

Translational Repression Mediates Activation of Nuclear Factor Kappa B by Phosphorylated Translation Initiation Factor 2

Jing Deng,¹ Phoebe D. Lu,¹ Yuhong Zhang,¹ Donalyn Scheuner,² Randal J. Kaufman,^{2,3}
Nahum Sonenberg,⁴ Heather P. Harding,⁵ and David Ron^{1*}

*Skirball Institute of Biomolecular Medicine¹ and Departments of Cell Biology, Medicine, and Pharmacology,⁵
New York University School of Medicine, New York, New York; Department of Biochemistry² and Howard
Hughes Medical Institute,³ University of Michigan School of Medicine, Ann Arbor, Michigan;
and Department of Biochemistry, McGill University, Montreal, Quebec, Canada⁴*

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Numerous stressful conditions activate kinases that phosphorylate the alpha subunit of translation initiation factor 2 (eIF2 α), thus attenuating mRNA translation and activating a gene expression program known as the integrated stress response. It has been noted that conditions associated with eIF2 α phosphorylation, notably accumulation of unfolded proteins in the endoplasmic reticulum (ER), or ER stress, are also associated with activation of nuclear factor kappa B (NF- κ B) and that eIF2 α phosphorylation is required for NF- κ B activation by ER stress. We have used a pharmacologically activable version of pancreatic ER kinase (PERK, an ER stress-responsive eIF2 α kinase) to uncouple eIF2 α phosphorylation from stress and found that phosphorylation of eIF2 α is both necessary and sufficient to activate both NF- κ B DNA binding and an NF- κ B reporter gene. eIF2 α phosphorylation-dependent NF- κ B activation correlated with decreased levels of the inhibitor I κ B α protein. Unlike canonical signaling pathways that promote I κ B α phosphorylation and degradation, eIF2 α phosphorylation did not increase phosphorylated I κ B α levels or affect the stability of the protein. Pulse-chase labeling experiments indicate instead that repression of I κ B α translation plays an important role in NF- κ B activation in cells experiencing high levels of eIF2 α phosphorylation. These studies suggest a direct role for eIF2 α phosphorylation-dependent translational control in activating NF- κ B during ER stress.

Diverse stressful conditions lead to the phosphorylation of translation initiation factor 2 on its alpha subunit (eIF2 α). Phosphorylated eIF2 inhibits its guanine nucleotide exchange factor, eIF2B, and thereby inhibits the exchange reaction required to generate active GTP-bound eIF2. As a consequence, regulated phosphorylation of eIF2 α serves to modulate mRNA translation rates (18, 20). In addition to its negative impact on global protein synthesis, eIF2 phosphorylation also promotes gene-specific upregulation of the translation of certain mRNAs. The two known examples of this involve the yeast transcription factor GCN4 (19) and the mammalian transcription factor ATF4 (12). Regulated gene expression appears to be an important consequence of eIF2 α phosphorylation, as mutations that interfere with eIF2 α phosphorylation lead to an important defect in stress-induced gene expression (16, 28, 39).

Four known eIF2 α kinases couple seemingly unrelated stressful conditions to the aforementioned common translational regulatory event. PKR responds to double-stranded RNA in virally infected cells (23), GCN2 is activated by uncharged tRNAs in amino acid-starved cells (20), HRI is activated by heme depletion in erythroid precursor cells (3), and PERK is activated by unfolded proteins in the endoplasmic reticulum (ER), or ER stress (37). Mutations in each of these four kinases have been produced, and their phenotypes reveal the importance of eIF2 α phosphorylation in stressed cells (6).

Nuclear factor kappa B (NF- κ B) encompasses a family of

stress-induced transcription factors. Like the more ancient eIF2 α phosphorylation-dependent signaling, NF- κ B signaling is also triggered by diverse stressful conditions, and activated NF- κ B has broad effects on gene expression (38). Several studies have suggested cross talk between the eIF2 α phosphorylation pathway and NF- κ B activation. The double-stranded-RNA-activated eIF2 α kinase PKR was noted to phosphorylate the NF- κ B inhibitor, I κ B (26), and genetic and pharmacological interventions that interfere with PKR activity attenuated NF- κ B activation by cytokines (4, 27, 47) or viruses (9, 43). There is some uncertainty regarding the role of eIF2 α phosphorylation in NF- κ B activation by PKR, as the latter contributes to NF- κ B activation by both kinase-dependent (9) and kinase-independent (8) mechanisms.

Conditions that promote accumulation of unfolded proteins in the endoplasmic reticulum lead to high levels of eIF2 α phosphorylation (34, 35), which is mediated by the ER-localized kinase PERK (14, 15). These same conditions activate NF- κ B (32). A recent study has found that ER stress-mediated NF- κ B activation was attenuated both in *PERK*^{-/-} cells and, importantly, in cells bearing two mutant alleles of *EIF2A* in which serine 51 (the substrate of the stress-inducible kinases) had been mutated to an alanine. These mutant eIF2 α ^{A/A} cells were also defective in NF- κ B activation by amino acid starvation, as were cells lacking GCN2 (21), the kinase that phosphorylates eIF2 α in amino acid-starved cells.

Together these observations point to a nonredundant role for eIF2 α phosphorylation in NF- κ B activation under various stress conditions. But they provide little insight into the mechanisms involved. One of the best-characterized aspects of

* Corresponding author. Mailing address: New York University Medical Center, SI 3-10, 540 First Ave., New York, NY 10016. Phone: (212) 263-7786. Fax: (212) 263-8951. E-mail: ron@saturn.med.nyu.edu.

NF- κ B regulation is the phosphorylation-dependent, proteasome-mediated degradation of its inhibitor, I κ B. However, it is not clear if and how eIF2 α phosphorylation ties in to I κ B levels. Because the stressful conditions used to promote eIF2 α phosphorylation have multiple other effects (reviewed in reference 17), it is not even known whether eIF2 α phosphorylation plays a permissive role or an instructive role in NF- κ B activation, nor is it known whether the phosphorylated form of eIF2 α is affecting NF- κ B activation as a modified translation initiation factor or by some other means. In an effort to answer some of these questions, we have probed NF- κ B activation in an experimental system that uncouples eIF2 α phosphorylation from stress signaling and discovered that translational repression of I κ B can account for activation of NF- κ B under conditions of eIF2 α phosphorylation.

MATERIALS AND METHODS

Cell culture, cell transfection, and treatment. The wild-type cells and *EIF2A*^{A/A} mutant cells in which the serine at position 51, the regulatory phosphorylation site, had been replaced by an alanine have been previously described (39). They were cultured in Dulbecco's modified Eagle's medium supplemented with glutamine, nonessential amino acids, 55 μ M β -mercaptoethanol, and 10% fetal calf serum. The establishment of stable clones of mouse fibroblasts expressing Fv2E-PERK with defined *EIF2A* genotypes has been previously described (28).

The Fv2E-PERK⁺ wild-type mouse embryonic fibroblasts described above were transiently transfected using Fugene lipid-based gene transfer reagent (catalog no. 1814443; Roche, Indianapolis, Ind.) with luciferase reporter plasmids containing a minimal rat angiotensinogen promoter driven by four wild-type or mutant NF- κ B binding sites from the rat angiotensinogen gene, as previously described (36). One day later the cells were treated for 1 h with the indicated concentration of AP20187 (gift of ARIAD Pharmaceuticals, Cambridge, Mass.), washed free of the activator (to allow translation to recover), and harvested for use in a luciferase assay 24 h later.

