Double-stranded RNA-dependent Protein Kinase Phosphorylation of the \(\alpha\)-Subunit of Eukaryotic Translation Initiation Factor 2 Mediates Apoptosis\(^5\)

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As the molecular processes of complex cell stress signaling pathways are defined, the subsequent challenge is to elucidate how each individual event influences the final biological outcome. Phosphorylation of the translation initiation factor 2 (eIF2\(\alpha\)) at Ser\(^51\) is a molecular signal that inhibits translation in response to activation of any of four diverse eIF2\(\alpha\) stress kinases. We used gene targeting to replace the wild-type Ser\(^51\) allele with an Ala in the eIF2\(\alpha\) gene to test the hypothesis that translational control through eIF2\(\alpha\) phosphorylation is a central death stimulus in eukaryotic cells. Homozygous eIF2\(\alpha\) mutant mouse embryo fibroblasts were resistant to the apoptotic effects of dsRNA, tumor necrosis factor-\(\alpha\), and serum deprivation. TNF\(\alpha\) treatment induced eIF2\(\alpha\) phosphorylation and activation of caspase 3 primarily through the dsRNA-activated eIF2\(\alpha\) kinase PKR. In addition, expression of a phospho-mimetic Ser\(^51\) to Asp mutant eIF2\(\alpha\)-activated caspase 3, indicating that eIF2\(\alpha\) phosphorylation is sufficient to induce apoptosis. The proapoptotic effects of PKR-mediated eIF2\(\alpha\) phosphorylation contrast with the anti-apoptotic response upon activation of the PKR-related endoplasmic reticulum eIF2\(\alpha\) kinase, PERK. Therefore, divergent fates of death and survival can be mediated through phosphorylation at the same site within eIF2\(\alpha\). We propose that eIF2\(\alpha\) phosphorylation is fundamentally a death signal, yet it may promote either death or survival, depending upon coincident signaling events.

One of the most perplexing problems in modern biology is to understand how the cell chooses between adaptation and apoptotic demise in response to stressful insults. Because there are multiple interacting anti-apoptotic and pro-apoptotic signaling pathways, it is assumed that the sum of these signaling cascades dictates the final outcome. When one pathway becomes predominant, a delicate balance is perturbed and either an adaptive or a lethal response ensues. Advances in our knowledge of how this commitment occurs will lead to a greater understanding of cell growth and differentiation as well as the etiology of various disease states.

Numerous phosphorylation events are known to regulate the overall rate of protein synthesis or translation of selective mRNAs. However, the most dominant influence is mediated through phosphorylation at Ser\(^51\) on the \(\alpha\)-subunit of heterotrimeric eukaryotic translation initiation factor 2 (eIF2\(\alpha\))\(^3\). eIF2\(\alpha\) is required to deliver Met\(\text{tRNA}\) to the 40 S ribosomal subunit. Physiological conditions that induce eIF2\(\alpha\) Ser\(^51\) phosphorylation regulate global as well as specific mRNA translation. Phosphorylation of eIF2\(\alpha\) at Ser\(^51\) inactivates eIF2\(\alpha\) and reduces the efficiency of AUG initiation codon recognition, thereby attenuating translation initiation. However, reduced AUG initiation codon recognition can increase the initiation efficiency at selective AUG codons, thereby altering initiation site utilization to regulate both the quantity and quality of proteins produced (2).

Four protein kinases phosphorylate eIF2\(\alpha\) at Ser\(^51\) in response to different stress stimuli: 1) the dsRNA-activated protein kinase PKR is a major component of the interferon-mediated antiviral response and is activated by binding to dsRNA produced during viral infection (3); 2) the general control of nitrogen metabolism kinase GCN2 responds to amino acid depletion (4); 3) the heme-regulated inhibitor kinase HRI responds to heme deprivation to couple globin synthesis with available heme (5); and 4) the PKR-related endoplasmic reticulum (ER) kinase PERK responds to the accumulation of unfolded proteins in the ER in a subpathway of the unfolded protein response (6). Generally, eIF2\(\alpha\) phosphorylation provides a fundamental mechanism to couple the rate of protein synthesis with the capacity to fold proteins under conditions of

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\(^{3}\) The abbreviations used are: eIF2\(\alpha\), eukaryotic translation initiation factor 2 \(\alpha\)-subunit; PKR, protein kinase dsRNA-dependent; HRI, heme-regulated inhibitor protein kinase; GCN2, general control of nitrogen protein kinase; ER, endoplasmic reticulum; PERK, PKR-like ER kinase; NF-kB, nuclear factor \(\kappa\)B; TNF\(\alpha\), tumor necrosis factor-\(\alpha\); IFN, interferon; IKK, inhibitor of NF-\(\kappa\)B kinase; TNFR1, tumor necrosis factor-\(\alpha\) receptor 1; Act D, actinomycin D; CHX, cycloheximide; IAPs, inhibitors of apoptosis; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; UPR, unfolded protein response; MEF, mouse embryo fibroblasts; CHAPS, 4-morpholinoethane-sulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium; dsRNA, double-stranded RNA.
Phosphorylation of eIF2α Subunit Mediates Apoptosis

Different physiological stress, such as nutrient deprivation or viral infection.

Although the mechanism by which phosphorylation of eIF2α inhibits protein synthesis is well characterized, the cellular responses to eIF2α phosphorylation remain elusive. Recent studies support the idea that eIF2α phosphorylation promotes survival under conditions of oxidative stress and accumulation of unfolded proteins in the lumen of the ER (7, 8). In contrast, eIF2α phosphorylation was proposed to mediate apoptosis in response to PKR activation (9–12). In this study, we addressed how eIF2α phosphorylation influences the balance between survival and apoptosis upon activation of PKR.

