Biochemical and Biophysical Research Communications **280,** 293–300 (2001)
doi:10.1006/bbrc.2000.4103, available online at http://www.idealibrary.com on IDEAL®

The Double-Stranded RNA-Activated Protein Kinase PKR Is Dispensable for Regulation of Translation Initiation in Response to either Calcium Mobilization from the Endoplasmic Reticulum or Essential Amino Acid Starvation

Scot R. Kimball,* Michael J. Clemens,† Vivienne J. Tilleray,† Ronald C. Wek,‡ Rick L. Horetsky,* and Leonard S. Jefferson*

**Department of Cellular and Molecular Physiology, Pennsylvania State University, College of Medicine, Hershey, Pennsylvania 17033; †Department of Biochemistry and Immunology, Cellular and Molecular Sciences Group, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, United Kingdom; and ‡Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202*

Received November 30, 2000

The α-subunit of eukaryotic initiation factor eIF2 is a preferred substrate for the double-stranded RNA-activated protein kinase, PKR. Phosphorylation of eIF2α converts the factor from a substrate into a competitive inhibitor of the guanine nucleotide exchange factor, eIF2B, leading to a decline in mRNA translation. Early studies provided evidence implicating PKR as the kinase that phosphorylates eIF2α under conditions of cell stress such as the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum, i.e., the *u*nfolded *p*rotein *r*esponse (UPR). However, the recent identification of a *trans*-microsomal membrane eIF2α kinase, termed PEK or PERK, suggests that this kinase, and not PKR, might be the kinase that is activated by misfolded protein accumulation. Similarly, genetic studies in yeast provide compelling evidence that a kinase termed GCN2 phosphorylates eIF2α in response to amino acid deprivation. However, no direct evidence showing activation of the mammalian homologue of GCN2 by amino acid deprivation has been reported. In the present study, we find that in fibroblasts treated with agents that promote the UPR, protein synthesis is inhibited as a result of a decrease in eIF2B activity. Furthermore, the reduction in eIF2B activity is associated with enhanced phosphorylation of eIF2α. Importantly, the magnitude of the change in each parameter is identical in wildtype cells and in fibroblasts containing a chromosomal deletion in the PKR gene (PKR-KO cells). In a similar manner, we find that during amino acid deprivation the inhibition of protein synthesis and extent of increase in eIF2α phosphorylation are identical in wildtype and PKR-KO cells. Overall, the results show that PKR is not required for increased eIF2α phosphorylation or inhibition of protein synthesis under conditions promoting the UPR or in response to amino acid deprivation.

Key Words: **translation initiation; eukaryotic initiation factor; eIF2; eIF2B; PKR.**

The endoplasmic reticulum is the focal point for the folding and processing of newly synthesized proteins destined for secretion, incorporation into the plasma membrane, or localization into other intracellular compartments such as the lysosome (1). Conditions that foster misfolding of proteins in the lumen of the endoplasmic reticulum, such as treatment of cells with reducing agents such as DTT or mobilization of calcium from the ER, result in an inhibition of cytosolic protein synthesis. The reduction in protein synthesis protects the cells from further accumulation of misfolded proteins in the secretory pathway and may enhance preferential expression of certain genes required to remedy the ER stress. The inhibition of protein synthesis that occurs under these conditions is the result of phosphorylation of the α-subunit of eukaryotic initiation factor eIF2 (reviewed in 2). eIF2, as a ternary complex with GTP and initiator methionyl-tRNA$_i$, mediates the first step in translation initiation, the binding of Met-tRNA$_i$ to the 40S ribosomal subunit. At a later step in initiation, the GTP bound to eIF2 is hydrolyzed and eIF2 is released from the ribosome as an eIF2 · GDP binary complex. Prior to binding Met-tRNA$_i$ and participating in another cycle of initiation, the GDP bound to eIF2 must be exchanged for GTP. A second initiation factor, eIF2B, catalyzes the guanine nucleotide exchange re-

0006-291X/01 $35.00

action on eIF2. A predominant mechanism for regulation of eIF2B involves phosphorylation of eIF2 on its α-subunit, where phosphorylation converts it from a substrate into an inhibitor of eIF2B. Thus, phosphorylation of eIF2α causes a decline in protein synthesis due to an inhibition of eIF2B activity.

The protein kinase that phosphorylates eIF2α in response to accumulation of misfolded proteins was initially reported to be the double-stranded RNA-dependent protein kinase, PKR (3–5). However, several more recent studies have identified a novel eIF2α kinase that is resident in the membrane of the endoplasmic reticulum (6–8). This kinase (termed PEK or PERK) has, in addition to a cytosolic kinase domain with homology to other eIF2α kinases, an intraluminal domain that exhibits homology to the yeast protein IRE1p (6, 8). IRE1p is a trans-ER membrane protein kinase that is activated by the unfolded protein response and is thought to be the sensor that detects the presence of unfolded proteins and initiates the unfolded protein response. The studies cited above suggest that PEK/PERK may be activated during the unfolded protein response and might therefore mediate phosphorylation of eIF2α under these conditions. Moreover, the identification of PEK/PERK brings into question the involvement of PKR in the unfolded protein response in mammalian cells.

Phosphorylation of eIF2α is also observed in eukaryotic cells deprived of essential amino acids (reviewed in 9). The kinase that phosphorylates eIF2α in amino acid-deprived mammalian cells has not been identified. In *Saccharomyces cerevisiae*, amino acid deprivation activates the eIF2α kinase, GCN2 (10, 11). The recent identification of a mammalian homologue of GCN2, mGCN2 (12, 13), suggests that this kinase may also be involved in regulating eIF2α phosphorylation in higher eukaryotes in response to amino acid starvation. However, although mGCN2 is reportedly activated by serum starvation (12), its regulation by amino acids is still undefined.