Cells were treated with thapsigargin (catalog no. T9033; Sigma, St. Louis, Mo.) at a final concentration of 400 nM or cycloheximide (catalog no. C7698; Sigma) at 20 μ g/ml. Unless otherwise indicated, AP20187 was used at a concentration of 10 nM. Cells were treated with 20 ng of tumor necrosis factor alpha (TNF- α ; catalog no. T7539; Sigma)/ml with or without the proteasome inhibitor MG132 (catalog no. 474790; Calbiochem-Novobiochem, San Diego, Calif.) at 10 μ M.

Immunoblotting and immunoprecipitation. Total I κ B α was detected with a purified rabbit immune serum (catalog no. 9242; Cell Signaling, Beverly, Mass.), and I κ B α phosphorylated on serine 32 and 36 was detected with an epitope-specific antiserum (catalog no. 9246; Cell Signaling). GADD34 was detected with an antiserum directed to the N terminus of the mouse protein raised in our lab (30). PERK was detected with a 1:1 mixture of two rabbit antisera (NY97, which detects the unphosphorylated form of the protein, and NY201, which detects predominantly the hyperphosphorylated forms of the protein) as described previously (2). Total eIF2 α was detected with a monoclonal antibody to human eIF2 α , a gift of the late Edward Henshaw (40), and phosphorylated eIF2 α was detected with an epitope-specific antiserum (catalog no. RG0001; Research Genetics, Huntsville, Ala.).

Pulse-chase labeling experiments were carried out in the Fv2E-PERK⁺ wild-type mouse embryonic fibroblasts described above. Cells were switched to Dulbecco's modified Eagle's medium containing 10% of the normal content of methionine and cysteine (these levels of methionine and cysteine are sufficient to suppress activation of the eIF2 α kinase GCN2 yet are compatible with high-level incorporation of labeled amino acids into newly synthesized proteins) 15 min before addition of TRANSLabel (MP Biomedical, Irvine, Calif.) ³⁵S-labeled methionine-cysteine mixture at 200 μ Ci/ml for 10 min. The labeling pulse was terminated by washing the unincorporated label and flooding the cells with complete medium. Following the indicated chase period, during which cells were exposed to AP20187 and/or MG132, the cells were lysed in RIPA buffer (20 mM Tris [pH 8.5], 100 mM NaCl, 0.2% sodium deoxycholate, 0.2% NP-40, 0.2% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 4 μ g of aprotinin/ml, 2 μ g of pepstatin/ml), and the lysate was clarified by centrifugation at 14,000 \times g for 15 min, precleared on protein A-Sepharose beads (catalog no. 10-1042; Zymed, South San Francisco, Calif.), and subjected to immunoprecipitation with prebound anti-I κ B α

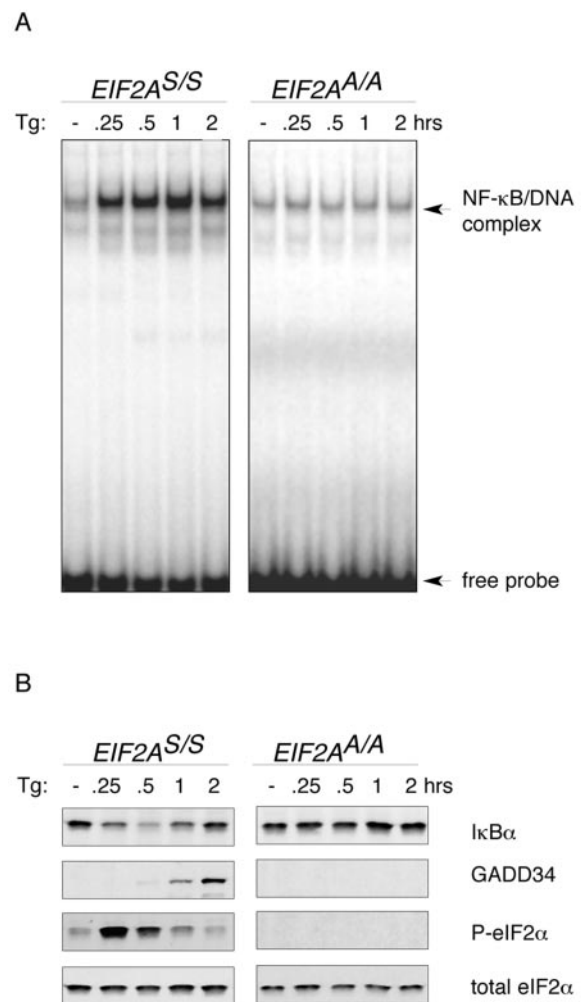


FIG. 1. NF- κ B activation during ER stress depends on eIF2 α phosphorylation and is associated with declining levels of the NF- κ B inhibitor I κ B α . (A) Autoradiogram of an NF- κ B EMSA performed with nuclear extracts of thapsigargin-treated mouse fibroblasts (Tg) with wild-type (*EIF2A*^{S/S}) or mutant (*EIF2A*^{A/A}) *EIF2A* genotypes. The free radiolabeled probe and the labeled NF- κ B/DNA complex are indicated. (B) Immunoblots of I κ B α , GADD34, phosphorylated eIF2 α , and total eIF2 α from extracts of the cells shown in panel A, detected with specific antibodies.

rabbit immunoglobulin G (catalog no. SC-371 AC; Santa Cruz Biotech, Santa Cruz, Calif.). Radiolabeled proteins found in the immunoprecipitate were resolved by reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the dried gel was exposed to autoradiography using a phosphorimaging cassette (Molecular Dynamics, Sunnyvale, Calif.).

EMSA. NF- κ B DNA binding activity in nuclear extracts was detected by an electrophoretic mobility shift assay (EMSA) performed as previously described (21, 36). The indicated molar excess of unlabeled competitor probe or 1 μ l of purified anti-p65 (catalog no. SC-7151; Santa Cruz Biotech) or anti-CHOP antiserum (45) was added to the binding reaction together with the radiolabeled probe.

RESULTS

To confirm the previously reported role of eIF2 α phosphorylation in NF- κ B activation (21), we performed EMSA on nuclear extracts prepared from unstressed cells and cells that had been treated with thapsigargin (Fig. 1A). Thapsigargin-mediated ER calcium depletion leads to rapid onset of ER

stress, eIF2 α phosphorylation (detected here by immunoblotting with an antiserum specifically reactive with the phosphorylated form), and subsequent ATF4-mediated activation of downstream gene expression, measured here by accumulation of the GADD34 target gene. A protein complex rapidly formed on the NF- κ B binding site in nuclear extracts of treated wild-type cells but not in extracts from cells homozygous for the *EIF2A*^{A/A} mutation that substitutes the serine at position 51 of eIF2 α with an alanine and thereby prevents regulatory phosphorylation. Reduced levels of the NF- κ B inhibitory protein I κ B α , detected by immunoblotting, preceded the induction of NF- κ B EMSA activity in thapsigargin-treated cells. The recovery of I κ B α levels at longer treatment points correlated with the induction of the GADD34 phosphatase and the dephosphorylation of eIF2 α (Fig. 1B).

