly in the strain expressing K3L protein (Figure 9). By comparison, cells containing the parent plasmid or p349 still exhibited a slow-growth phenotype in the galactose-containing medium. These results indicate that K3L protein can function as an inhibitor of PEK and support the idea that this kinase recognizes its eIF-2α substrate by mechanisms shared with other members of the eIF-2α kinase family.

DISCUSSION

In this report, we show that a recently described eIF-2α kinase, PEK, is present in diverse multicellular organisms, including humans, *D. melanogaster* and *C. elegans*. These PEK homologues share sequence features in their kinase regions, as well as in their extended N-termini thought to be located in the lumen of the ER (Figure 1). Human PEK mRNA was found to be expressed in all tissues examined, with elevated levels in tissues that specialize in polypeptide secretion, including pancreas, salivary gland, stomach and pituitary gland (Figures 2–4). In cultured cells, the eIF-2α kinase activity of PEK was induced in response to ER stress coincident with elevated levels of eIF-2α phosphorylation *in vivo* (Figure 5). Activation of PEK required the ER luminal sequences of this eIF-2α kinase (Figure 6). Furthermore, in this first reported characterization of an eIF-2α kinase from *C. elegans*, we showed that CePEK can phosphorylate the regulatory site, Ser-51, in eIF-2α *in vitro* and *in vivo* and can function in translational control in the yeast model (Figures 7 and 8).

Furthermore, using this yeast translation system, we showed that a viral product, vaccinia K3L, inhibits the activity of PEK (Figure 9). These results suggest that PEK mediates translational control in response to ER stress by inhibition of eIF-2B from nematodes to mammals.

Role of PEK in translational control in response to cellular stress

Cellular stress that contributes to accumulation of misfolded protein in the ER triggers an unfolded protein response involving increased transcription of genes whose products, such as ER chaperones GRP78/BiP and GRP94 (where GRP is glucose-regulated protein), facilitate correct protein maturation in the lumen of the ER [36–38]. A second response is a reduction in protein synthesis by phosphorylation of eIF-2α [2,39]. Given that the expression of HsPEK mRNA is elevated in pancreas and salivary gland, cell types that contain an expanded ER network and are specialized for protein secretion, it is inviting to consider a role for PEK in translational control in response to ER stress. Recently, Harding et al. [14] observed that the mouse homologue of PEK, designated PERK, is glycosylated and associated with the ER. Furthermore, we found that ER stress brought about by exposure to thapsigargin or A23187 stimulates the eIF-2α kinase activity of PEK, suggesting that PEK impairs protein synthesis in response to ER stress.

The topological arrangement of PEK draws many parallels to IRE1 protein, another component of the unfolded protein response. IRE1, described in both yeast and mammalian systems, is required for transcriptional activation of genes in response to ER stress [28,29,40,41]. IRE1 is also a type-1 transmembrane protein, with the N-terminal regulatory domain in the lumen of the ER. The N-terminal portion of the PEK homologues, which is also thought to reside in the ER lumen, has limited sequence similarity with IRE1 [14]. Using the BLAST2 program, we found the most significant sequence similarity to be between a 120-residue segment of CePEK from positions 43 to 160 and the predicted IRE1 protein from *C. elegans*.

The association of PEK with the ER, and the fact that its autophosphorylation and eIF-2α kinase activity are stimulated in response to ER stress, suggests a model whereby the N-terminal sequences of PEK function as a sensor for accumulation of unfolded protein in the ER lumen that result from cellular stresses. The ensuing activation of the PEK catalytic domain, located on the cytoplasmic side of the ER membrane, would lead to elevated phosphorylation of eIF-2α and reduced translation initiation. Decreased protein synthesis would provide the cell with an opportunity to elevate expression of ER chaperones via the transcriptional-response pathway prior to introducing newly synthesized proteins into the secretory pathway. To elicit the transcriptional response, the kinase and endonuclease functions of IRE1 mediate transcription of genes such as GRP78 and GRP94, required to remedy the ER stress.

The fact that PEK and IRE1 are both ER transmembrane protein kinases whose catalytic activities are induced during ER stress conditions would suggest overlapping modes of regulation. In the example of IRE1 in yeast, oligomerization involving the N-terminal sequences followed by trans-autophosphorylation are thought to be important steps leading to activation of the kinase [38]. Consistent with the idea that the N-terminus of PEK is important for mediating activation of this eIF-2α kinase, we found that deletion of these sequences, including the region similar to IRE1, greatly reduced the catalytic activity of PEK. We also found that enhanced PEK autophosphorylation is linked closely with increased eIF-2α kinase activity in response to ER stress. It should be noted that a different conclusion was drawn by a recent report that compared the activities of wild-type PEK and a kinase-mutant version expressed in Sf9 insect cells [26]. After immunoprecipitation, the mutant version of PEK was found to autophosphorylate despite being defective for eIF-2α kinase activity, leading to the idea that these two catalytic activities could be uncoupled during the regulation of PEK. We are uncertain about the underlying basis for these different experimental observations. One clue may lie in the fact that the mutant version of PEK was expressed at significantly elevated levels in the Sf9 cells compared with the wild-type eIF-2α kinase. Thus the comparison between mutant and wild-type PEK may be compromised by the large differences in enzyme concentrations used in the immunoprecipitation kinase assays.