In the present study, we have utilized fibroblasts containing a chromosomal deletion in the PKR gene (PKR-KO cells) to examine the role of this kinase in mediating eIF2α phosphorylation under conditions that deplete calcium from the ER or conditions of amino acid deprivation. We find that protein synthesis, eIF2α phosphorylation, and eIF2B activity are affected equally in wildtype and PKR-KO cells under these conditions. The results suggest that PKR is not required for the manifestation of the unfolded protein response or the inhibition of translation following deprivation of essential amino acids.

MATERIALS AND METHODS

Materials. ECL Detection Reagents and horseradish peroxidase-conjugated, sheep anti-mouse Ig and donkey anti-rabbit Ig were purchased from Amersham Life Sciences. PVDF membrane was obtained from BioRad. [^{35}S]Easytag Express Protein Labeling Mix was from NEN Research Products. Antibodies against eIF2α and an anti-phosphopeptide antibody that specifically recognizes eIF2α phosphorylated at Ser^{51} were kindly provided by Dr. Richard Panniers, National Institutes of Health and Drs. Gary S. Krause and Donald J. DeGracia, Wayne State University School of Medicine, respectively. The antibody to PKR was purchased from Santa Cruz Biotechnology, Inc. (#sc-6282) and was raised against the full length mouse protein.

Culture of mouse embryo fibroblasts. Wildtype mouse embryo fibroblasts and fibroblasts containing a chromosomal deletion of exons 2 and 3 of the mouse PKR gene were a generous gift of Dr. Charles Weissmann (University of Zurich). Cells were maintained in DMEM containing 20% fetal calf serum. For the calcium mobilization experiments, cells at approximately 80% confluence were deprived of serum for 15 min prior to addition of 2,5-di-(*tert*-butyl)-hydroquinone (tBuHQ) and EGTA as described in the legend to Fig. 2. For the amino acid deprivation studies, cells were incubated in the presence of 10% dialysed fetal calf serum in complete medium or medium lacking leucine, glutamine, or both amino acids for 45 min prior to labeling.

Measurement of protein synthesis. Protein synthesis in mouse embryo fibroblasts was monitored by measuring the incorporation of [^{35}S]methionine and [^{35}S]cysteine into protein as described previously (14).

Protein immunoblot analysis. Blots were developed as described previously using an ECL Western Blotting Kit (15) or by diaminobenzidine (DAB)/peroxide staining (16). Films were scanned using a Microtek ScanMaker V scanner equipped with a transparent media adapter connected to a Macintosh PowerMac 9600 computer. Images were obtained using the ScanWizard Plugin (Microtek) for Adobe Photoshop and quantitated using NIH Image software. Densitometry of DAB-stained blots was performed as described in (17).

Measurement of eIF2B activity. Cells (approximately 2×10^6) were washed with ice-cold PBS and then lysed in 400 μl of ice-cold buffer B (45 mM Hepes, pH 7.4, 0.375 mM magnesium acetate, 0.075 mM EDTA, 95 mM potassium acetate, 2.5 mg/ml digitonin, and 10% glycerol). The homogenates were centrifuged at 10,000*g* for 10 min at 4°C and the supernatants were assayed for the exchange of [^{3}H]GDP bound to eIF2 for unlabeled GDP as previously described (18).

Determination of eIF2α phosphorylation state. Cells were maintained in culture as described above with the exception that they were harvested by scraping in SDS sample buffer at 90°C as described previously (19). The relative amount of eIF2α in the phosphorylated form was quantitated by protein immunoblot analysis using an affinity-purified antibody that specifically recognizes eIF2α phosphorylated at Ser^{51} [eIF2(αP)] (20). For this analysis, samples were resolved by electrophoresis using a 12.5% SDS-polyacrylamide gel and the proteins in the gel were electrophoretically transferred to a PVDF membrane as described previously (15). The membranes were incubated with the rabbit polyclonal antibody that recognizes specifically eIF2(αP) and blots were developed as described above. The horseradish peroxidase coupled to the anti-rabbit secondary antibody was then inactivated by incubating the blot in 15% H_2O_2 for 30 min at room temperature. The total amount of eIF2α in the samples was determined by reprobing the blot with a monoclonal antibody that recognizes equally the phosphorylated and unphosphorylated forms of eIF2α (21) followed by an anti-mouse secondary antibody. Values obtained using the anti-eIF2(αP) antibody were normalized for the total amount of eIF2α present in the sample.

Examination of 4E-BP1 phosphorylation in extracts of mouse embryo fibroblasts. Aliquots of cell homogenates were immunoprecipitated using a monoclonal anti-4E-BP1 antibody using a previously described method (22). The immunoprecipitates were solubilized

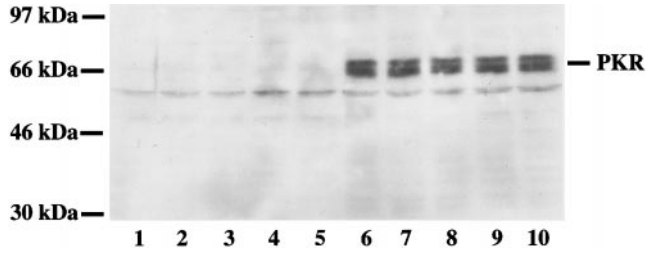

FIG. 1. Quantitation of PKR in wildtype and PKR-KO fibroblasts. Either PKR-KO (lanes 1–5) or wildtype (lanes 6–10) fibroblasts were harvested and homogenized as described under Materials and Methods. Equal amounts of protein from each dish of cells were resolved by electrophoresis on an SDS-polyacrylamide gel and the proteins in the gel were transferred to a PVDF membrane. The membrane was then probed with an antibody to full-length mouse PKR as described under Materials and Methods. The results are representative of three experiments.

with SDS sample buffer and then subjected to protein immunoblot analysis using a rabbit anti-rat 4E-BP1 antibody (22).