To more closely examine the role of eIF2 α phosphorylation in NF- κ B activation, we made use of an experimental system that uncouples eIF2 α phosphorylation from stress signaling. PERK, the ER stress-inducible eIF2 α kinase, is normally activated by oligomerization in the plane of the ER membrane (2). We fused PERK's eIF2 α kinase domain to a protein module with two high-affinity binding sites for the otherwise inert bivalent compound AP20187. When expressed in cells, this artificial kinase, Fv2E-PERK, is subordinate to AP20187 treatment (28) and is activated independently of any stress signaling. AP20187 treatment led to high-level eIF2 α phosphorylation in Fv2E-PERK⁺ cells but had no effect on the parental cells lacking the artificial kinase (Fig. 2A). Fv2E-PERK was readily activated in mutant *EIF2A*^{A/A} cells, but this predictably failed to induce eIF2 α phosphorylation. EMSA of nuclear extracts showed that AP20187 induced NF- κ B activity in Fv2E-PERK⁺ wild-type (*EIF2A*^{S/S}) cells but not in the mutant *EIF2A*^{A/A} cells (Fig. 2B). Homologous competition binding assays and antibody supershift experiments confirmed the identity of the NF- κ B protein-DNA complex detected in the assay (Fig. 2C).

To gauge the functional significance of Fv2E-PERK-mediated eIF2 α phosphorylation and activation of NF- κ B DNA binding activity, we measured the activity of a transfected reporter gene driven by four copies of a wild-type NF- κ B binding site. A brief (60-min) pulse of AP20187 induced marked activation of the wild-type reporter gene (measured 24 h later [Fig. 3]). No activation of a reporter gene driven by mutant NF- κ B sites was observed. In addition, endogenous NF- κ B target genes, such as those encoding the major histocompatibility complex heavy chains (H2-Q8, H2-2KF, H2-K2, and H2-D1) and β 2 microglobulin (Qb-1), were induced in the Fv2E-PERK⁺ cells by AP20187 treatments and in wild-type mouse fibroblasts by exposure to tunicamycin (National Center for Biotechnology Information Gene Expression Omnibus [GEO] data set GDS405).

Fv2E-PERK-mediated eIF2 α phosphorylation and NF- κ B activation correlated with a time-dependent decrease in I κ B α levels that was not observed in the mutant *EIF2A*^{A/A} cells (Fig. 4A). Interestingly, Fv2E-PERK activation had no measurable effect on levels of the p65 NF- κ B subunit, which is consistent with the known stability of that protein (24) and with the induction of NF- κ B binding activity that we observe. eIF2 α levels were similarly stable, attesting to the effect's specificity to I κ B α (Fig. 4B). Canonical activators of NF- κ B access signal

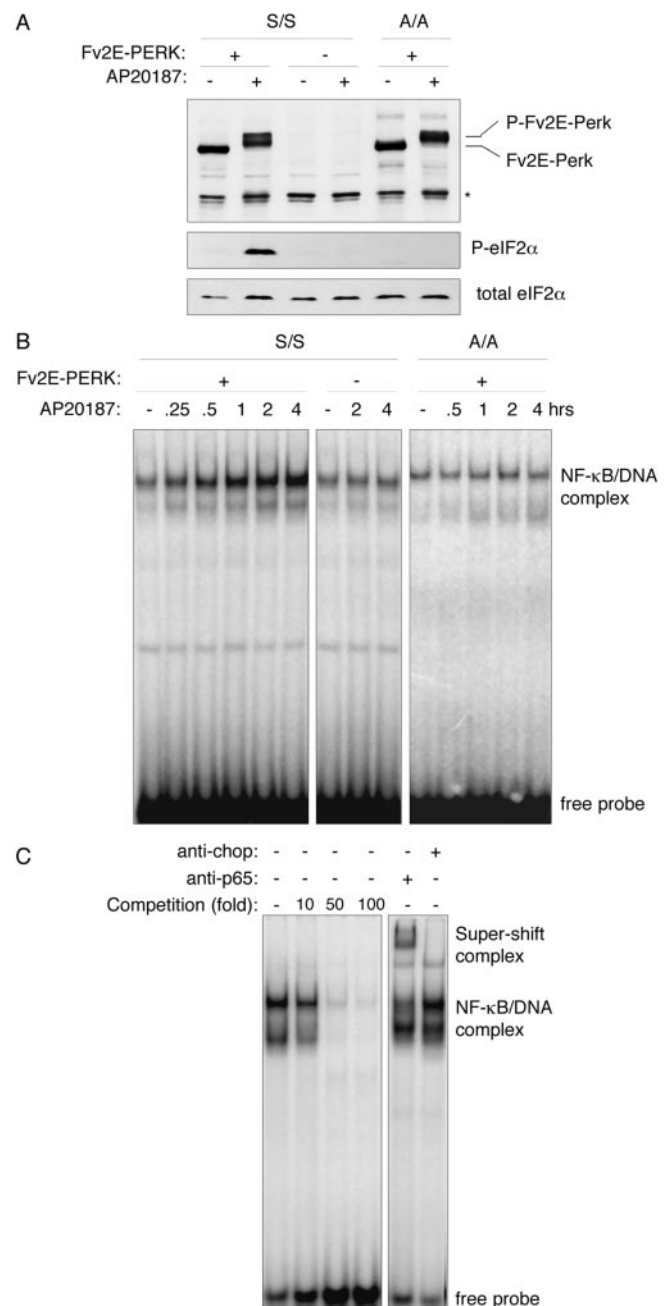


FIG. 2. Phosphorylation of eIF2 α on serine 51 is sufficient to activate NF- κ B DNA binding activity in vivo. (A) Immunoblots of ligand-activable Fv2E-PERK (upper panel), phosphorylated eIF2 α (P-eIF2 α ; middle panel), and total eIF2 α (lower panel) in extracts of mouse fibroblasts of wild-type (S/S) and *EIF2A*^{A/A} mutant (A/A) genotypes that do and do not stably express the chimeric eIF2 α kinase, Fv2E-PERK. Where indicated, the cells had been treated with the Fv2E ligand, AP20187. Endogenous PERK is not detected at this exposure. The asterisk marks the position of a nonspecific band reactive with the anti-PERK sera. (B) Autoradiogram of an NF- κ B EMSA performed with nuclear extracts of cells treated as described for panel A. (C) Autoradiogram of an NF- κ B EMSA with nuclear extract obtained from AP20187-treated Fv2E-PERK⁺ cells performed in the presence of the indicated excess of an unlabeled homologous competitor oligonucleotide (left panel) or in the presence of antisera to CHOP (a negative control) or p65 (a component of the NF- κ B DNA binding complex) (right panel). The positions of the free radiolabeled probe, the NF- κ B/DNA complex, and antiserum supershifted complex are indicated.

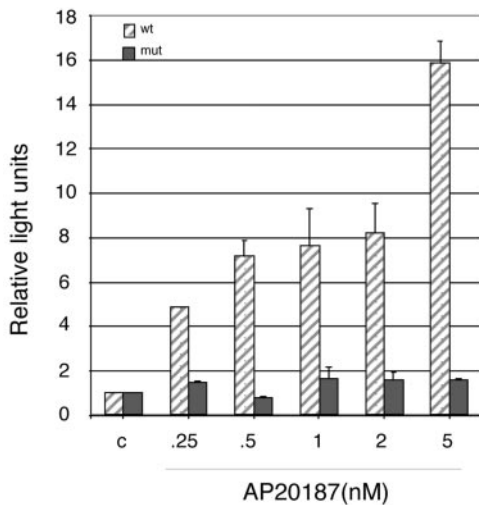


FIG. 3. eIF2 α phosphorylation is sufficient to activate an NF- κ B reporter gene. The activity of a transiently transfected reporter gene consisting of a minimal promoter driven by four wild-type (wt) or mutant (mut) NF- κ B binding sites in mouse fibroblasts stably expressing Fv2E-PERK is shown following treatment with the indicated concentration of the activating ligand AP20187. The results are expressed as relative light units, and the activity of the reporter in untreated cells is arbitrarily set at 1. Shown are means and standard errors of the means of results from an experiment performed in triplicate and reproduced twice.

transduction pathways that promote phosphorylation of the inhibitor I κ B α on serines 32 and 36 (38). A ubiquitin ligase complex recognizes the phosphorylated form of I κ B α , and polyubiquitinated I κ B α is degraded by the proteasome. Fv2E-PERK activation by AP20187 did not promote a measurable increase in levels of phosphorylated I κ B α , which remained undetectable. However, phosphorylated I κ B α was readily detectable in lysates of cells treated with the proteasome inhibitor, MG132, which stabilizes the phosphorylated form of the protein (Fig. 4B).