The molecular details by which PEK or IRE1 sense protein misfolding during ER stress is currently not understood. An interesting possibility is that an ER protein such as GRP78 associates with the N-terminus of IRE1 and PEK, and regulates their catalytic activity. For example, during ER stress, GRP78 may bind to unfolded proteins that accumulate in the lumen of the ER, leaving PEK free to dimerize and trans-autophosphorylate. Overexpression of GRP78 in Chinese hamster ovary cells reduced the unfolded protein response and partially relieved the translation inhibition during treatment with A23187 [42]. Following the logic of our model, the elevated levels of GRP78 may result in this ER chaperone retaining its association with PEK, even during ER stress, preventing PEK dimerization and trans-autophosphorylation.

Relationships between PEK and PKR in translational control

In addition to its role in the anti-viral defence pathway, PKR was proposed to mediate translational control in response to ER stress [39,43]. Two lines of evidence support this role for PKR. First, PKR immunoprecipitated from NIH 3T3 cells treated with double-stranded RNA and calcium-mobilizing agents exhibited elevated autophosphorylation activity compared with cells treated with double-stranded RNA alone [43]. A second line of support involves overexpression of wild-type and mutant versions of the PKR in cultured NIH 3T3 cells. Overexpression of wild-type PKR further exacerbated the protein synthesis defect associated with ER stress, whereas expression of a dominant-negative mutant of PKR partially alleviated the translation inhibition resulting from ER stress [44]. Together, these results suggested that PKR functioned to mediate translational control in response to conditions leading to impaired protein folding in the ER. However, embryo fibroblast cells derived from a mouse deleted for PKR retained the ability to reduce translation in response to treatment with tunicamycin or thapsigargin [14]. This result argues against PKR being the sole eIF-2α kinase responding to ER stress, although it is still possible that PKR functions in combination with PEK.

There are many parallels between the studies addressing the function of PKR in response to ER stress and its role in the control of cell proliferation. Expression of dominant-negative mutant forms of PKR in NIH 3T3 cells were found to lead to a transformed phenotype and when injected into nude mice these transfected cells contributed to tumours [5,6]. However, mice deleted for PKR did not display any tumour formation [45,46]. Perhaps another eIF-2α kinase, such as PEK, has overlapping regulatory functions with PKR. For example, overexpression of a dominant-negative mutant version of PKR in cultured cells might interfere with PEK by competing for regulatory factors. In this report, we observed that the viral protein K3L also inhibits PEK activity (Figure 9), demonstrating that a common regulatory

protein can interact with either PKR or PEK and alter their activity.

PKR has a well-characterized role in cellular anti-viral defence and many different viral RNAs and proteins were described that interact with PKR and impair its kinase activity [4,31,32,47]. Viral infection can also affect protein processing in the ER [39]. For example, rotavirus infection or expression of E2 protein of hepatitis C virus increases the expression of GRP78 and GRP94, suggesting these ER chaperones function in viral maturation in the ER lumen [48,49]. Furthermore, poliovirus infection alters host secretory pathways [50]. Such virally induced ER stress may stimulate PEK activity, in combination with activation of PKR, leading to reduced protein synthesis. Given that many virally encoded proteins reduce PKR activity by direct interaction with PKR, it is inviting to speculate that some of these products may also impact the eIF-2α kinase activity of PEK. This idea is consistent with our observation that vaccinia virus K3L, a protein that associates with the catalytic domain of PKR, can impede PEK control of translation.

Identification of new eIF-2α kinases in the model systems

The genome-sequencing projects involving different eukaryotic model systems have provided an important means to survey for potential eIF-2α kinases. As illustrated in this report, we used sequencing information from *C. elegans* and *D. melanogaster* to identify PEK homologues. Using each of the eIF-2α kinase sequences as queries to search the completed *C. elegans* genomic sequence, we also identified a second probable eIF-2α kinase, a GCN2 homologue, encoded in cosmid clone y81G3A, accession number AL034453. The putative *C. elegans* GCN2 contains a protein kinase domain related to yeast GCN2 juxtaposed to a histidyl-tRNA synthetase-like sequence that is the hallmark feature of GCN2 from *S. cerevisiae* [1,51]. GCN2 kinase is also present in *D. melanogaster* [52,53] and mammals [54]. Given the role of GCN2 in responding to amino acid starvation in yeast, this eIF-2α kinase may serve as a cytoplasmic counterpart to PEK, impacting protein synthesis in response to nutritional stresses in multicellular organisms.

Whereas the multicellular organisms discussed above regulate translation initiation by at least two different eIF-2α kinases, *S. cerevisiae* only has a single eIF-2α kinase, GCN2. In contrast to the mammalian eIF-2α kinases shown to regulate global protein synthesis, the function of GCN2 in yeast has been adapted to induce translation of a single species of mRNA, encoding GCN4, in response to starvation of amino acids [1]. It is not known currently whether gene-specific regulation occurs in higher eukaryotes in response to modest levels of eIF-2α phosphorylation. Given the genetic and biochemical tools available to yeast, this model system may prove useful in future work to delineate the mechanisms regulating PEK in response to ER stress.

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