RESULTS

In the present study, cells containing a chromosomal deletion in the gene encoding PKR were used to examine the requirement for the kinase in mediating eIF2α phosphorylation under conditions that cause mobilization of calcium from the lumen of the endoplasmic reticulum or during amino acid deprivation. The genome of these cells lacks exons 2 and 3 of the PKR gene which encodes 15 nucleotides of the 5′-untranslated region of the mRNA as well as amino acids 1–80 of the coding region. If the truncated gene is transcribed, the most upstream methionine codon in the correct reading frame could potentially encode a truncated variant of PKR which would have a calculated molecular mass of 43 kDa. To confirm the lack of PKR, the amount of the kinase present in PKR-KO cells was compared to that present in wildtype cells by protein immunoblot analysis. As shown in Fig. 1, PKR was readily detected in homogenates of wildtype cells as a doublet, but was undetectable in homogenates of PKR-KO cells. Furthermore, no proteins with a molecular mass of approximately 43 kDa were detected in either wildtype or PKR-KO cells, suggesting that the truncated PKR gene is either not transcribed or not translated in PKR-KO cells.

Protein synthesis is dramatically reduced in a variety of conditions that cause mobilization of calcium from the endoplasmic reticulum. We reported previously that inhibition of the microsomal ATP-dependent calcium pump by a combination of tBuHQ and EGTA results in an inhibition of protein synthesis in perfused rat liver (23, 24). In the present study, protein synthesis was reduced to the same extent in both wildtype cells and PKR-KO cells following treatment with tBuHQ/EGTA (Fig. 2). Thus, the inhibition of protein synthesis caused by calcium depletion occurs in the absence of PKR.

Earlier studies have shown that the inhibition of protein synthesis by calcium mobilization is the result of increased phosphorylation of eIF2α (14, 23, 25, 26). To determine whether calcium depletion can cause eIF2α phosphorylation in the absence of PKR, wildtype and PKR-KO cells were incubated in the presence or absence of tBuHQ/EGTA and the relative phosphorylation state of eIF2α was quantitated by protein immunoblot analysis. For these analyses, blots were probed with a polyclonal antibody that only recognizes eIF2α when it is phosphorylated at Ser[51] (20). The blots were then reprobed with a monoclonal antibody that recognizes equally both phosphorylated and unphosphorylated forms of the protein (21). As shown in Fig. 3 (top) and quantitated in Fig. 3 (bottom), treatment with tBuHQ/EGTA stimulated eIF2α phosphorylation approximately 3-fold in either wildtype or PKR-KO cells. Furthermore, the total amount of eIF2α was the same in both cell types and was not affected by tBuHQ/EGTA treatment. The results indicate that PKR is not required for eIF2α phosphorylation caused by calcium mobilization from the endoplasmic reticulum.

Phosphorylated eIF2 inhibits protein synthesis by acting as a competitive inhibitor of eIF2B (reviewed in 27). As shown in Fig. 4, the guanine nucleotide exchange activity of eIF2B was reduced by tBuHQ/EGTA

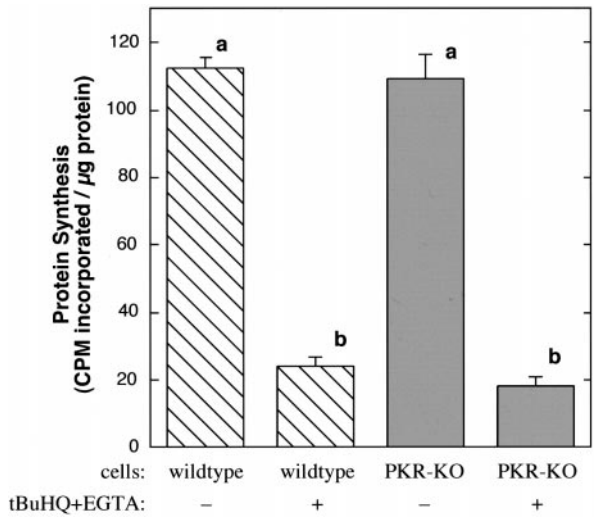

FIG. 2. Effect of calcium depletion on protein synthesis in wildtype and PKR-KO fibroblasts. Wildtype and PKR-KO cells were incubated for 15 min in the presence or absence of 20 μM tBuHQ and 3 mM EGTA. All dishes received 5 μl of [^{35}S]Easytag Express Protein Labeling Mix (11 mCi/ml). Thirty minutes later cells were harvested and the incorporation of [^{35}S]methionine and [^{35}S]cysteine into protein was determined as described under Materials and Methods. The results represent the mean ± SEM of 9 dishes of cells per condition. Values not sharing the same superscript are significantly different ($P < 0.001$) by the Tukey–Kramer Multiple Comparisons Test. Hatched bars, wildtype fibroblasts; solid grey bars, PKR-KO fibroblasts.

