The eIF2α Kinases PERK and PKR Activate Glycogen Synthase Kinase 3 to Promote the Proteasomal Degradation of p53*  
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Phosphorylation of eukaryotic initiation factor 2α (eIF2α) is mediated by a family of kinases that respond to various forms of environmental stress. The eIF2α kinases are critical for mRNA translation, cell proliferation, and apoptosis. Activation of the tumor suppressor p53 results in cell cycle arrest and apoptosis in response to various types of stress. We previously showed that, unlike the majority of stress responses that stabilize and activate p53, induction of endoplasmic reticulum stress leads to p53 degradation through an Mdm2-dependent mechanism. Here, we demonstrate that the endoplasmic reticulum-resident eIF2α kinase PERK mediates the proteasomal degradation of p53 independently of translational control. This role is not specific for PERK, because the eIF2α kinase PKR also promotes p53 degradation in response to double-stranded RNA. We further establish that the eIF2α kinases induce glycogen synthase kinase 3 to promote the nuclear export and proteasomal degradation of p53. Our findings reveal a novel cross-talk between the eIF2α kinases and p53 with implications in cell proliferation and tumorigenesis.

The tumor suppressor p53 is a transcription factor mutated in ~50% of human cancers (1). In normal cells, p53 plays a pivotal role in controlling cell cycle, apoptosis, and DNA repair in response to various forms of genotoxic stress (2, 3). The regulation of p53 is complex and occurs mainly at the post-translational level (4). This is mediated by various post-translational modifications, such as phosphorylation and acetylation, which contribute to its stabilization and activation (5). The stability of p53 is regulated by its interaction with Hdm2 (human Mdm2), an E3-ubiquitin ligase that acts as an antagonist limiting p53 tumor suppressor function (6). Both p53 and Hdm2 are in an autoregulatory feedback loop in which p53 induces Hdm2 expression at the transcriptional level. The Hdm2 protein then binds to and ubiquitinates p53 in the nucleus, a process that allows the nuclear export and the cytoplasmic proteasome-dependent degradation of the tumor suppressor (6). In addition to Hdm2, other ubiquitin ligases, such as COP1 (7) and Pirh2 (8), have been shown to disrupt p53 stability. However, compared with Hdm2, little is currently known about how these ligases act on p53 (9).

The majority of stress responses that activate p53 requires its nuclear accumulation and function (10). This is mediated mainly through inactivation of the Hdm2-dependent degradation pathway as well as through interactions with nuclear proteins that promote post-translational modifications of p53 leading to its stabilization and activation (10). The current interest in p53 is underscored by the tremendous therapeutic benefits of its reactivation in cancer cells. Small molecules or peptides that restore the function of mutant p53 proteins have a great anti-tumor potential by enhancing the apoptotic sensitivity of tumor cells (11–13). Because p53 activity is influenced by many factors, targeting of proteins that regulate p53 function may also be necessary to ensure its ability to switch on its apoptotic programs (1, 6). Thus, more information is needed about the partners of p53 and their role in regulating signaling pathways that modulate p53 tumor suppressor function in both normal and tumor cells.

Regulation of gene expression at the translational level plays a critical role in cell growth, proliferation, and tumor development (14, 15). Translation can be controlled at each of the three steps: initiation, elongation, and termination (16). However, most regulation is exerted at the level of initiation, when the ribosome is recruited to an mRNA and positioned at the initiation codon (17). A critical event in this process is the phosphorylation of the α subunit of translation initiation factor eIF2α at serine 51 (Ser51), a modification that blocks initiation (18). This is because phosphorylated eIF2α acts as a dominant inhibitor of the guanine exchange factor eIF2B and prevents recycling of

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The abbreviations used are: eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; dsRNA, double-stranded RNA; TG, thapsigargin; FRAT1, frequently rearranged in advanced T cell lymphomas 1; GSK-3, glycogen synthase kinase-3; GS, glycogen synthase; GFP, green fluorescent protein; DAPI, 4,6-diamidino-2-phenylindole; CREB, CAMP-response element-binding protein; siRNA, small interfering RNA; RT, reverse transcriptase; MEF, mouse embryonic fibroblast; shRNA, short hairpin RNA; GydB, gyrase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphatidylinositol 3-kinase; KD, protein kinase domain.
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eLF2 by successive rounds of protein synthesis. Phosphorylation of elf2α is mediated by kinases that respond to distinct forms of stress (18). The elf2α kinase family includes the heme-regulated inhibitor (HRI), whose activity is prevented by heme in vitro and in vivo, and which becomes activated when cells are deficient in iron or heme or exposed to oxidative stress (19).