Because it is rapidly degraded, signal-dependent accumulation of phosphorylated I κ B α is difficult to detect, rendering an Fv2E-PERK-mediated increase in I κ B α phosphorylation potentially easy to miss. Therefore, to determine if the eIF2 α phosphorylation-dependent decline in I κ B α levels correlated with any increased phosphorylation on serines 32 and 36, we exposed the AP20187-treated cells to the proteasome inhibitor MG132. As expected, proteasome inhibition markedly increased the levels of phosphorylated I κ B α in tumor necrosis factor alpha-treated cells (Fig. 5A). Interestingly, proteasome inhibition led to only modest stabilization of total I κ B α , an observation that is consistent with the existence of proteasome-independent mechanisms for I κ B α degradation (5, 11).

MG132 treatment led to a progressive increase in phosphorylated I κ B α levels in cells that were otherwise unperturbed (Fig. 5A, compare lanes 1 and 3, and B, compare lane 1 with lanes 2, 4, 6, 8, and 10). This observation is consistent with a relatively high basal phosphorylation-dependent turnover of I κ B α in these cells. The decline in I κ B α levels effected by Fv2E-PERK was only slightly attenuated by proteasome inhibition (compare Fig. 4, lanes 4 to 6, with 5B, lanes 7, 9, and 11). Furthermore, proteasome inhibition promoted some eIF2 α

phosphorylation (Fig. 5, lanes 8 and 10), presumably mediated by proteotoxic stress. Remarkably, however, Fv2E-PERK activation and eIF2 α phosphorylation not only failed to increase I κ B α phosphorylation but also significantly attenuated the accumulation of phosphorylated I κ B α in proteasome-inhibited cells (Fig. 5B, compare odd- and even-numbered lanes). These observations indicate that eIF2 α phosphorylation does not activate NF- κ B by accessing one of the canonical I κ B α phosphorylation-promoting pathways and must use a different mechanism.

The original descriptions of I κ B emphasized the lability of the factor, as translational inhibitors were noted to promote NF- κ B DNA binding activity (1, 42). Given that eIF2 α phosphorylation also inhibits protein synthesis, we decided to explore this facet of NF- κ B activation in more detail. NF- κ B DNA binding activity was increased by cycloheximide treatment of wild-type cells, as previously reported (42), and this correlated with reduced levels of the inhibitor, I κ B α (Fig. 6A). Cycloheximide treatment led to no measurable decrease in p65 or eIF2 α protein levels, attesting to the stability of these proteins. The effects of cycloheximide on levels of phosphorylated I κ B α also resembled those of Fv2E-PERK activation (Fig. 4B) in that no increase in the phosphorylated protein was observed

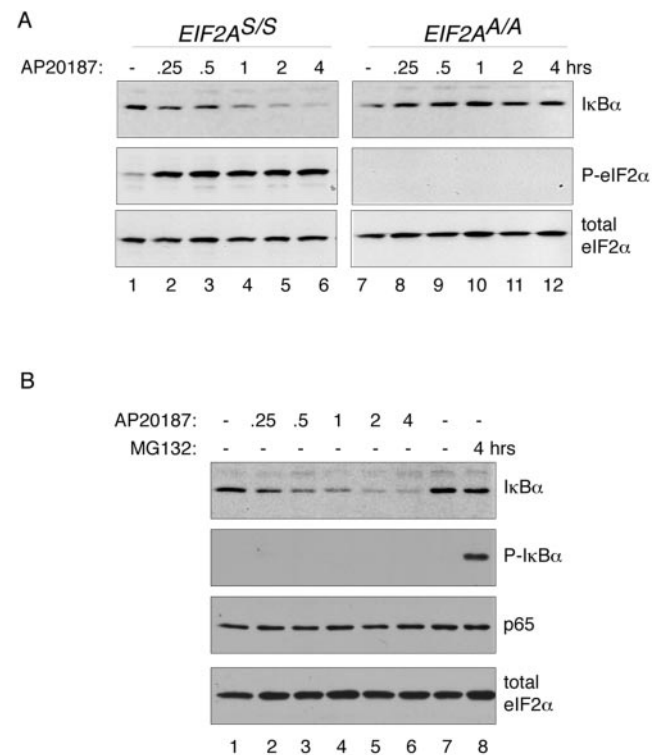


FIG. 4. eIF2 α phosphorylation reduces cellular levels of I κ B α . (A) Immunoblots of total I κ B α (upper panel), phosphorylated eIF2 α (P-eIF2 α ; middle panel), and total eIF2 α (lower panel) in extracts of wild-type (EIF2A^{S/S}) and mutant (EIF2A^{A/A}) Fv2E-PERK⁺ mouse fibroblasts following treatment with the activating ligand AP20187 for the indicated periods of time. (B) Immunoblots of total I κ B α , phosphorylated I κ B α (P-I κ B α), p65 NF- κ B subunit, and total eIF2 α in extracts of wild-type (EIF2A^{S/S}) Fv2E-PERK⁺ mouse fibroblasts following treatment with the activating ligand AP20187 or the proteasome inhibitor (MG132) for the indicated periods of time.

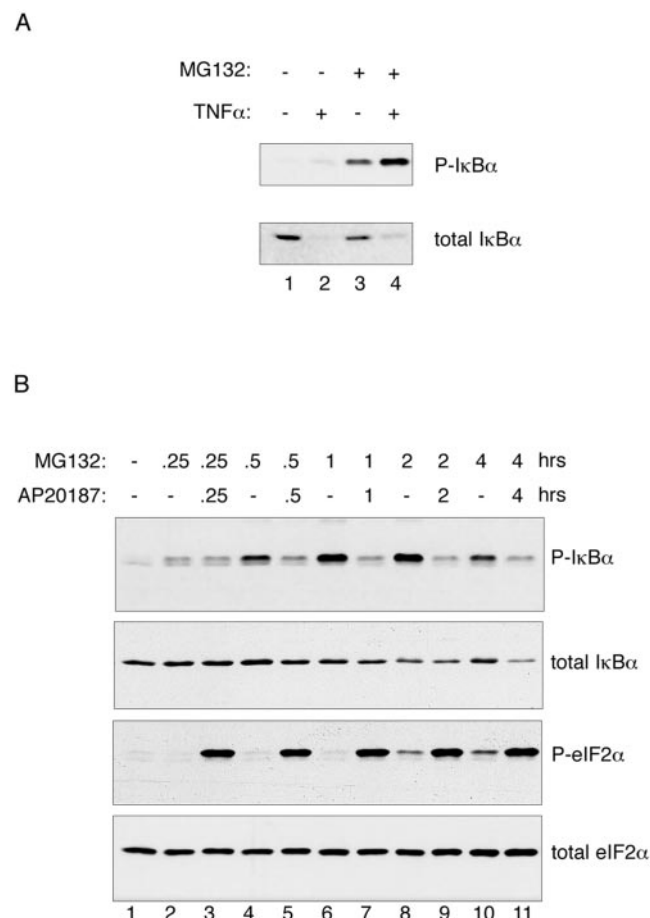


FIG. 5. Reduction in levels of I κ B α in cells with elevated eIF2 α phosphorylation occurs independently of I κ B α phosphorylation. (A) Immunoblots of I κ B α phosphorylated on serines 32 and 36 (P-I κ B α ; upper panel) and total I κ B α (lower panel) in extracts of mouse fibroblasts treated with TNF- α and/or the proteasome inhibitor MG132. (B) Immunoblots of phosphorylated I κ B α (P-I κ B α), total I κ B α , phosphorylated eIF2 α (P-eIF2 α), and total eIF2 α in extracts of Fv2E-PERK⁺ mouse fibroblasts treated with the activating ligand AP20187 and/or the proteasome inhibitor MG132 for the indicated periods of time.

in cells treated with cycloheximide alone. Proteasome inhibitor, by itself, led to a progressive increase in levels of phosphorylated I κ B α , whereas the addition of cycloheximide strongly attenuated this increase (Fig. 6B).