Treatment of cells with interferon and dsRNA is cytotoxic, and data support the hypothesis that this toxicity is mediated by PKR activation and induction of apoptosis (13). Although a number of different signal transduction and transcriptional programs are influenced through PKR activation (for review see Ref. 14), the most well characterized PKR substrate is eIF2α. The growth suppressing activity mediated through eIF2α phosphorylation is an evolutionarily well conserved cell response. Either inactivation of the PKR pathway (12, 15–22) or overexpression of a nonphosphorylatable S51A mutant eIF2α (10, 12) protects from stress-mediated apoptosis. These studies provide compelling evidence for an anti-proliferative effect of PKR-mediated eIF2α phosphorylation in growth inhibition. However, the interpretation of these results is confounded because of the diverse effects that PKR activation has on multiple stress signaling pathways. Therefore, to date there is no direct evidence to support the hypothesis that eIF2α phosphorylation is necessary and/or sufficient for apoptosis.

We propose that PKR activation with subsequent eIF2α phosphorylation is a primary mechanism that 1) initiates inhibition of protein synthesis, and 2) contributes to apoptosis in response to a variety of physiological and environmental stimuli. To test this hypothesis, we have studied apoptosis induced by dsRNA, TNFα, or serum deprivation in cells that harbor a homozygous S51A knock-in mutation at the phosphorylation site in eIF2α (23). Here, we show that apoptosis induced by TNFα, the interferon pathway, and serum deprivation requires PKR-mediated phosphorylation of eIF2α. In addition, expression of a Ser51 to Asp phospho-mimetic mutant of eIF2α was sufficient to activate caspase 3 in the absence of any apoptosis-inducing stimuli. The results demonstrate that transnational inhibition through eIF2α phosphorylation contributes to and can be sufficient to activate an apoptotic response.

MATERIALS AND METHODS

Plasmid DNA Transfection—The poly(ADP-ribose) polymerase (PARP) cDNA cloned in pCDNA3 was kindly provided by Dr. M. Keifer, (LXR Biotechnologies). The eIF2α expression vectors were previously described (24). Transfection of eIF2α expression plasmids into HeLa cells was performed as described (25). After transfection, the cells were washed twice with Dulbecco’s modified essential medium (DMEM) and incubated at 5% CO2 for 2 days in DMEM with 10% fetal bovine serum-containing antibiotics. The cells were washed twice with phosphate-buffered saline (PBS), harvested using Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 8.0) with complete protease inhibitors (Roche), and incubated on ice for 15 min followed by centrifugation at 10,000 rpm for 10 min. The supernatant was collected, and protein concentrations were determined by the Bradford method (26).

In Vitro Transcription and Translation of PARP—In vitro transcription and translation of PARP was performed in the presence of [35S]methionine/cysteine (Redivue PRO-MIX, Amersham Biosciences.) using the TnT kit (Promega Biotech) following the manufacturer’s instructions. Cleavage of in vitro translated PARP was previously described (27). The reaction products were analyzed by SDS-PAGE and autoradiography using ENHANCE (Dupont). Briefly, a 50-μl reaction containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10% sucrose, 20 μg of cell extract protein from transfected COS-1 cells, and 2 μl of in vitro translated [35S]methionine/cysteine-labeled PARP were mixed and incubated at 37 °C for 2 h. Then, 25 μl of 3× SDS-PAGE sample buffer was added to each sample followed by heating at 90 °C for 5 min. The reaction products were analyzed by SDS-PAGE under reducing conditions. After electrophoresis, the gels were fixed, soaked for 45 min in ENHANCE (Dupont), dried, and subjected to autoradiography. ImageJ (Version 1.31 for Mac OS X, NIH) was used to quantitate band intensities.

Mouse Embryonic Fibroblasts—MEFs from wild-type, eIF2α S51A mutant, Hri−/−, and Pkr−/− 14.5 day embryos were prepared from breeding heterozygous mutant mice as previously described (23). MEFs isolated from wild-type littermates were used as controls. Pkr−/− mice were provided by Dr. Bryan Williams, (28) (Cleveland Clinic). Hri−/− mice were provided by Jane-Jane Chen (5)(MIT). Perk−/− (29) and Gcn2−/− (30) MEFs and their wild-type control MEFs were kindly provided by Drs. David Ron and Heather Harding (New York University). Cells were cultured in DMEM with 10% fetal bovine serum, penicillin-streptomycin, essential and unessential amino acids.

Immunoblot Analysis—Cells were treated as described and harvested using Nonidet P-40 lysis buffer containing 150 mM NaCl, complete protease inhibitors (Roche), and 100 μg/ml phenylmethylsulfonyl fluoride. Lysis buffer additionally included 500 mM β-glycerol phosphate, 50 mM sodium orthovanadate, and 1× phosphatase inhibitor (Sigma P2850) (Fig. 3, E and F; supplemental Fig. S2). Samples were centrifuged at 10,000 rpm, and supernatants were collected for SDS-PAGE and transfer to nitrocellulose. The eIF2α Ser51 phosphospecific antibody was obtained from BioSource (Camarillo, CA) and PKR antibody was kindly provided by Dr. Bryan Williams (Cleveland Clinic). Phosphospecific PKR antibody (3075) and PKR antibody (3072), (Fig. 3, E and F, supplemental Fig. S2), were obtained from Cell Signaling. The antibody that recognizes total eIF2α was previously described (23). Anti-TNFRI antibody (SC-8436) was obtained from Santa Cruz Biotechnology. All Western blotting was performed with chemiluminescence detection and quantitation of film band intensities was performed with ImageJ (Version 1.31 for Mac OS X, NIH).