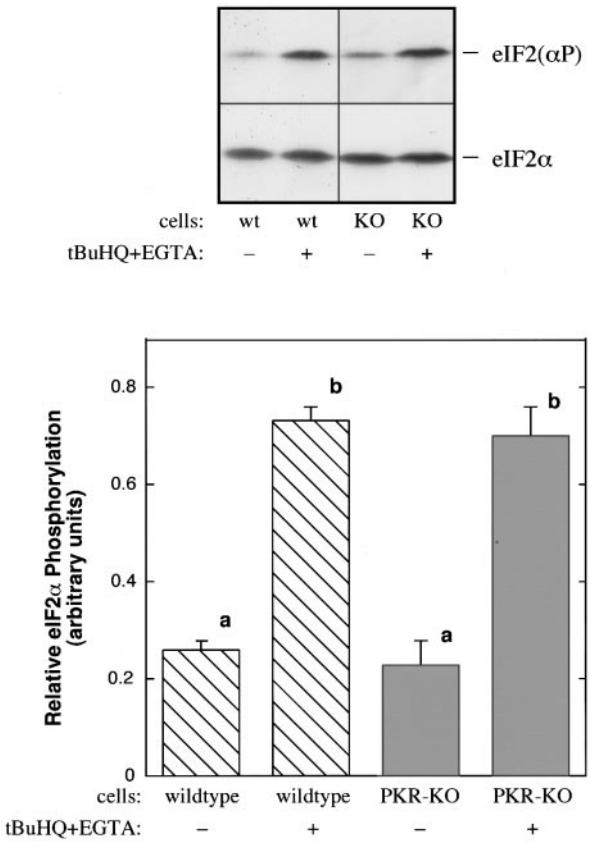

FIG. 3. Effect of calcium depletion on eIF2α phosphorylation in wildtype and PKR-KO fibroblasts. Wildtype and PKR-KO cells were incubated in the presence or absence of tBuHQ and EGTA as described in the legend to Fig. 2. Cells were harvested in SDS-sample buffer at 90°C and homogenates were resolved by SDS-polyacrylamide gel electrophoresis as described under Materials and Methods. eIF2α was quantitated by protein immunoblot analysis as described under Materials and Methods. The results from representative blots are shown in the top panel. eIF2α(P), blot probed with an antibody that specifically recognizes eIF2α phosphorylated at Ser[51]; eIF2α, blot probed with a monoclonal antibody that recognizes equally the phosphorylated and unphosphorylated forms of eIF2α. The results shown in the bottom panel represent the mean ± SEM of 8–9 dishes of cells per condition. Values not sharing the same superscript are significantly different ($P < 0.001$) by the Tukey–Kramer Multiple Comparisons Test. Hatched bars, wildtype fibroblasts; solid gray bars, PKR-KO fibroblasts.

in both wildtype and PKR-KO cells. Furthermore, the magnitude of the change was similar in both cell types. Therefore, changes in eIF2B activity were inversely proportional to alterations in eIF2α phosphorylation and did not require PKR.

Because the studies described above suggested that PKR is not required for phosphorylation of eIF2α by tBuHQ/EGTA, we attempted to measure PEK/PERK activity in PKR-KO cells using our previously described assay (8). In this assay, PEK/PERK is immunoprecipitated using a polyclonal anti-rat PEK/PERK antibody and eIF2α kinase activity present in the immunoprecipitate is measured. However, we were unable to detect PEK/PERK activity using this assay. We also were unable to detect PEK/PERK by Western blot analysis, suggesting that the amount of the kinase in these cells is very low.

Under conditions that generate the unfolded protein response, the synthesis of a number of resident endoplasmic reticulum proteins is preferentially increased, even though total protein synthesis is decreased to 10–15% of the control value (3, 5). One mechanism for preferentially increasing the translation of a subset of mRNAs is by increasing the amount of eIF4E present in the active complex with eIF4G (28). The amount of eIF4E available to form the eIF4G-eIF4E complex is regulated by the association of eIF4E with the translational repressor, 4E-BP1. Binding of eIF4E to 4E-BP1 prevents its association with eIF4G. The binding of eIF4E to 4E-BP1 is regulated by phosphorylation of 4E-BP1 where hypophosphorylated 4E-BP1 (i.e., the α and β forms) binds to eIF4E but the hyperphosphorylated form (i.e., the γ form) does not. However, as shown in Fig. 5, tBuHQ/EGTA had no significant effect on the amount of 4E-BP1 in the γ-form in either wildtype or PKR-KO cells, suggesting that mobilization of calcium from the endoplasmic reticulum has no effect on eIF4E availability.

eIF2α is also phosphorylated when cells are deprived of essential amino acids. Because the kinase that is activated in mammalian cells under such conditions has not been positively identified, we examined the effect of glutamine and/or leucine deprivation on pro-

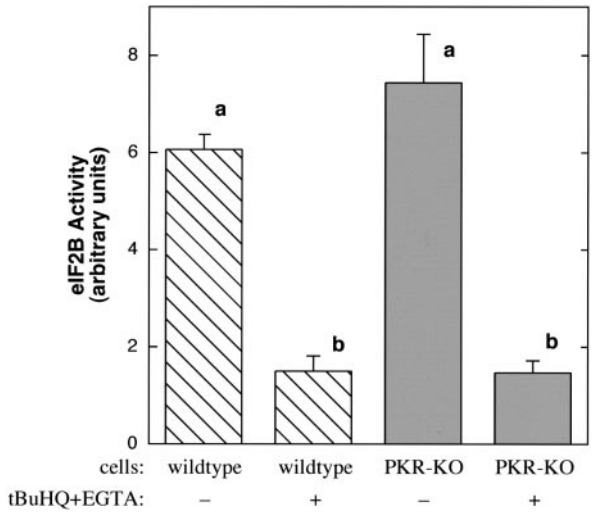

FIG. 4. Effect of calcium depletion on eIF2B activity in wildtype and PKR-KO fibroblasts. Wildtype and PKR-KO cells were incubated in the presence or absence of tBuHQ and EGTA as described in the legend to Fig. 2. The guanine nucleotide exchange activity of eIF2B was measured in cell extracts as described under Materials and Methods. The results represent the mean ± SEM of 6 dishes of cells per condition. Values not sharing the same superscript are significantly different ($P < 0.001$) by the Tukey–Kramer Multiple Comparisons Test. Hatched bars, wildtype fibroblasts; solid gray bars, PKR-KO fibroblasts.