As previously noted, cycloheximide treatment induced eIF2 α phosphorylation (21, 22) (Fig. 6A), an effect that might be attributed to loss of the labile eIF2 α phosphatase CREP (22). To study the role of eIF2 α phosphorylation in cycloheximide-mediated activation of NF- κ B, we treated mutant *EIF2A*^{A/A} cells with the protein synthesis inhibitor and studied NF- κ B activation by EMSA and I κ B α levels by immunoblotting. The *EIF2A*^{A/A} genotype, which inhibits regulatory phosphorylation of eIF2 α , had no measurable effect on NF- κ B activation, I κ B α phosphorylation, or total I κ B α levels in cycloheximide-treated cells (Fig. 6C). These observations suggest that inhibition of new protein synthesis can adequately explain the effects of cycloheximide on NF- κ B activity without evoking an additional role for eIF2 α phosphorylation.

Induced degradation of I κ B α plays an important role in

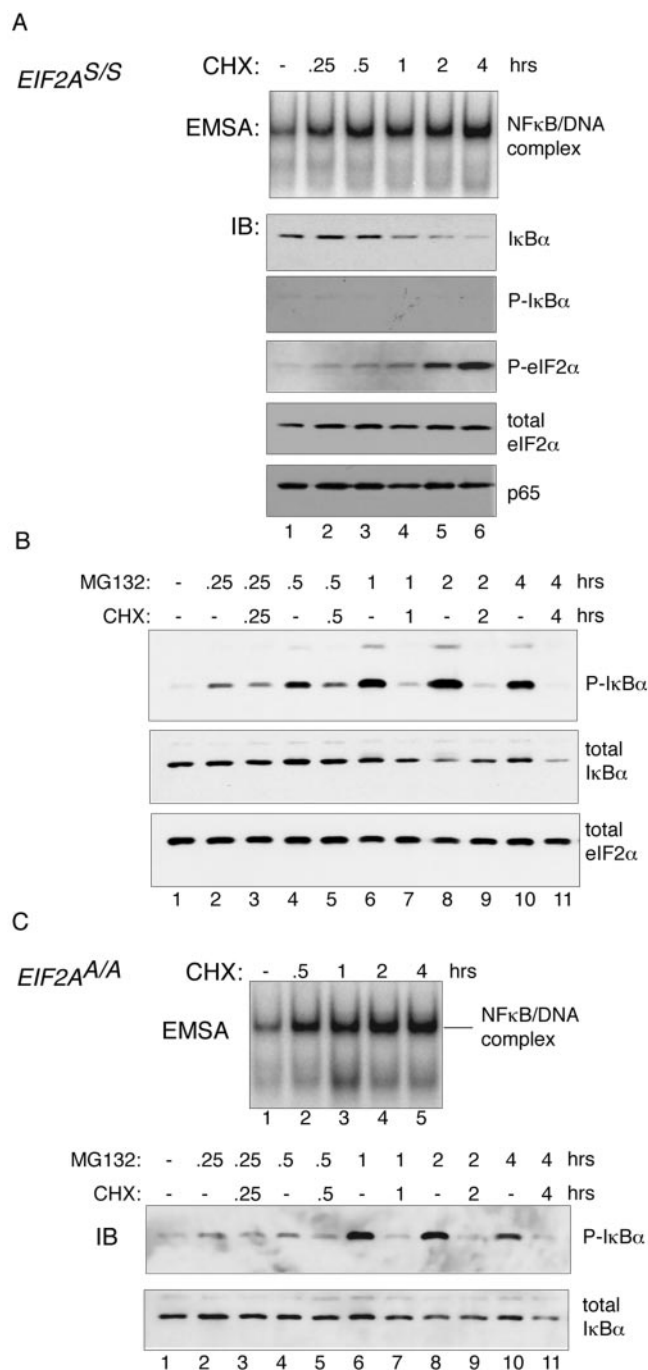


FIG. 6. Reduction in levels of I κ B α in cells treated with the protein synthesis inhibitor cycloheximide occurs independently of I κ B α phosphorylation or eIF2 α phosphorylation. (A) The top panel is an autoradiogram of an NF- κ B EMSA from nuclear extracts of untreated and cycloheximide (CHX)-treated mouse fibroblasts. The lower panels are immunoblots (IB) of total I κ B α , phosphorylated I κ B α (P-I κ B α), phosphorylated eIF2 α (P-eIF2 α), total eIF2 α , and the p65 NF- κ B subunit from the same cells. (B) Immunoblots of phosphorylated I κ B α (P-I κ B α), total I κ B α , and total eIF2 α in extracts of wild-type (*EIF2A*^{S/S}) Fv2E-PERK⁺ mouse fibroblasts treated with cycloheximide and/or the proteasome inhibitor MG132 for the indicated periods of time are shown. (C) The same assays as shown in panels A and B were conducted with mutant (*EIF2A*^{A/A}) Fv2E-PERK⁺ cells.

canonical activation of NF- κ B. To address the possibility that eIF2 α phosphorylation might affect this aspect of I κ B α metabolism (independently of I κ B α phosphorylation), we performed pulse-chase labeling experiments, tracking the fate of newly synthesized I κ B α . The basal turnover of I κ B α in murine fibroblasts proved very high. Less than 30% of the signal measured at the end of the 10-min labeling pulse was present after a 20-min chase. Furthermore, activation of Fv2E-PERK during the chase had no measurable effect on the decay of the I κ B α signal (Fig. 7A). Addition of proteasome inhibitor during the chase stabilized I κ B α somewhat; however, in that context, too, activation of Fv2E-PERK during the chase did not accelerate I κ B α degradation and may have even contributed modestly to its stability (Fig. 7B). We conclude that I κ B α turns over rapidly in murine fibroblasts and that eIF2 α phosphorylation does not exert its effects on the levels of the inhibitor by further enhancing its degradation.

Next we compared the rates of synthesis of I κ B α in untreated cells with those in cells treated with AP20187, cycloheximide, the ER stress-promoting agent thapsigargin, and the canonical NF- κ B activator TNF- α . The amount of radiolabeled I κ B α immunoprecipitated with a specific antibody following a short labeling pulse was markedly diminished by activation of the eIF2 α kinase Fv2E-PERK by AP20187, by treatment with cycloheximide, or by exposure to conditions that cause ER stress (thapsigargin) (Fig. 7C). The effect of thapsigargin on I κ B α synthesis depended on eIF2 α phosphorylation, since it was abolished in the *EIF2A*^{A/A} mutant cells (Fig. 7C), and the decline in I κ B α synthesis paralleled the global inhibition in protein synthesis in the cells exposed to conditions promoting eIF2 α phosphorylation (Fig. 7D). By contrast, exposure to the canonical NF- κ B activator, TNF- α , increased I κ B α synthesis, suggesting a completely different mechanism of action. These observations are consistent with a role for inhibited synthesis of I κ B α in mediating the effects of eIF2 α phosphorylation on NF- κ B activation both in ER-stressed cells and following activation of Fv2E-PERK.