Measurement of Translation Rates—MEFs were cultured as described above. After overnight culture, subconfluent cultures were treated with culture medium containing TNFα, okadaic acid (OA), or poly(rI-C) as described. Cells were washed two
times with PBS and incubated in methionine/cysteine-free medium including 200 μCi/ml [35S]methionine/cysteine (Redivue PRO-MIX, Amersham Biosciences) in the continued presence of the described stimulus for 15 min. Cells were washed twice with ice-cold PBS and cell lysates were prepared in Nonidet P-40 lysis buffer containing complete protease inhibitors as described above for immunoblot analysis. Protein concentration was determined by the Bradford method (26). Trichloroacetic acid precipitation was performed by spotting samples on Whatman filter paper with subsequent washing in ice-cold 20% trichloroacetic acid, 10% trichloroacetic acid, and 100% ethanol. Filters were dried and liquid scintillation counting was performed.

Real-time Quantitative RT-PCR—Total RNA was isolated from MEFs at 12 h after cell plating using the TRIzol method (Invitrogen), and RNA was dissolved in diethylpyrocarbonate-treated water containing 1 unit/μl RNase inhibitor (Roche). Reverse transcription reactions were performed with i-Script (Bio-Rad) and then diluted 25-fold with water for real-time PCR in an i-Cycler machine using 9 μl of diluted reverse transcriptase product and iQ SYBR Green Supermix in a 20-μl reaction (Bio-Rad). The amplification primers used for TNFR1 detection were forward (5′-CATCCCAAGCAGTGCTATG-3′) and reverse (5′-GCTACAGGTTAGCTGAC-3′) and the primers used for β-actin amplification were forward (5′-CTCTATGCCAACACAGTGC-3′) and reverse (5′-GTACTTGCCGTCAGGAG-3′).

Cell Survival—Cells were cultured on 10-cm tissue culture dishes. At 24 h after plating, apoptosis was induced by treatment with culture medium containing 100 pg/ml poly(rI-C) (Amersham Biosciences) for 16–18 h or 1 ng/ml TNFα (Invitrogen) for 18–21 h including 50 ng/ml actinomycin D (Act D) (Sigma) during both incubations. Serum deprivation was performed by washing cells three times with serum-free DMEM followed by 14–23 h of incubation in DMEM containing 0.01% serum. In the morphological studies, cells were cultured on coverslips coated with 1% gelatin, treated as described, and fixed with 10% formalin (Sigma) prior to phase contrast microscopy. Cell viability was quantified by trypan blue dye exclusion. For nuclear staining, cells were plated and fixed with 10% formalin (Sigma) prior to phase contrast microscopy. Cell viability was quantified by trypan blue dye exclusion.

Caspase 3 Assay—Adherent and floating cells were washed three times with PBS, collected at 1,200 × g and resuspended in 100–200 μl of 25 mM HEPES, pH 7.5, 5 mM MgCl2, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The samples were lysed by four freeze-thaw cycles, centrifuged at 10,000 rpm, and the supernatant was collected for caspase 3 fluorescent assay using 70–80 μg of protein extracts as described by the supplier (Promega CaspACE Fluorometric Assay System, Madison, WI). Addition of the supplied caspase 3 inhibitor peptide to the cell lysates inhibited all activity (supplemental Fig. S1). A SPECTRAMax Gemini XS spectrophotometer (Molecular Devices, Sunnyvale, CA) was used with excitation, emission, and cutoff wavelengths of 368 nm, 467 nm, and 420 nm respectively. Protein concentrations were determined using a detergent-compatible assay (Bio-Rad).

IKK Assay—IKK activity was measured by an immune complex kinase assay as previously described (31, 32). Briefly, cell lysates were immunoprecipitated with anti-IKKα antibody and the immune complexes used for phosphorylation of a GST-IκBα(1–54) peptide substrate.

RESULTS

Phosphorylation of eIF2α Is Sufficient to Activate Caspase 3—To elucidate whether PKR activation and/or eIF2α phosphorylation are sufficient to activate apoptosis through activation of caspase 3, wild-type, and mutant forms of eIF2α and PKR were transiently transfected into HeLa cells in the presence of a procaspase 3 expression vector. Because transiently co-transfected cells express both plasmid DNAs, using this approach it is possible to measure caspase 3 activation in the subpopulation of co-transfected cells that transiently express wild-type or mutant forms of PKR or eIF2α.

Western blot analysis demonstrated that significant levels of procaspase 3 were detected only in cells that received the procaspase 3 expression vector (Fig. 1A, lanes 1–3, 7–9 versus 4–6). However, the total amount of procaspase 3 was 3-fold lower in cells co-transfected with either wild-type PKR or S51D phospho-mimetic mutant eIF2α expression vectors compared with the other transfectants (Fig. 1A, lanes 2 and 9). This is consistent with cleavage and activation of procaspase 3 or with the translational inhibition observed upon overexpression of wild-type PKR or the S51D mutant eIF2α (33). At 48-h post-transfection, caspase 3 activity...
Wild-type and mutant MEFs were pretreated with 400 units/ml interferon to total eIF2 $\alpha$. Blue dye exclusion indicated that the viable cell counts of treated wild-type MEFs and dsRNA-induced cell death. MEFs were treated with poly(rI-C) (0.1 A) and wild-type MEFs, the activation of caspase 3 was significantly impaired in MEFs that harbor the homozygous S51A mutant eIF2 $\alpha$ (Fig. 2A, lanes 3 and 4), consistent with the presence of the homozygous eIF2 $\alpha$ mutation in these cells. Where treatment of Pkr $^{-/-}$ MEFs with interferon, and poly(rI-C) also increased levels of eIF2 $\alpha$ phosphorylation 1.4-fold over that observed under control conditions, there was no increase in the Pkr $^{-/-}$ MEFs (Fig. 2A, lanes 5–8). Western blot analysis confirmed that the Pkr $^{-/-}$ MEFs did not express PKR (Fig. 2A). These results support the notion that PKR is the major eIF2 $\alpha$ kinase activated under these conditions.