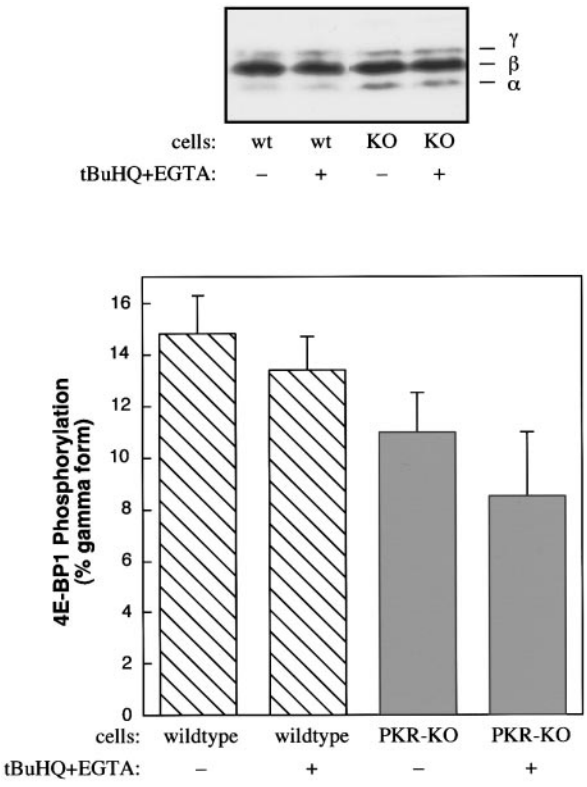

FIG. 5. Effect of calcium depletion on 4E-BP1 phosphorylation in wildtype and PKR-KO fibroblasts. Wildtype and PKR-KO cells were incubated in the presence or absence of tBuHQ and EGTA as described in the legend to Fig. 2. Cell extracts were immunoprecipitated with a monoclonal antibody to 4E-BP1 and the phosphorylation state of 4E-BP1 was examined by protein immunoblot analysis as described under Materials and Methods. The results of a typical blot are shown in the top panel. The proportion of 4E-BP1 in the most highly phosphorylated form was assessed by densitometric analysis of the blots. The results represent the mean ± SEM of 8–9 dishes of cells per condition. Hatched bars, wildtype fibroblasts; solid gray bars, PKR-KO fibroblasts.

tein synthesis in wildtype and PKR-KO cells. As shown in Fig. 6A, protein synthesis is reduced in wildtype cells deprived of either leucine or glutamine. Deprivation of both amino acids had no additional effect beyond that caused by removal of glutamine alone. Protein synthesis was also depressed in PKR-KO cells deprived of leucine and/or glutamine, and the magnitude of the effect was similar to that observed in wildtype cells. The inhibition of protein synthesis caused by glutamine deprivation was associated with increased phosphorylation of eIF2α (Fig. 6B). This effect was observed equally well in both wildtype and PKR-KO cells, suggesting that PKR is not required for eIF2α phosphorylation under conditions of amino acid deprivation.

DISCUSSION

Several previous studies have identified PKR as the kinase that phosphorylates eIF2α under a variety of cellular stresses, including conditions that promote the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (3–5). The conclusion drawn from these studies was supported by the following observations. First, the stimulation of eIF2α phosphorylation caused by the calcium ionophore, A23187, or an inhibitor of the microsomal ATP-dependent calcium pump, thapsigargin, is magnified when cells are treated with interferon to induce PKR synthesis (4). Second, in extracts of cells treated with either A23187 (4) or arsenite (3), eIF2α kinase activity is increased in