DISCUSSION

Signaling through stress-induced phosphorylation of eIF2 α is conserved among the eukaryotes and represents one of the oldest pathways for stress-induced gene expression. Furthermore, eIF2 α phosphorylation is concerned mostly with autonomous cell adaptations to stress. NF- κ B signaling, on the other hand, is found in metazoans, and canonical activators of NF- κ B signaling, such as cytokines, are intercellular signaling molecules. However, over the years evidence that autonomous cell phenomena, such as ER stress, are also associated with NF- κ B activation has accrued, with the suggestion that ancient, autonomous cell signaling pathways might be linked to NF- κ B activation.

This study confirms the established role of eIF2 α phosphorylation in NF- κ B activation by ER stress (21). Using an inducible system that uncouples eIF2 α phosphorylation from other stress signals, we find that eIF2 α phosphorylation can have an instructive role in NF- κ B activation. In other words, activation of an eIF2 α kinase provides a signal sufficient for NF- κ B activation in cultured mouse fibroblasts. Our study also reveals significant differences between the mechanism used by canon-

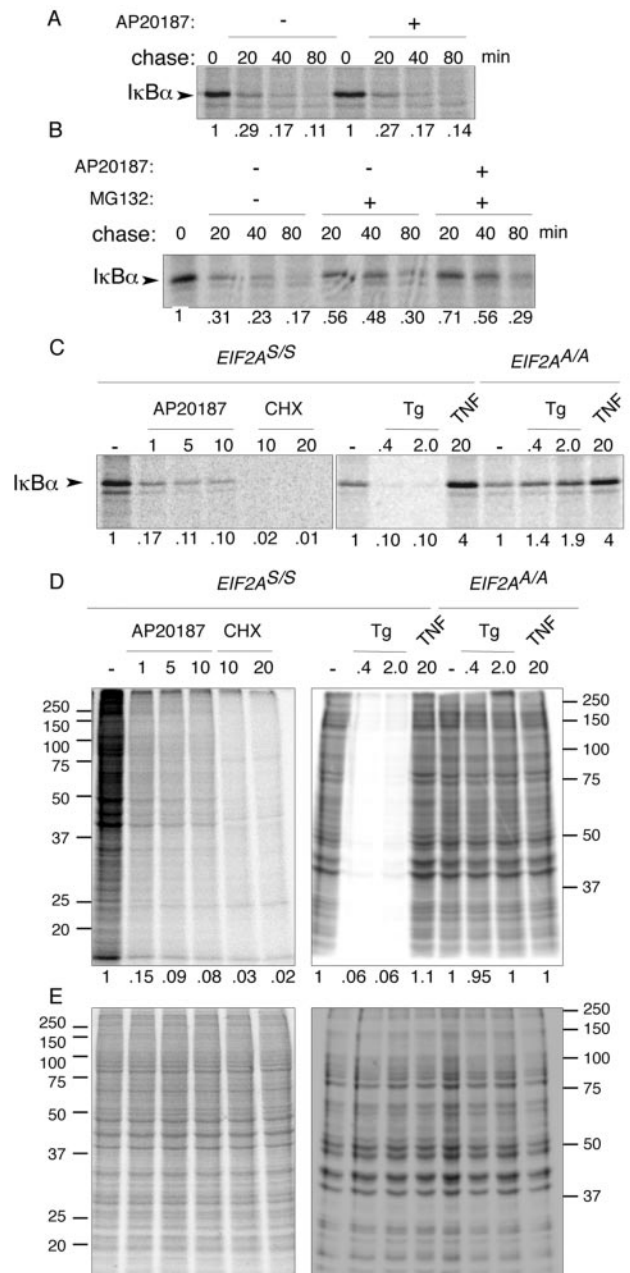


FIG. 7. eIF2 α phosphorylation inhibits synthesis of I κ B α but does not destabilize the preexisting protein. (A) Autoradiogram of I κ B α immunoprecipitated from wild-type (*EIF2A*^{S/S}) Fv2E-PERK⁺ mouse fibroblasts following a brief, 10-min [³⁵S]methionine- and cysteine-labeling pulse and cold chase of the indicated duration. The chase was conducted in the presence or absence of the activating ligand AP20187. The I κ B α signal intensity is expressed as a fraction of that present at the end of the labeling pulse and is depicted beneath each lane. (B) Same assay as shown in panel A except that the proteasome inhibitor, MG132, was included during the chase where indicated. (C) Autoradiogram of the radiolabeled I κ B α present at the end of the 10-min labeling pulse in wild-type (*EIF2A*^{S/S}) or mutant (*EIF2A*^{A/A}) Fv2E-PERK⁺ mouse fibroblasts treated with the indicated concentration of AP20187 ligand (in nM), cycloheximide (in μ g/ml), thapsigargin (in μ M), or TNF- α (in ng/ml) starting 30 min before and continuing throughout the pulse. (D) Autoradiogram ([³⁵S]methionine) of equal fractions of the cell lysates used in panel C. The right panel is of a gel that was run longer than the left panel, accounting for differences in appearance of the two. (E) Coomassie stain of the gels shown in panel D.

ical inducers of NF- κ B and the consequences of eIF2 α phosphorylation. Unlike canonical inducers of NF- κ B, eIF2 α phosphorylation promoted neither phosphorylation nor degradation of I κ B α . Instead, our data argue that the major impact of eIF2 α phosphorylation on NF- κ B activation is inhibition of the synthesis of the labile inhibitor I κ B α .

The mechanism uncovered in this study suggests that the link between eIF2 α phosphorylation and NF- κ B activation depends on the lability of the inhibitor, which, in turn, likely depends on basal levels of signaling through the canonical pathway(s) that activates NF- κ B. Indeed, the rapid accumulation of phosphorylated I κ B α in mouse fibroblasts treated with proteasome inhibitor is consistent with high basal levels of I κ B α kinase activity in these cells. It is worth noting that both eIF2 α phosphorylation and cycloheximide treatment disproportionately reduced the levels of phosphorylated I κ B α , compared with their effect on the levels of total I κ B α . Inhibited protein synthesis may attenuate basal activity of an I κ B α kinase and account for some of this effect. Alternatively, newly synthesized I κ B α might constitute a preferred substrate for its kinases. The plausibility of the latter explanation is supported by evidence for the existence of multiple pools of I κ B α in cells (25, 33, 41). The existence of more than one pool of I κ B α might also explain the discrepancy between the short half-life of newly synthesized I κ B α (measured by the pulse-chase method [Fig. 7A and B]) and the much longer half-life inferred from the gradual decline in total I κ B α protein levels in the cycloheximide-treated and Fv2E-PERK-activated cells (Fig. 4, 5B, and 6A and B). However, these potential complexities of I κ B α metabolism do not weaken our conclusion that attenuated synthesis of the inhibitor plays a major role in mediating activation of NF- κ B by eIF2 α phosphorylation in mouse fibroblasts.

Our findings are at odds with those reported by Jiang and colleagues, who found no decrease in steady-state I κ B α levels in thapsigargin-treated cells and instead uncovered evidence for dissociation of the I κ B α -NF- κ B complex under those conditions (see Fig. 6 in reference 21). We have no explanation for these differences; however, we do note that since the submission of the present study Wu and colleagues have reported that induction of NF- κ B DNA binding activity in cells exposed to UV light is also associated with eIF2 α phosphorylation-dependent repression of I κ B α synthesis (46).