Morphological analysis of cells treated with poly(rI-C) indicated a distinct difference in survival. Act D was included at a low concentration to prevent the anti-apoptotic response mediated by NF-$\kappa$B activation under these conditions (11). Act D is necessary to elicit an apoptotic response in cultured MEFs. Cycloheximide is also an apoptotic sensitizer that may be used in conjunction with TNF$\alpha$ (34). Compared with both wild-type Pkr $^{+/+}$ and wild-type eIF2 $\alpha$ S/S MEFs that did not survive this treatment, the survival of homozygous eIF2 $\alpha$A/A mutant MEFs was not compromised (Fig. 2B). Analysis of viability by trypan blue dye exclusion was consistent with the morphological observations (Fig. 2B, legend). These results demonstrate that inactivation of either the eIF2 $\alpha$ kinase PKR or mutation at the Ser$^{51}$ phosphorylation site in eIF2 $\alpha$ produced substantial resistance to poly(rI-C)-induced death.

To quantitatively monitor a direct marker of apoptosis, caspase 3 activity was measured in prepared cell lysates. Where a 16-h treatment with poly(rI-C) increased caspase 3 activity in wild-type MEFs, the activation of caspase 3 was significantly impaired in MEFs that harbor the homozygous S51A mutant eIF2 $\alpha$ (A/A) (Fig. 2C). In addition, caspase 3 activation was reduced 50% in the Pkr $^{-/-}$ MEFs, consistent with earlier findings described under "Materials and Methods." Caspase 3 activation was significantly reduced in eIF2 $\alpha$ A/A MEFs and Pkr $^{-/-}$ MEFs compared with their respective controls. ***, $p < 0.001.$

**Phosphorylation of eIF2 $\alpha$ Subunit Mediates Apoptosis**

**FIGURE 2. dsRNA-induced apoptosis requires PKR-mediated eIF2 $\alpha$ phosphorylation.** A, Ser$^{51}$ phosphorylation of eIF2 $\alpha$ is not detected in eIF2 $\alpha$A/A MEFs and dsRNA does not induce eIF2 $\alpha$ phosphorylation in Pkr $^{-/-}$ MEFs. Wild-type and mutant MEFs were pretreated with 400 units/ml interferon-$\alpha$ overnight and then with poly(rI-C) for 8 h. Cell extracts were prepared for Western blot analysis with anti-phosphopeptide-specific eIF2 $\alpha$, total anti-eIF2 $\alpha$, or anti-PKR antibodies. The intensities of eIF2 $\alpha$ phosphorylation relative to total eIF2 $\alpha$ levels are indicated. B, Pkr $^{-/-}$ and eIF2 $\alpha$ A/A MEFs are resistant to dsRNA-induced cell death. MEFs were treated with poly(rI-C) (0.1 mg/ml) and Act D (10 ng/ml) for 16 h and then analyzed by light microscopy. Act D treatment alone did not significantly affect cell morphology. Trypan blue dye exclusion indicated that the viable cell counts of treated wild-type Pkr $^{-/-}$ and eIF2 $\alpha$ S/S MEFs were $\approx 20\%$ of control vehicle-treated cultures. In contrast, the viable cell counts were $\approx 60$ and $45\%$ in the Pkr $^{-/-}$ and the eIF2 $\alpha$ A/A mutant MEFs, respectively. C, procaspase 3 activation is reduced in eIF2 $\alpha$ A/A and Pkr $^{-/-}$ MEFs. MEFs were treated with poly(rI-C) and Act D for 20 h, and then cell extracts were prepared for analysis of caspase 3 activity as measured in cell lysates using a PARP cleavage assay. In the presence of vector alone or in the presence of vectors expressing K296P trans-dominant-negative mutant kinase PKR or S51A non-phosphorylatable mutant eIF2 $\alpha$, a low level of PARP cleavage was detected (Fig. 1B, lanes 1 and 2 and 10 and 13). In contrast, co-transfection with vectors that express either wild-type PKR or S51D mutant eIF2 $\alpha$, increased the amount of PARP cleavage 3–5-fold (Fig. 1B, lanes 3 and 12). These results show that expression of either wild-type PKR or a S51D mutant eIF2 $\alpha$ activates caspase 3 compared with expression of K296P mutant PKR or S51A mutant eIF2 $\alpha$. Therefore, we conclude that phosphorylation of eIF2 $\alpha$ is sufficient to activate caspase 3.