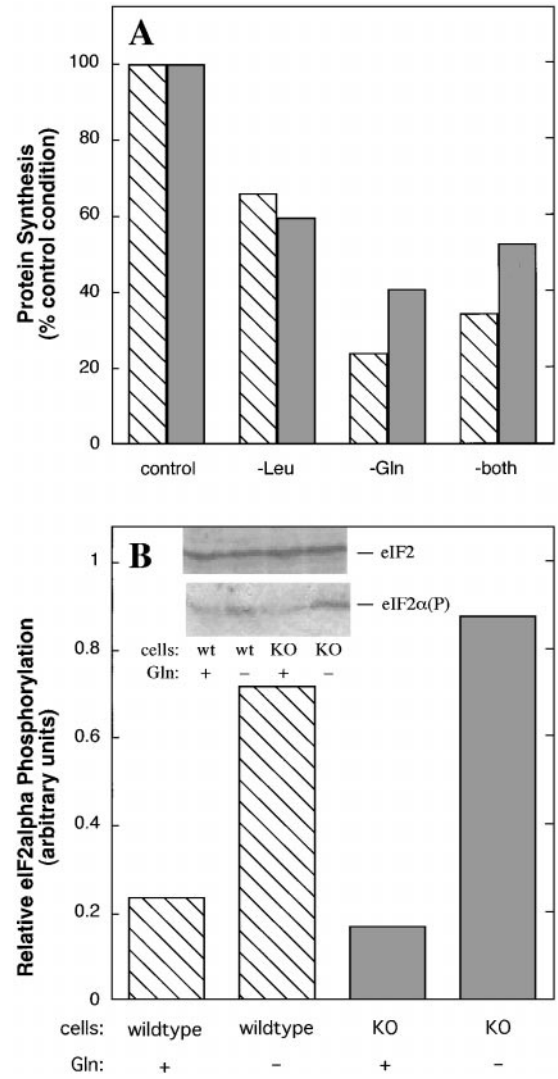

FIG. 6. Effect of depletion of leucine or glutamine on protein synthesis and eIF2α phosphorylation in wildtype and PKR-KO fibroblasts. Wildtype and PKR-KO cells were incubated in the presence or absence of leucine or glutamine or both amino acids as described under Materials and Methods. Protein synthesis (A) and eIF2α phosphorylation (B) were measured as described under Materials and Methods. The results represent the average of 3–4 replicates per condition. Hatched bars, wildtype fibroblasts; solid gray bars, PKR-KO fibroblasts. Blots of the total and phosphorylated forms of eIF2α are shown as an inset in B.

the presence of double-stranded RNA, an activator of PKR. Third, in NIH-3T3 cells expressing a dominant interfering variant of PKR, phosphorylation of eIF2α in response to A23187, ionomycin, dithiothreitol, or arsenite is repressed compared to non-transfected cells (3, 5). Finally, overexpression of wildtype PKR in L929 cells increases the sensitivity of protein synthesis to inhibition by A23187 (5). Overall, the results of these studies provide strong support for a role for PKR in mediating the inhibition of protein synthesis during physiological stress and the unfolded protein response.

In contrast to the above reports, in the present study we found that the magnitude of the changes in protein synthesis, eIF2B activity, and eIF2α phosphorylation caused by calcium depletion was the same in cells lacking PKR as in wildtype cells, suggesting that PKR is not required for manifestation of the unfolded protein response. A possible explanation for this apparent anomaly is that either PKR or PEK/PERK might be able to mediate eIF2α phosphorylation during the unfolded protein response. If this assumption is true, then under most circumstances PEK/PERK would likely be the predominant kinase that is activated during the unfolded protein response, because in uninduced cells the amount of PKR is normally low (reviewed in 29, 30). Thus, little PKR autophosphorylation is observed in extracts of A23187- or thapsigargin-treated HeLa or NIH-3T3 cells unless the cells are pretreated with interferon and double-stranded RNA is added to the extracts, even though eIF2α phosphorylation is dramatically increased (4). Furthermore, even though PKR expression is increased five-fold in interferon-treated HeLa cells, eIF2α phosphorylation caused by either A23187 or thapsigargin is the same in interferon-treated as in non-treated cells (4), suggesting that PKR is not the primary kinase involved in the unfolded protein response.

The finding that expression of a dominant interfering variant of PKR partially prevents the stimulation of eIF2α phosphorylation under conditions that favor misfolding of proteins in the endoplasmic reticulum (3, 5) would seem to provide compelling evidence for a role for the kinase in the unfolded protein response. However, during activation of PKR, the kinase dimerizes and autophosphorylates (reviewed in 29, 30). The dominant interfering variant of PKR [PKR(K296P)] used in the studies of Brostrom *et al.* (3) and Srivastava *et al.* (5) contains a mutation that changes Lys^{296} to Pro, which abrogates the activity of the kinase. Such inactive variants of PKR are thought to prevent activation of the endogenous kinase through the binding of the inactive variant to the wildtype kinase, resulting in formation of disfunctional dimers (31). The ability to dimerize seems to be a common feature in the regulation of eIF2α kinases since, in addition to PKR, both the nutrient-regulated eIF2α kinase, GCN2 (32), and the heme-regulated eIF2α kinase, HRI (33), form dimers. Whether or not the various eIF2α kinases are able to form heterodimers is unknown. However, if dominant interfering variants of PKR are able to form inactive complexes with PEK/PERK, then the results from earlier studies implicating PKR in the unfolded protein response (3, 5) might have been misinterpreted. Thus, one possible explanation for these results is that, in addition to binding to wildtype PKR, PKR (K296P) binds to PEK/PERK and forms inactive heterodimers, and through this interaction is able to minimize the phosphorylation of eIF2α under conditions promoting the accumulation of misfolded proteins in the lumen of the ER. A second, perhaps more likely, possibility is that the overexpressed PKR variant binds to a positive regulatory protein that also activates PEK/PERK. Such an explanation might help account for the observation that overexpression of dominant negative PKR results in oncogenic transformation whereas disruption of the PKR gene has no impact on cell growth.

The results from studies in which PKR expression is increased, either by treating cells with interferon (4) or by transfection with an expression plasmid containing the PKR cDNA (5), suggest that PKR can augment the unfolded protein response. This result is not surprising considering that previous studies have shown that either mammalian PKR or HRI can functionally substitute for GCN2 in the general control response in yeast. In yeast deprived of amino acids, the eIF2α kinase GCN2 is thought to be activated as a result of the accumulation of uncharged tRNA (reviewed in 34). Activation of GCN2, and the subsequent phosphorylation of eIF2α, initiates a cascade of events that lead to increased translation of the mRNA encoding GCN4, a transcription factor that activates transcription of genes encoding enzymes that synthesize amino acids. The overall response allows yeast to grow in the absence of an exogenous supply of amino acids. In yeast containing a chromosomal deletion of GCN2, eIF2α is not phosphorylated during amino acid starvation, enhanced translation of GCN4 does not occur, and the amino acid biosynthetic genes are not induced, leading to a drastic reduction in cell growth and proliferation (35). Expression of PKR or HRI restores the general control response, resulting in increased GCN4 expression and maintenance of cell growth during amino acid starvation (36). Since either PKR or HRI can functionally substitute for GCN2, it is possible that all the eIF2α kinases are to some extent interchangeable and that PKR might also be able to function similarly to PEK/PERK to augment the unfolded protein response in cells overexpressing PKR.