Our study does not address the physiological significance of the link between eIF2 α phosphorylation and NF- κ B activation. It is worth noting that we have but an incomplete understanding of the relative significance of regulated protein synthesis versus activation of gene expression programs as readouts of eIF2 α phosphorylation. In yeast it is fairly clear that mutations in the transcription factor GCN4 phenocopy mutations in the upstream kinase GCN2 or in the gene encoding its substrate SUI2 (yeast eIF2 α) (6, 7). In mammalian cells too, some of the phenotypes of loss of *PERK* gene function or the *EIF2A*^{A/A} genotype are mimicked by mutations in the gene encoding the downstream transcription factor ATF4 (16, 29, 39). Furthermore, in both yeast and mammalian cells, translation activation of the transcription factors GCN4 and ATF4 occurs at levels of eIF2 α phosphorylation that have only a modest impact on global protein synthesis (7, 44; Lu et al., unpublished observation). By contrast, our proposed mechanism of cross talk be-

tween eIF2 α phosphorylation and NF- κ B signaling is proportional to the repression of I κ B α translation. Such levels of repression are easily attained in thapsigargin-treated cells (14) or in Fv2E-PERK⁺ cells activated by AP20187 (28) and are clearly sufficient to activate NF- κ B in cultured mouse fibroblasts (Fig. 1A and 2B) (21).

The extent to which translational repression contributes to NF- κ B activation in more physiological contexts in which eIF2 α kinases are activated is not known. However, we note that endogenous proinflammatory NF- κ B target genes, such as those encoding the major histocompatibility complex heavy and light chains, the interleukin 17 receptor, and a complement receptor-related protein, were all induced in the Fv2E-PERK⁺ cells by AP20187 treatment and in wild-type mouse fibroblasts by exposure to tunicamycin (National Center for Biotechnology Information GEO data set GDS405). The PERK-dependent induction of NF- κ B target genes by tunicamycin is potentially significant, as global repression of mRNA translation is relatively modest under those conditions (14), mimicking physiological stress situations. Furthermore, loss-of-function mutations in the eIF2 α kinase PERK or HRI or the *EIF2A*^{A/A} genotype all predispose cells to programmed cell death under physiologically stressful conditions (10, 13, 14, 39, 48); however, the role of defective activation of NF- κ B in this phenotype, if any, remains to be explored.

Translational repression in response to activation of eIF2 α kinases tends to be transient (34, 35). Translational recovery is mediated in part by activation of GADD34, an eIF2 α -specific regulatory subunit of a holophosphatase complex (30, 31), which is itself a target of the eIF2 α phosphorylation-dependent gene expression program, the integrated stress response (16, 29, 30). GADD34-mediated translational recovery is therefore likely to reestablish I κ B α translation and reverse the effects of eIF2 α phosphorylation on NF- κ B activity, since the stress response is attenuated (Fig. 1B). Furthermore, while activation of NF- κ B proceeds through utilization of preformed components, the response in terms of target gene expression depends on new protein synthesis. Thus, the biphasic nature of the inhibition of protein synthesis, which is inherent to stressful conditions that promote eIF2 α phosphorylation, is also predicted to contribute to the expression of NF- κ B target genes.

In conclusion, our study indicates that the pathways promoting eIF2 α phosphorylation and those that activate NF- κ B interact through translational repression of the inhibitor I κ B α . Our study also suggests that the importance of this link is likely to be influenced by signaling through canonical NF- κ B activation pathways that define the turnover rate of I κ B α . As such, eIF2 α phosphorylation and the consequent inhibition of eIF2B might modulate NF- κ B signaling by parallel pathways active in stressed cells.