Interferon $\alpha$- and dsRNA-induced Apoptosis Requires eIF2 $\alpha$ Phosphorylation—We then tested whether PKR-mediated apoptosis requires eIF2 $\alpha$ phosphorylation by studying MEFs that harbor a knock-in replacement of Ser$^{51}$ for Ala in the endogenous eIF2 $\alpha$ gene (23). Wild-type (S/S) and homozygous (A/A) eIF2 $\alpha$ mutant MEFs were treated with interferon $\alpha$ and poly(rI-C) to strongly activate the PKR pathway. Whereas treatment with interferon $\alpha$ and poly(rI-C) increased the level of phosphorylated Ser$^{51}$ eIF2 $\alpha$ in the wild-type S/S MEFs 1.9-fold (Fig. 2A, lanes 1 and 2), phosphorylated eIF2 $\alpha$ was not detected in the homozygous A/A mutant MEFs (Fig. 2A, lanes 3 and 4), consistent with the presence of the homozygous eIF2 $\alpha$ mutation in these cells. Where treatment of Pkr $^{+/+}$ MEFs with interferon $\alpha$ and poly(rI-C) also increased levels of eIF2 $\alpha$ phosphorylation 1.4-fold over that observed under control conditions, there was no increase in the Pkr $^{-/-}$ MEFs (Fig. 2A, lanes 5–8). Western blot analysis confirmed that the Pkr $^{-/-}$ MEFs did not express PKR (Fig. 2A). These results support the notion that PKR is the major eIF2 $\alpha$ kinase activated under these conditions. **TABLE 2. dsRNA-induced apoptosis was compared with the respective controls.***, $p < 0.001.$
Phosphorylation of eIF2α Subunit Mediates Apoptosis
In contrast, the same treatment produced no apoptotic nuclei (41/271 fragmented apoptotic nuclei, 57/271 picnotic nuclei). Perk—cells are resistant to apoptotic stimulation (9–12, 18). These data suggest that eIF2α phosphorylation contributes to PKR-mediated cell death, although it is not absolutely required.

**eIF2α Phosphorylation Is Required for TNFα-induced Apoptosis**—To determine the role of eIF2α phosphorylation in response to another inducer of apoptosis, the response to TNFα was measured. Although previous studies suggest that TNFα induces eIF2α phosphorylation (9–11), this has not been directly demonstrated nor has the role of PKR and possibly other eIF2α kinases in this process been established. Treatment of subconfluent wild-type MEFs with TNFα induced apoptotic bodies in 36% of the cells analyzed by propidium iodide staining (41/271 fragmented apoptotic nuclei, 57/271 picnotic nuclei). In contrast, the same treatment produced no apoptotic nuclei derived from C57Bl/6, and the eIF2α S/S and eIF2α A/A MEFs were derived from C57Bl/6 X 129/Sv.

To verify the validity of the protective effect of the eIF2α mutation, two additional wild-type MEF isolates were derived from independent litters of the eIF2α mouse strain (S/S-2 and S/S-3) and analyzed. Both lines displayed remarkably similar degrees of caspase 3 activation upon treatment with TNFα compared with the original control MEFs (eIF2α S/S, S/S-1) (Fig. 3C). Additionally, an alternative preparation of homozygous eIF2α mutant MEFs (A/A-2) was also markedly impaired in caspase 3 activation in response to TNFα.

To evaluate the requirement for the three additional eIF2α kinases in TNFα signaling of eIF2α phosphorylation and apoptosis, caspase 3 activity was analyzed in Perk−/− (29), Hri−/− (5), and Gcn2−/− (35) MEFs and their respective wild-type counterparts.

![Graph A](image1.png)  
**FIGURE 4.** TNFα-induced apoptosis requires translational attenuation. A and B, inhibition of protein synthesis complements the defect in eIF2α phosphorylation to induce apoptosis. The indicated MEFs were treated with TNFα (1 ng/ml) and CHX (10 μg/ml) for 2–7 h (A and B). Act D is not required for induction of caspase 3 activity under these conditions. In comparison to wild-type control MEFs, caspase 3 activation was significantly reduced in Pkr−/−, and eIF2α A/A MEFs ***, p < 0.001. C, proteasome inhibition protects from TNFα-induced apoptosis. Cells were treated same as above (Fig. 3, A and B), except lactacystin (LC) was present during the TNFα treatment. Lactacystin treatment decreased caspase 3 activation in wild-type MEFs treated with TNFα and Act D. ***, p < 0.001.

![Graph B](image2.png)  
![Graph C](image3.png)

![Graph D](image4.png)  
**FIGURE 3.** TNFα-induced apoptosis requires phosphorylation of eIF2α. A, nuclear fragmentation is extensive in wild-type MEFs and does not occur in homozygous S51A eIF2α A/A mutant MEFs. Wild-type eIF2α S/S and homozygous eIF2α A/A MEFs were incubated 18 h with TNFα/Act D as described under “Materials and Methods.” Nuclear morphology was visualized by staining with propidium iodide and fluorescence microscopy. B–D, caspase 3 activation in response to TNFα requires eIF2α phosphorylation and the eIF2α kinase PKR. The indicated MEFs were treated with TNFα/Act D for 18 h, and caspase 3 activation was measured as described under “Materials and Methods.” Caspase 3 activity was significantly reduced in eIF2α A/A MEFs and Pkr−/− MEFs compared with their respective wild-type MEFs ***, p < 0.01; ***, p < 0.001. Analysis of independently derived wild-type eIF2α S/S MEFs, S/S-1, S/S-2, S/S-3, and a second eIF2α A/A MEF isolate, A/A-2, is shown in C. The Perk−/− and Gcn2−/− MEFs are in the same genetic C57Bl/6 background so only wild-type Perk−/− MEFs are shown (D). E and F, TNFα mediates eIF2α phosphorylation through PKR activation. Wild-type and Pkr−/− MEFs were treated with TNFα (10 ng/ml), poly,r(C) (100 μg/ml), and okadaic acid (OA, 100 nM) for 0–4 h and then harvested for Western blot analysis. The blot was sequentially probed with anti-PKR-Thr^172-, anti-eIF2α-Ser^51P antibody and anti-actin antibody. Phosphorylated species were quantified and expressed relative to the actin level. Act D was not included during these studies or those shown in G. G, TNFα inhibits protein synthesis in a PKR-dependent manner. Cells were treated as described in E and F and labeled with [35S]methionine/cysteine for 15 min in the continued presence of stimuli. Incorporation was measured and corrected for the amount of protein as described under “Materials and Methods.” Data are mean ± S.E. of triplicate measurements, Pkr−/− versus Pkr−/−; ***, p < 0.01; ***, p < 0.001.
Phosphorylation of eIF2α Subunit Mediates Apoptosis