In addition to calcium mobilization from the endoplasmic reticulum, deprivation of essential amino acids also results in increased phosphorylation of eIF2α in mammalian cells (reviewed in 9). As discussed above,

in yeast deprived of amino acids the eIF2α kinase, GCN2, is responsible for the enhanced phosphorylation of eIF2α (reviewed in 37). A variety of evidence has led to the proposal that an accumulation of uncharged tRNA during amino acid deprivation is the stimulus for GCN2 activation. Based on these observations, and the recent identification of mGCN2 in mammalian cells, it might be expected that the latter enzyme is responsible for increased phosphorylation of eIF2α in amino acid-deprived cells. However, although genetic evidence strongly supports a mechanism involving activation of GCN2 in amino acid-regulation of eIF2α phosphorylation in yeast, the activity of the kinase has not been shown to be elevated in extracts of amino acid-deprived cells or by addition of uncharged tRNA to purified GCN2. Furthermore, although serum-deprivation has been shown to activate mGCN2 (12), amino acid deprivation so far has not. Thus, in the present study, we examined the possibility that PKR might be required for stimulation of eIF2α phosphorylation in amino acid-deprived mouse fibroblasts. It was found that eIF2α phosphorylation was increased to the same extent in wildtype and PKR-KO cells, indicating that a kinase other than PKR is responsible for enhanced eIF2α phosphorylation under these conditions.

In summary, we have shown that depletion of endoplasmic reticulum calcium stores results in a reduction in protein synthesis through inhibition of eIF2B activity which in turn is caused by phosphorylation of eIF2α on Ser^{51}. Likewise, deprivation of essential amino acids inhibits protein synthesis though increased phosphorylation of eIF2α. The magnitude of each of the changes is the same in cells containing a chromosomal deletion of the PKR gene as in wildtype cells, suggesting that PKR is not required for the unfolded protein response in mammalian cells or for the inhibition of protein synthesis caused by amino acid deprivation.

ACKNOWLEDGMENTS

The authors are grateful to Joan McGwire for technical help. We also thank Dr. C. Weissmann for the mouse embryo fibroblasts and Drs. D. J. DeGracia, G. S. Krause, and R. Panniers for eIF2α antibodies. This work was supported by National Institute of Health Grants DK-13499 (L.S.J.), DK-15658 (L.S.J.), and GM49164 (R.C.W.), by American Cancer Society Grant RPG MBC-87806 (R.C.W.), and by funding from the Welcome Trust (Grant 056778) (M.J.C.), the Leukemia Research Fund (M.J.C.), and the Cancer Prevention Research Trust (M.J.C.).

REFERENCES

1. Hegde, R. S., and Lingappa, V. R. (1999) Regulation of protein biogenesis at the endoplasmic reticulum membrane. *Trends Cell Biol.* **9,** 132–137.
2. Kaufman, R. J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls. *Genes Dev.* **13,** 1211–1233.
3. Brostrom, C. O., Prostko, C. R., Kaufman, R. J., and Brostrom, M. A. (1996) Inhibition of translation initiation by activators of the glucose-regulated stress protein and heat shock protein stress response systems. Role of the interferon-inducible double-stranded RNA-activated eukaryotic initiation factor 2α kinase. *J. Biol. Chem.* **271,** 24995–25002.
4. Prostko, C. R., Dholakia, J. N., Brostrom, M. A., and Brostrom, C. O. (1995) Activation of the double-stranded RNA-regulated protein kinase by depletion of endoplasmic reticular calcium stores. *J. Biol. Chem.* **270,** 6211–6215.
5. Srivastava, S. P., Davies, M. V., and Kaufman, R. J. (1995) Calcium depletion from the endoplasmic reticulum activates the double-stranded RNA-dependent protein kinase (PKR) to inhibit protein synthesis. *J. Biol. Chem.* **270,** 16619–16624.
6. Harding, H. P., Zhang, Y., and Ron, D. (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397,** 271–274.
7. Mori, K., Ma, W., Gething, M.-J., and Sambrook, J. (1999) A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus. *Cell* **74,** 743–756.
8. Shi, Y., Vattem, K. M., Sood, R., An, J., Liang, J., Stramm, L., and Wek, R. C. (1998) Identification and characterization of pancreatic eukaryotic initiation factor 2 α-subunit kinase, PEK, involved in translational control. *Mol. Cell. Biol.* **18,** 7499–7509.
9. Kimball, S. R., and Jefferson, L. S. (2000) Regulation of translation initiation in mammalian cells by amino acids. *In* Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.), pp. 561–579 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Wek, S. A., Zhu, S., and Wek, R. C. (1995) The histidyl-tRNA synthetase-related sequence in the eIF-2α protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. *Mol. Cell. Biol.* **15,** 4497–4506.
11. Zhu, S., Sobolev, A. Y., and Wek, R. C. (1996) Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate in vitro phosphorylation of eIF-2. *J. Biol. Chem.* **271,** 24989–24994.
12. Berlanga, J. J., Santoyo, J., and de Haro, C. (1999) Characterization of a mammalian homolog of GCN2 eukarotic initiation factor 2α kinase. *Eur. J. Biochem.* **265,** 754–762.
13. Sood, R., Porter, A. C., Olsen, D., Cavener, D. R., and Wek, R. C. (2000) A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2α. *Genetics* **154,** 787–801.
14. Kimball, S. R., and Jefferson, L. S. (1990) Mechanism of inhibition of protein synthesis by vasopressin in rat liver. *J. Biol. Chem.* **265,** 16794–16798.
15. Kimball, S. R., Karinch, A. M., Feldhoff, R. C., Mellor, H., and Jefferson, L. S. (1994) Purification and characterization of eukaryotic initiation factor eIF-2B from liver. *Biochem. Biophys. Acta* **1201,** 473–481.
16. Jeffrey, I. W., Kadereit, S., Meurs, E. F., Metzger, T., Bachmann, M., Schwemmle, M., Hovanessian, A. G., and Clemens, M. J. (1995) Nuclear localization of the interferon-inducible protein kinase PKR in human cells and transfected mouse cells. *Exp. Cell Res.* **218,** 17–27.
17. Clemens, M. J., Bushell, M., and Morley, S. J. (1998) Degradation of eukaryotic polypeptide chain initiation factor (eIF) 4G in response to induction of apoptosis in human lymphoma cell lines [Review]. *Oncogene* **17,** 2921–2931.
18. Kimball, S. R., Everson, W. V., Flaim, K. E., and Jefferson, L. S. (1989) Initiation of protein synthesis in a cell-free system prepared from rat hepatocytes. *Am. J. Physiol.* **256,** C28–C34.
19. Mellor, H., Kimball, S. R., and Jefferson, L. S. (1994) Brefeldin A