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REFERENCES

1. Baeuerle, P. A., and D. Baltimore. 1988. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 242:540-546.

2. Bertolotti, A., Y. Zhang, L. Hendershot, H. Harding, and D. Ron. 2000. Dynamic interaction of BiP and the ER stress transducers in the unfolded protein response. *Nat. Cell Biol.* **2**:326–332.
3. Chen, J. 2000. Heme-regulated eIF2 α kinase, p. 529–546. *In* N. Sonenberg, J. W. B. Hershey, and M. B. Mathews (ed.), *Translational control of gene expression*. CSHL Press, Cold Spring Harbor, N.Y.
4. Cheshire, J. L., B. R. Williams, and A. S. Baldwin, Jr. 1999. Involvement of double-stranded RNA-activated protein kinase in the synergistic activation of nuclear factor-kappaB by tumor necrosis factor-alpha and gamma-interferon in preneural cells. *J. Biol. Chem.* **274**:4801–4806.
5. Cuervo, A. M., W. Hu, B. Lim, and J. F. Dice. 1998. I kappa B is a substrate for a selective pathway of lysosomal proteolysis. *Mol. Biol. Cell* **9**:1995–2010.
6. Dever, T. E. 2002. Gene-specific regulation by general translation factors. *Cell* **108**:545–556.
7. Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. F. Donahue, and A. G. Hinnebusch. 1992. Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell* **68**:585–596.
8. Donze, O., J. Deng, J. Curran, R. Sladek, D. Picard, and N. Sonenberg. 2004. The protein kinase PKR: a molecular clock that sequentially activates survival and death programs. *EMBO J.* **23**:564–571.
9. Gil, J., J. Rullas, M. A. Garcia, J. Alcami, and M. Esteban. 2001. The catalytic activity of dsRNA-dependent protein kinase, PKR, is required for NF-kappaB activation. *Oncogene* **20**:385–394.
10. Han, A. P., C. Yu, L. Lu, Y. Fujiwara, C. Browne, G. Chin, M. Fleming, P. Lebouloch, S. H. Orkin, and J. J. Chen. 2001. Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J.* **20**:6909–6918.
11. Han, Y., S. Weinman, I. Boldogh, R. K. Walker, and A. R. Brasier. 1999. Tumor necrosis factor-alpha-inducible I kappa Balpha proteolysis mediated by cytosolic m-calpain. A mechanism parallel to the ubiquitin-proteasome pathway for nuclear factor-kappaB activation. *J. Biol. Chem.* **274**:787–794.
12. Harding, H., I. Novoa, Y. Zhang, H. Zeng, R. C. Wek, M. Schapira, and D. Ron. 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* **6**:1099–1108.
13. Harding, H., H. Zeng, Y. Zhang, R. Jungreis, P. Chung, H. Plesken, D. Sabatini, and D. Ron. 2001. Diabetes Mellitus and exocrine pancreatic dysfunction in *Perk*^{-/-} mice reveals a role for translational control in survival of secretory cells. *Mol. Cell* **7**:1153–1163.
14. Harding, H., Y. Zhang, A. Bertolotti, H. Zeng, and D. Ron. 2000. *Perk* is essential for translational regulation and cell survival during the unfolded protein response. *Mol. Cell* **5**:897–904.
15. Harding, H., Y. Zhang, and D. Ron. 1999. Translation and protein folding are coupled by an endoplasmic reticulum resident kinase. *Nature* **397**:271–274.
16. Harding, H., Y. Zhang, H. Zeng, I. Novoa, P. Lu, M. Calfon, N. Sadri, C. Yun, B. Popko, R. Paules, D. Stojdl, J. Bell, T. Hettmann, J. Leiden, and D. Ron. 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* **11**:619–633.
17. Harding, H. P., M. Calfon, F. Urano, I. Novoa, and D. Ron. 2002. Transcriptional and translational control in the mammalian unfolded protein response. *Annu. Rev. Cell Dev. Biol.* **18**:575–599.
18. Hershey, J. W. B., and W. C. Merrick. 2000. The pathway and mechanism of initiation of protein synthesis, p. 33–88. *In* N. Sonenberg, J. W. B. Hershey, and M. B. Mathews (ed.), *Translational control of gene expression*. CSHL Press, Cold Spring Harbor, N.Y.
19. Hinnebusch, A. 1996. Translational control of *GCN4*: gene-specific regulation by phosphorylation of eIF2, p. 199–244. *In* J. Hershey, M. Mathews, and N. Sonenberg (ed.), *Translational control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. Hinnebusch, A. G. 2000. Mechanism and regulation of initiator methionyl-tRNA binding to ribosomes, p. 185–243. *In* N. Sonenberg, J. W. B. Hershey, and M. B. Mathews (ed.), *Translational control of gene expression*. CSHL Press, Cold Spring Harbor, N.Y.
21. Jiang, H. Y., S. A. Wek, B. C. McGrath, D. Scheuner, R. J. Kaufman, D. R. Cavener, and R. C. Wek. 2003. Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 is required for activation of NF-kappaB in response to diverse cellular stresses. *Mol. Cell. Biol.* **23**:5651–5663.
22. Jousse, C., S. Oyadomari, I. Novoa, P. D. Lu, Y. Zhang, H. P. Harding, and D. Ron. 2003. Inhibition of a constitutive translation initiation factor 2 α phosphatase, CREP, promotes survival of stressed cells. *J. Cell Biol.* **163**:767–775.
23. Kaufman, R. J. 2000. The double-stranded RNA-activated protein kinase PKR, p. 503–527. *In* N. Sonenberg, J. W. B. Hershey, and M. B. Mathews (ed.), *Translational control of gene expression*. CSHL Press, Cold Spring Harbor, N.Y.
24. Krappmann, D., and C. Scheidereit. 1997. Regulation of NF-kappa B activity by I kappa B alpha and I kappa B beta stability. *Immunobiology* **198**:3–13.
25. Krappmann, D., F. G. Wulczyn, and C. Scheidereit. 1996. Different mechanisms control signal-induced degradation and basal turnover of the NF-kappaB inhibitor I kappa B alpha in vivo. *EMBO J.* **15**:6716–6726.
26. Kumar, A., J. Haque, J. Lacoste, J. Hiscott, and B. R. Williams. 1994. Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. *Proc. Natl. Acad. Sci. USA* **91**:6288–6292.
27. Kumar, A., Y. L. Yang, V. Flati, S. Der, S. Kadereit, A. Deb, J. Haque, L. Reis, C. Weissmann, and B. R. Williams. 1997. Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: role of IRF-1 and NF-kappaB. *EMBO J.* **16**:406–416.
28. Lu, P. D., C. Jousse, S. J. Marciniak, Y. Zhang, I. Novoa, D. Scheuner, R. J. Kaufman, D. Ron, and H. P. Harding. 2004. Cytoprotection by pre-emptive conditional phosphorylation of translation initiation factor 2. *EMBO J.* **23**:169–179.
29. Ma, Y., and L. M. Hendershot. 2003. Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. *J. Biol. Chem.* **278**:34864–34873.
30. Novoa, I., H. Zeng, H. Harding, and D. Ron. 2001. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2 α . *J. Cell Biol.* **153**:1011–1022.
31. Novoa, I., Y. Zhang, H. Zeng, R. Jungreis, H. P. Harding, and D. Ron. 2003. Stress-induced gene expression requires programmed recovery from translational repression. *EMBO J.* **22**:1180–1187.
32. Pahl, H., and P. Baeuerle. 1995. A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by the transcription factor NF- κ B. *EMBO J.* **14**:2580–2588.
33. Pando, M. P., and I. M. Verma. 2000. Signal-dependent and -independent degradation of free and NF-kappa B-bound I kappa Balpha. *J. Biol. Chem.* **275**:21278–21286.
34. Prostko, C. R., M. A. Brostrom, and C. O. Brostrom. 1993. Reversible phosphorylation of eukaryotic initiation factor 2 alpha in response to endoplasmic reticular signaling. *Mol. Cell. Biochem.* **127**:255–265.
35. Prostko, C. R., M. A. Brostrom, E. M. Malara, and C. O. Brostrom. 1992. Phosphorylation of eukaryotic initiation factor (eIF) 2 alpha and inhibition of eIF-2B in GH3 pituitary cells by perturbants of early protein processing that induce GRP78. *J. Biol. Chem.* **267**:16751–16754.
36. Ron, D., A. R. Brasier, K. A. Wright, J. E. Tate, and J. F. Habener. 1990. An inducible 50-kilodalton NFkB-like protein and a constitutive protein both bind the acute-phase response element of the angiotensinogen gene. *Mol. Cell. Biol.* **10**:1023–1032.
37. Ron, D., and H. Harding. 2000. PERK and translational control by stress in the endoplasmic reticulum, p. 547–560. *In* J. Hershey, M. Mathews, and N. Sonenberg (ed.), *Translational control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. Rothwarf, D. M., and M. Karin. 1999. The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. *Sci. STKE* **1999**:RE1.
39. Scheuner, D., B. Song, E. McEwen, P. Gillespie, T. Saunders, S. Bonner-Weir, and R. J. Kaufman. 2001. Translational control is required for the unfolded protein response and in-vivo glucose homeostasis. *Mol. Cell* **7**:1165–1176.
40. Scorsone, K. A., R. Panniers, A. G. Rowlands, and E. C. Henshaw. 1987. Phosphorylation of eukaryotic initiation factor 2 during physiological stresses which affect protein synthesis. *J. Biol. Chem.* **262**:14538–14543.
41. Scott, M. L., T. Fujita, H. C. Liou, G. P. Nolan, and D. Baltimore. 1993. The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. *Genes Dev.* **7**:1266–1276.
42. Sen, R., and D. Baltimore. 1986. Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism. *Cell* **47**:921–928.
43. Taddeo, B., T. R. Luo, W. Zhang, and B. Roizman. 2003. Activation of NF-kappaB in cells productively infected with HSV-1 depends on activated protein kinase R and plays no apparent role in blocking apoptosis. *Proc. Natl. Acad. Sci. USA* **100**:12408–12413.
44. Tzamaras, D., I. Roussou, and G. Thireos. 1989. Coupling of GCN4 mRNA translational activation with decreased rates of polypeptide chain initiation. *Cell* **57**:947–954.
45. Ubeda, M., X.-Z. Wang, H. Zinszner, I. Wu, J. Habener, and D. Ron. 1996. Stress-induced binding of transcription factor CHOP to a novel DNA-control element. *Mol. Cell. Biol.* **16**:1479–1489.
46. Wu, S., M. Tan, Y. Hu, J. L. Wang, D. Scheuner, and R. J. Kaufman. 2004. Ultraviolet light activates NFkB through translational inhibition of I kB α synthesis. *J. Biol. Chem.* **279**:34898–34902.
47. Zamanian-Daryoush, M., T. H. Mogensen, J. A. DiDonato, and B. R. G. Williams. 2000. NF- κ B activation by double-stranded-RNA-activated protein kinase (PKR) is mediated through NF- κ B-inducing kinase and I kB kinase. *Mol. Cell. Biol.* **20**:1278–1290.
48. Zhang, P., B. McGrath, S. Li, A. Frank, F. Zambito, J. Reinert, M. Gannon, K. Ma, K. McNaughton, and D. R. Cavener. 2002. The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol. Cell. Biol.* **22**:3864–3874.