FIGURE 5. eIF2α phosphorylation is not required for PKR-mediated activation of IKK. Cells expressing wild-type or designated mutants of PKR or eIF2α were assayed for IKK activity after treatment with poly(rI-C), TNFα, or IL-1 (A), Real-time quantitative RT-PCR for TNFR1 mRNA (B) and Western blot analysis (C) for TNFR1 and eIF2α was performed using lysates from logarithmically growing cells as described under "Materials and Methods."

control MEFs. TNFα treatment significantly elevated caspase 3 activity in both the wild-type and all knock-out MEFs, indicating that the TNFα-dependent caspase 3 activity does not require PERK, HRI, or GCN2 eIF2α kinases (Fig. 3D).

Next we explored the relationship between TNFα receptor signaling, PKR activation, and eIF2α phosphorylation. We tested whether TNFα treatment leads to PKR activation, eIF2α phosphorylation, and translational inhibition. Western blot analysis using a phosphopeptide-specific antibody detected an ~2.5-fold increase in activated PKR-Thr451-P in wild-type MEFs treated with TNFα for 2 or 4 h (Fig. 3E, lanes 3 and 4). These treatments did not alter the steady state level of PKR (data not shown and supplemental Fig. S2). PKR-Thr451-P was not detected in Pkr−/− MEFs indicating specificity of the antibody (Fig. 3E, lane 1). In addition, TNFα stimulation induced an ~2.5-fold increase in eIF2α-Ser51-P (Fig. 3E). The TNFα-mediated increases in PKR-Thr451-P and eIF2α-Ser51-P were similar to those observed upon treatment of MEFs with poly(rI-C), which is a very strong stimulus for PKR activation (Fig. 3E, lane 6). These increases in phospho-PKR and phospho-eIF2α, were reproducibly detected in independent experiments (supplemental Fig. S2). When TNFα stimulation was performed in the presence of the phosphatase inhibitor okadaic acid, slightly larger increases in PKR phosphorylation and eIF2α phosphorylation were observed. In contrast, poly(rI-C), and TNFα did not increase eIF2α phosphorylation in Pkr−/− MEFs (Fig. 3F). These results demonstrate that TNFα signaling activates PKR and is required to elicit eIF2α phosphorylation under these conditions.

Because TNFα induces PKR activation and eIF2α phosphorylation, we asked whether protein synthesis is inhibited upon TNFα treatment. After 4 h, TNFα inhibited protein synthesis to ~40% in wild-type MEFs, but had little effect in Pkr−/− MEFs (Fig. 3G). Treatment with okadaic acid alone or okadaic acid with TNFα reduced protein synthesis to ~25% in wild-type MEFs, consistent with the increased eIF2α phosphorylation observed in the presence of okadaic acid. In contrast, TNFα treatment only modestly reduced protein synthesis to ~90% in the Pkr−/− MEFs, consistent with the reduced level of eIF2α phosphorylation measured (Fig. 3F). Overall, the same conditions that elicit eIF2α phosphorylation measured by Western blot analysis also inhibit translation in a PKR-dependent manner. Therefore, TNFα treatment inhibits protein synthesis through the PKR-eIF2α pathway.

TNFα-induced Apoptosis Requires Protein Synthesis Inhibition—Phosphorylation of eIF2α inhibits protein synthesis at the level of initiation. Our results support the hypothesis that phosphorylation of eIF2α is required for apoptosis induced by poly(rI-C) and TNFα. To test the requirement for protein synthesis in the TNFα apoptotic response, we measured the effect of protein synthesis elongation inhibition on caspase 3 activation induced by TNFα. Increasing time of cycloheximide (CHX) treatment in the presence of TNFα very rapidly increased caspase 3 activation in the eIF2α wild-type and Pkr−/− MEFs (Fig. 4, A and B). In contrast, caspase 3 activation was significantly reduced in the eIF2α A/A and Pkr−/− mutant MEFs. However, in these mutant MEFs, 6 h of CHX treatment restored activation of caspase 3. Therefore, the protective effect of the S51A mutant eIF2α allele or PKR deletion could be partially reversed by general inhibition of protein synthesis.