inhibits protein synthesis through the phosphorylation of the α-subunit of eukaryotic initiation factor-2. *FEBS Lett.* **350,** 143–146.

20. DeGracia, D. J., Sullivan, J. M., Neuman, R. W., Alousi, S. S., Hikade, K. R., Pittman, J. E., Rafols, J. A., and Krause, G. S. (1997) Effect of brain ischemia and reperfusion on the localization of phosphorylated eukaryotic initiation factor 2α. *J. Cereb. Blood Flow Metab.* **17,** 1291–1302.
21. Scorsone, K. A., Panniers, R., Rowlands, A. G., and Henshaw, E. C. (1987) Phosphorylation of eukaryotic initiation factor 2 during physiological stresses which affect protein synthesis. *J. Biol. Chem.* **262,** 14538–14543.
22. Kimball, S. R., Horetsky, R. L., and Jefferson, L. S. (1998) Signal transduction pathways involved in the regulation of protein synthesis by insulin in L6 myoblasts. *Am. J. Physiol.* **274,** C221–C228.
23. Kimball, S. R., and Jefferson, L. S. (1991) Inhibition of microsomal calcium sequestration causes an impairment of initiation of protein synthesis in perfused rat liver. *Biochem. Biophys. Res. Commun.* **177,** 1082–1086.
24. Kimball, S. R., and Jefferson, L. S. (1992) Regulation of protein synthesis by modulation of intracellular calcium in rat liver. *Am. J. Physiol.* **263,** E958–E964.
25. Prostko, C. R., Brostrom, M. A., and Brostrom, C. O. (1993) Reversible phosphorylation of eukaryotic initiation factor 2α in response to endoplasmic reticular signalling. *Mol. Cell. Biochem.* **127–128,** 255–265.
26. Brostrom, M. A., Prostko, C. R., Gmitter, D., and Brostrom, C. O. (1995) Independent signaling of *grp78* gene transcription and phosphorylation of eukaryotic initiation factor 2α by the stressed endoplasmic reticulum. *J. Biol. Chem.* **270,** 4127–4132.
27. Pain, V. M. (1996) Initiation of protein synthesis in eukaryotic cells. *Eur. J. Biochem.* **236,** 747–771.
28. Raught, B., and Gingras, A.-C. (1999) eIF4E activity is regulated at multiple levels. *Int. J. Biochem. Cell Biol.* **31,** 43–57.
29. Clemens, M. J. (1997) PKR—A protein kinase regulated by double-stranded RNA. *Int. J. Biochem. Cell Biol.* **29,** 945–949.
30. Kaufman, R. J. (2000) Double-stranded RNA-activated protein kinase PKR. *In* Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.), pp. 503–527. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. Romano, P. R., Green, S. R., Barber, G. N., Mathews, M. B., and Hinnebusch, A. G. (1995) Structural requirements for double-stranded RNA binding, dimerization, and activation of the human eIF2α kinase DAI in Saccaromyces cerevisiae. *Mol. Cell. Biol.* **15,** 365–378.
32. Qiu, H., Garcia-Barrio, M. T., and Hinnebusch, A. G. (1998) Dimerization by translation initiation factor 2 kinase GCN2 is mediated by interactions in the C-terminal ribosome-binding region and the protein kinase domain. *Mol. Cell. Biol.* **18,** 2697–2711.
33. Chen, J.-J. (2000) Heme-regulated eIF2α kinase. *In* Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.), pp. 529–546. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. Hinnebusch, A. G. (2000) Mechanism and regulation of initiator methionyl-tRNA binding to ribosomes. *In* Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.), pp. 185–243. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. *Cell* **68,** 585–596.
36. Dever, T. E., Chen, J.-J., Barber, G. N., Cigan, A. M., Feng, L., Donahue, T. F., London, I. M., Katze, M. G., and Hinnebusch, A. G. (1993) Mammalian eukaryotic initiation factor 2α kinases functionally substitute for GCN2 protein kinase in the GCN4 translational control mechanism in yeast. *Proc. Natl. Acad. Sci. USA* **90,** 4616–4620.
37. Hinnebusch, A. G. (1997) Translational regulation of yeast *GCN4*—A window on factors that control initiator-tRNA binding to the ribosome. *J. Biol. Chem.* **272,** 21661–21664.