The general control non-derepressible-2 is activated by uncharged tRNA as a result of amino acid starvation resulting in the induction of amino acid biosynthetic genes (20, 21). The activity of the endoplasmic reticulum (ER)-resident protein kinase PERK/PEK is induced by the presence of unfolded proteins in the ER and results in a decrease of protein synthesis to prevent the accumulation of incorrectly folded or unfolded proteins (22, 23). Finally, the interferon-inducible protein kinase PKR, the prototype of the elf2α kinases, is activated by double-stranded (ds) RNA produced during virus replication and results in the inhibition of viral and host protein synthesis (24, 25). Each of these enzymes exhibit a number of significant sequence similarities between them, particularly in the protein kinase domain (KD) (18). This may account for the common substrate specificity toward elf2α and indicate the conserved properties of the kinases throughout evolution (18).

In the past years, there has been much progress in identifying new conditions that induce the elf2α phosphorylation pathway. For example, the cloning and characterization of PERK and mammalian general control non-derepressible-2 have revealed the important role of translational control in cells subjected to ER stress and nutrient deprivation (26). ER stress typically switches on cytoprotective measures that help cells adapt to this form of stress and induces apoptosis only when adaptation is not possible (27). One of these adaptive mechanisms requires the down-regulation of p53 (28). Specifically, we demonstrated that ER stress inhibits the apoptotic function of p53 (29). It does so by promoting the nuclear export and degradation of p53 through the activation of glycogen synthase kinase-3β (GSK-3β) (29). Further, we showed that ER stress enhances the Hdm2-dependent ubiquitination of p53, which requires GSK-3β-mediated phosphorylation of p53 at Ser115 and Ser176 (29, 30). The negative role of GSK-3β in regulation of p53 is supported by other studies showing the ability of nuclear GSK-3β to phosphorylate Hdm2 and promote p53 degradation (31). Thus, unlike DNA damage and other types of stress that stabilize p53, ER stress is the only type of stress described so far that leads to p53 destabilization.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—NIH 3T3 and derived cell lines, and HT1080 and derived cell lines were maintained in Dulbecco’s modified Eagle’s medium plus 10% calf serum (Invitrogen) and antibiotics (penicillin/streptomycin, 100 units/ml). Iso- genic elf2α S/S and elf2α A/A MEFs were maintained in Dulbecco’s modified Eagle’s medium plus heat-inactivated calf serum, essential and non-essential amino acids (Invitrogen), and antibiotics. A549, U2OS, and HCT116 cells were maintained in F12K medium (Cellgro). Dulbecco’s modified Eagle’s medium, and McCoy’s 5A medium (Cellgro), respectively, supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (penicillin/streptomycin, 100 units/ml). Tunicamycin (10 μg/ml, Sigma), thapsigargin (TG) (1 μM, Sigma), leptomycin B (3 μg/ml, Sigma), MG132 (10 μM, Biomol), and 1-azaken- pallone (1 μM, Calbiochem) were dissolved in dimethyl sulfoxide (Me2SO). LiCl (Sigma) was dissolved in water, dsRNA (poly(r-l-c)) (10 μg/ml) was dissolved in phosphate-buffered saline. The salubrinal derivative Sal003 (75 μM) and coumeryrin (100 ng/ml, Sigma) were used as described (32, 33). For transient transfections, Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer’s specifications.

Protein Extraction and Immunoblot Analysis—Cells were washed twice with ice-cold phosphate-buffered saline and proteins were extracted in ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl2, 1% Triton X-100, 3 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM dithiothreitol, 0.1 mM Na2VO4, and 1 mM phenylmethylsulfonyl fluoride. Extracts were kept on ice for 15 min, centrifuged at 10,000 × g for 15 min (4 °C), and supernatants were stored at −80 °C. Proteins were quantified by Bradford assay (Bio-Rad).