These results suggest that eIF2α phosphorylation may inhibit the translation of a short lived inhibitor of apoptosis, such as p53 (36) or inhibitors of caspase activation (IAPs). Most IAPs contain a C-terminal RING-Zinc finger domain that has ubiquitin ligase (E3) activity and is responsible for their rapid degradation mediated by the proteasome (37). Therefore, inhibition of proteasome activity to prevent p53 and/or IAP degradation may also protect cells from the caspase activation. Indeed, treatment with the proteasome inhibitor lactacystin did partially prevent caspase activation in the wild-type cells (Fig. 4C), as previously described (12).

eIF2α Phosphorylation Is Not Required for TNFα Signaling and Activation of IKK—Under conditions of ultraviolet light or ER stress, eIF2α phosphorylation facilitates activation of NF-κB by decreasing translation of IκB (38, 39). Because PKR is also known to activate IKK in response to dsRNA (40), we determined the requirement for eIF2α phosphorylation in this response. Treatment with poly(rI-C), dsRNA, TNFα, or IL-1, activated IKK to a similar degree in the wild-type and homozygous eIF2α mutant A/A MEFs (Fig. 5A). These results demonstrate that eIF2α phosphorylation is not required for IKK activation by these stimuli and are consistent with reports that the catalytic activity of PKR is not required to signal NF-κB activation and target gene activation (11, 40–42). In addition, transcriptional activation of a luciferase reporter gene under control of three NF-κB binding sites was not altered in the homozygous eIF2α A/A mutant MEFs (data not shown). These studies demonstrate that impaired receptor signaling is not the
reason eIF2α mutant A/A MEFs are resistant to TNFα-mediated apoptosis.

Previous studies suggested that TNFR1 mRNA is downregulated in cells that express a trans-dominant-negative PKR mutant (15). Indeed, mRNA analysis by real-time quantitative RT-PCR demonstrated that homozygous eIF2α A/A and Pkr−/− MEFs did express lower levels of TNFR1 mRNA (Fig. 5B). However, Western blot analysis of TNFR1 protein demonstrated similar levels of expressed TNFR1 protein, relative to the loading control eIF2α (Fig. 5C). In conclusion, these results demonstrate that although TNFR1 mRNA was reduced in the mutant MEFs, the levels of TNFR1 protein were not altered and that signaling from the TNFα receptor to NF-κB activation was functional in the mutant MEFs.

PKR-mediated eIF2α Phosphorylation Is Required for Serum Deprivation-induced Apoptosis—Because previous studies suggested that serum deprivation induces apoptosis through PKR-mediated phosphorylation of eIF2α (10), we analyzed the response to serum deprivation in the wild-type, Pkr−/−, and eIF2α A/A MEFs. Where serum deprivation induced eIF2α phosphorylation by greater than 5-fold in wild-type MEFs (Fig. 6A), significantly less eIF2α phosphorylation occurred (1.8-fold) in Pkr−/− MEFs. Serum deprivation activated caspase 3 by 5–7-fold in wild-type MEFs. In contrast, serum deprivation did not activate caspase 3 in the Pkr−/− or eIF2α A/A MEFs (Fig. 6, B and D). Therefore, PKR-mediated phosphorylation is required for apoptosis induced by a different stimulus in the absence of the transcriptional blockade with Act D.

DISCUSSION

Although it is known that PKR activation leads to apoptosis, there is controversy as to what events downstream of PKR activation mediate apoptosis. It has been proposed that PKR mediates apoptosis at the transcriptional level. For example, PKR signals through STAT1 and STAT3 (36, 43, 44), interferon regulatory factor 1 (IRF1) (16, 45), IRF3 (46), p53 (17, 47, 48), and the IKK complex to regulate transcription during proinflammatory (16, 17, 40, 45) and/or proapoptotic responses (12, 40, 45, 49). PKR-dependent apoptosis was also associated with Fas-associated death domain (FADD)-mediated activation of caspase 8 (50, 51) and up-regulation of Fas and Bax (18, 52, 53). In addition, PKR can signal through the mitogen-activated protein kinase cascade to activate p38 and JNK in the innate immune response to bacterial endotoxin (54). Alternative studies support the notion that PKR mediates apoptosis through phosphorylation of eIF2α and translational inhibition (10–12, 53). In addition to translational control through eIF2α phosphorylation, PKR was also implicated in regulation of the eIF4F cap-binding protein complex by phosphorylation of B56a, a regulatory subunit of protein phosphatase PP2A that dephosphorylates eIF4E to increase translation (55).
We have studied the role of eIF2α phosphorylation by analysis of cells that express S51A or S51D mutants of eIF2α. The following results support the hypothesis that eIF2α phosphorylation is alone sufficient to activate apoptosis and in addition, is required for the apoptotic response to PKR activation. First, transient overexpression of wild-type PKR or S51D mutant eIF2α-induced caspase 3 activation (Fig. 1). Second, caspase 3 activation in cells that harbor a knock-in S51A mutation in eIF2α was significantly reduced in response to TNFα, poly(rI-C), as well as serum deprivation (Figs. 2 and 3). Our studies directly demonstrate that TNFα activates PKR to phosphorylate eIF2α and inhibit translation. TNFα-mediated apoptosis required eIF2α phosphorylation, whereas TNFα-dependent activation of IKK did not require eIF2α phosphorylation. This is consistent with results that demonstrate PKR signals activation of IKK in a manner that does not require PKR kinase activity (11, 40–42). Furthermore, TNFα-induced eIF2α phosphorylation was exclusively dependent on PKR, and not any of the other known eIF2α kinases. Finally, treatment with CHX partially restored caspase activation in the S51A eIF2α A/A mutant cells, suggesting that eIF2α phosphorylation mediates its apoptotic effects through translational inhibition (Fig. 4). These findings support the idea that apoptosis does not require new protein synthesis and that all the machinery required for cell death preexists in the cell. This finding is consistent with a requirement for continued protein synthesis to maintain a pool of short-lived anti-apoptotic factors, such as p53 or IAPs (Fig. 7) (36). The latter was also supported by the protective effect observed by proteasomal inhibition, conditions that should stabilize short-lived protective molecules.