For immunoblotting, whole cell extracts (50 μg of protein) were resolved by SDS-PAGE and proteins were then electroblotted onto polyvinylidene difluoride membranes (Immobilon P, Millipore). Primary antibodies (1:1000 dilution, unless specified) used were as follows: rabbit polyclonal antibody to PERK (generous gift from D. Ron), rabbit polyclonal antibody to phosphosine 51-elf2α (1:5000 dilution, BioSource), rabbit polyclonal anti-elf2α (sc-11386, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody to PKR (B-10, sc-6282, Santa Cruz), mouse monoclonal antibody to actin (1:5000 dilution, Clone C4, ICN Biomedicals Inc.), mouse monoclonal antibody to human p53 (DO-1, 1B-6, Oncogene Science), a rabbit polyclonal antibody to mouse p53 (CM-5, Novocastra), rabbit polyclonal antibody to GSK-3β (number 9332, Cell Signaling, rabbit polyclonal antibody to phosphosine 641/645 of glycogen synthase (Upstate), mouse monoclonal antibody to glycogen synthase (Cell Signaling), rabbit polyclonal antibody to PUMA (3041, ProSci Inc.), and rabbit polyclonal antibody to NOXA (2437, ProSci Inc.). Anti-mouse IgG-horseradish peroxidase or anti-rabbit IgG-horseradish peroxidase-conjugated antibodies were used as secondary antibodies (1:1000 dilution). Proteins were visualized by enhanced chemiluminescence (ECL) according to the manufacturer’s specification (Amer sham Biosciences). Quantification of bands was performed by densitometry using the NIH Image 1.54 software.

Immunofluorescence Studies—The detection of GFP-p53, GSK-3β, or p53 by immunofluorescence was performed as previously described (29). For immunofluorescence, cells were stained with a 1:200 dilution for mouse monoclonal antibody to p53 (DO-1, Oncogene Science) or a 1:100 dilution for rabbit polyclonal antibody to GSK-3β (number 9332, Cell Signaling). The nucleus was visualized after staining with 0.05 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) (Sigma). Images were captured on a Zeiss microscope using equal exposure times. Nuclear/cytoplasmic ratios were determined by calculating total pixel intensity in a circle of 4 μm in diameter in the nucleus and the cytoplasm using AxioVision 4.5 software. The background was determined by calculating pixel intensity in a 4-μm diameter cell-free area. This area was then subtracted from the
nuclear and cytoplasmic measurements for each cell within the microscopy field. Experiments were performed in triplicate.

GSK-3β Kinase Assay—Protein extracts (100 μg) were mixed with 15 μl of kinase buffer (50 mM HEPES, pH 7.2, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 μCi of [γ-32P]ATP, 10 μM MgCl2) and 50 μM synthetic phospho-CREB substrate peptide (KRREILSRRP(pS)YR) with or without 1-azakenpaullone (1 μM). After 30 min of incubation at 30 °C, 10 μg/ml dsRNA for 4 h. Protein extracts were subjected to immunoblot analysis with anti-p53 (CM-5) (top panel), anti-actin (bottom panel) antibodies. The p53 protein levels (% p53) in A–D were quantified using Scion Image 4.0 software.

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FIGURE 1. The activation of eIF2α kinases results in p53 degradation. A, primary PERK+/+ and PERK−/− MEFs were treated with thapsigargin (TG, 1 μM) for 4 h. Protein extracts were subjected to immunoblot analysis with anti-p53 (CM-5) (top panel) and anti-actin (bottom panel) antibodies. B, HT1080 cells were subjected to PERK silencing by siRNA and treated with 1 μM TG for 4 h. Protein extracts were immunoblotted with anti-p53 (DO-1) (top panel), anti-phosphoserine 51-eIF2α (middle panel), and anti-eIF2α (bottom panel) antibodies. C, HT1080 cells were pre-treated or not with MG132 (10 μM) for 4 h. Protein extracts were subjected to immunoblot analysis with anti-p53 (CM-5) (top panel), anti-PKR (second panel), anti-phosphoserine 51-eIF2α (third panel), and anti-eIF2α (bottom panel) antibodies. The p53 protein levels (% p53) in A–D were quantified using Scion Image 4.0 software.

serum-free Dulbecco’s modified Eagle’s medium and replenished with medium containing 10% serum. Cells were incubated at 37 °C for an additional 72 h before being treated with thapsigargin or coumermycin.

Polysome Profile Analysis and RNA Extraction—Polysome profiling protocol used was previously described (34). The gradients were prepared with the ISCO model 160 Gradient Former and were fractionated into 20 fractions of 500 μl using the ISCO density gradient fractionation system Foxy Jr. Fraction Collector while measuring the absorbance at 254 nm. The RNA was isolated from each fraction using TRIzol (Invitrogen) and the manufacturer’s specifications.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—The extracted RNA (from each fraction) was used for reverse transcription using SuperScript II RNase H Reverse Transcriptase (90 units/reaction, 18064-014, Invitrogen) and 100 μM oligo(dT) primer (d12VN) according to the manufacturer’s instructions. The TaqPlus Precision Polymerase (2 units/reaction, 60012, Stratagene) was used for PCR according to the manufacturer’s specifications. The PCR program was as follows: 94 °C, 1 min; 58 °C, 1 min; and 72 °C, 1 min × 28 cycles. The following primers were used: p53 forward, 5′-AACCTACGAGGCGACCTAGC-3′, p53 reverse, 5′-CTTCCCTCTGACCGAGCTCTC-3′; ATF4 forward, 5′-GGCGGTGTGATGGGCTG-3′, and GAPDH forward, 5′-CATCATC-3′.
cells, which contain wild type p53 (29), treated with TG was rescued when endogenous PERK was targeted by siRNA (Fig. 1B). Although siRNA did not completely eliminate endogenous PERK, its partial inactivation was sufficient to prevent p53 degradation in response to TG treatment (Fig. 1B, lanes 3 and 4). These data revealed a negative effect of PERK on p53 in ER-stressed cells.