Our results indicate that eIF2α phosphorylation is necessary and sufficient for the PKR apoptotic response. These findings are in contrast to conclusions recently derived from observations using an inducible overexpression system to produce S51A and S51D mutants of eIF2α (56). These studies did not detect a complete reduction in protein synthesis that would be expected with S51D eIF2α expression (33). In addition, although cell number was significantly reduced at 24 h post-induction, apoptosis was not measured at this time. When analyzed at 6 days after induction of the S51D mutant eIF2α, apoptosis was not detected. These results suggest that the robust apoptotic effect of eIF2α phosphorylation may be transient, with subsequent survival of a subpopulation of cells that activate adaptive mechanisms. In contrast, our apoptosis studies using transient DNA transfection of S51D mutant eIF2α in HeLa cells were performed at 24-h post-transfection, early after expression of S51D eIF2α commenced. Previous studies support the idea that apoptosis through PKR activation is rapid, occurring within 24 h (9, 57). Our studies are consistent with additional findings that support the conclusion that PKR-mediated phosphorylation of eIF2α promotes apoptosis. First, overexpression of S51A mutant eIF2α protected from vaccinia virus-, TNFα-, and serum deprivation-induced apoptosis (10, 12). In addition, macrophages from S51A eIF2α homozygous mutant mice were resistant to apoptosis induced by lipopolysaccharide treatment in the presence of p38 MAPK inhibition (9). This apoptotic response is mediated through toll-like receptor 4 and PKR. In contrast to the requirement for eIF2α phosphorylation for apoptosis mediated through PKR activation, S51A mutation in eIF2α or deletion of the eIF2α kinase PERK in MEFs dramatically increased sensitivity to agents that disrupt protein folding and produce stress in the ER (23, 29). Therefore, it was surprising that the same S51A eIF2α A/A mutant MEFs were resistant to apoptotic stimuli that signal through PKR activation. It is unknown how two different stress stimuli that signal through phosphorylation at the same site in eIF2α result in opposing responses. We propose that upon ER stress, cells stimulate the death-inducing property of eIF2α phosphorylation. However, the outcome is survival because eIF2α phosphorylation decreases the protein-folding burden on the ER to relieve the stress. In addition, ER stress induces auxiliary pathways to reverse eIF2α phosphorylation so that the eIF2α phosphorylation is transient (58). Thus, the ER-stressed cell may benefit from acute reduction of biosynthetic load, while escaping apoptosis in the long term through eIF2α dephosphorylation.

We hypothesize that the delicate balance between cell survival and death upon a stress stimuli is determined by the strength of the primary death-inducing stimulus and the input of auxiliary and compensatory pathways that are coordinately activated. Some of these secondary signals may assist or be required for death while others may be protective. We propose that eIF2α phosphorylation is fundamentally a death-promoting signal. Our data show that TNFα-induced eIF2α phosphorylation inhibits translation and possibly mediates apoptosis by inhibiting the synthesis of anti-apoptotic cellular factors, such as IAPs (Fig. 7). Under these conditions, the primary death signal to elicit caspase activation is increased. In addition, eIF2α phosphorylation also thwarts the adaptive transcriptional
response through translational inhibition to prevent synthesis of protective factors.

Given the observations of the importance of PKR and eIF2α phosphorylation in apoptosis, it was interesting and curious that homozygous mutation of S51A in eIF2α, Pkr deletion, or expression of a trans-dominant-negative mutant Pkr did not have an obvious developmental phenotype in the mouse (23, 28, 59, 60). These findings would support the idea that eIF2α phosphorylation is not an essential apoptotic signal in mammalian embryonic development, a process where apoptosis plays a central role. This is in contrast to dramatic phenotypes observed in mice harboring deletions in essential caspase genes or key modulators of apoptosis (61, 62). However, it is consistent with absence of embryonic lethality in mice lacking the known TNFα receptor family members (63). Thus, death receptor signaling has a lesser role in development than essential apoptosis effectors.

Although TNFα receptor signaling is not required for embryonic apoptosis, there are a number of circumstances where TNFα-induced apoptosis is physiologically important including lipopolysaccharide-mediated apoptosis in the liver (64), hepatotoxicant-induced apoptosis (65), suppression of acute HSV-1 viral infection (66), limitation of T cell number during chronic LCMV viral infection (67), infarction induced myocardial rupture and ventricular dysfunction (68), and death of malformed embryos (69). Adenovirus delivery of TNFα induced apoptosis in esophageal cancer cells in a manner that required Pkr (70), suggesting the utility of this approach to promote apoptosis in transformed cells. The delineation of TNFα signaling to eIF2α phosphorylation and apoptosis established by our studies suggests eIF2α phosphorylation may be an important death signal in these physiologically important apoptotic events. Conditional homozygous eIF2α A/A mice with tissue specific expression will provide important tools for future studies on TNFα-induced apoptosis.

Regulation of eIF2α phosphorylation could provide an attractive target for therapeutic intervention. Agents that inhibit eIF2α phosphorylation could promote survival under desired conditions, for example to inhibit macrophage apoptosis upon viral infection (9) or prevent ischemic cell injury (71). Alternatively, direct targeting of therapeutic agents to induce eIF2α phosphorylation may accentuate apoptosis of virus-infected cells (72). Future analysis of potential therapeutics to increase eIF2α phosphorylation likely will lead to death promoting applications in anti-tumor or anti-microbial targeted therapeutics as well as to protective functions in ER stress-related disease.

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Phosphorylation of eIF2α Subunit Mediates Apoptosis

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