We also investigated whether other eIF2α kinases exert similar effects on p53 as PERK. When HT1080 cells were transfected with dsRNA, which activates PKR, we noticed that p53 levels were reduced by ~80% concomitantly with an induction of eIF2α phosphorylation (Fig. 1C, lane 3). Unlike dsRNA transfection, incubation of cells with dsRNA alone was insufficient to down-regulate p53 indicating that an intracellular pathway is required to decrease the levels of the tumor suppressor (Fig. 1C, lane 2). Down-regulation of p53 by transfected dsRNA was prevented by the presence of MG132 thus implicating the proteasome degradation machinery in this process (Fig. 1C, lane 6). It is of interest that MG132 decreased eIF2α phosphorylation in cells transfected with dsRNA indicating that p53 down-regulation may be proportional to eIF2α phosphorylation (Fig. 1C, compare lane 3 with lane 6). Given that dsRNA transfection activates several proteins in addition to PKR (35), we verified the role of the eIF2α kinase in NIH 3T3 cells, which contain wild type p53 and whose endogenous PKR levels were knocked-down by shRNA. We noticed that p53 protein levels were decreased by 80% in control shRNA-treated cells in response to dsRNA transfection (Fig. 1D, lane 2) as opposed to cells with inactivated PKR, in which p53 protein levels remained stable (Fig. 1D, lanes 3 with 4). Collectively, these findings supported a negative role of eIF2α kinases in p53 stabilization.

Activation of eIF2α Kinases Results in p53 Degradation Indepenently of eIF2α Phosphorylation—To better understand the mechanisms of p53 down-regulation by eIF2α kinases, we utilized a conditionally active form of PKR expressed in HT1080 cells (33). This form consists of the first 220 amino acids of the bacterial gyrase B (GyrB) protein fused to the kinase domain (KD) of the human PKR (GyrB-PKR) (36). Treatment of cells with the antibiotic coumermycin causes the activation of GyrB-PKR by dimerization leading to the phosphorylation of eIF2α at Ser51 and inhibition of protein synthesis (33, 36). Given that the high degree of homology between the KD of all eIF2α kinases (37), the GyrB-PKR system faithfully represents the consequences of activation of all eIF2α kinases. Treatment of GyrB-PKR-expressing HT1080 cells with coumermycin resulted in the down-regulation of p53 concomitantly with an induction of eIF2α phosphorylation (Fig. 2A, lane 2). On the other hand, expression of the catalytically inactive GyrB-PKR K296H in HT1080 cells did not affect p53 protein levels (Fig. 2A, lane 4). Treatment with MG132 prevented the down-regulation of p53 by the conditional activation of GyrB-PKR (Fig. 2B, compare lane 2 with lane 4) supporting a role of the proteasome pathway in this process. Taken together, these data demonstrated that the eIF2α kinase activity is both necessary and sufficient to mediate the proteasomal degradation of p53.

Given the fundamental role of eIF2α kinases in translational control (37), we next investigated a possible link between p53 destabilization and protein synthesis inhibition. Treatment of HT1080 cells (Fig. 2C) or NIH 3T3 cells (Fig. 2D) with Sal003, a derivative of salubrinal (38) that blocks eIF2α dephosphorylation (32), did not affect the overall levels of p53. These data suggested that induction of eIF2α phosphorylation alone is not capable of inducing the destabilization of p53.

Translation of p53 mRNA Resists to eIF2α Phosphorylation—The above data favored a post-translational regulation of p53 by the eIF2α kinases. To verify this prediction, we measured the translatability of p53 mRNA by polysome profile analysis, a technique that allows the separation of monosomes from polyribosomes by sucrose density centrifugation (39). Efficiently translated mRNAs are bound to polyribosomes, whereas mRNAs that are poorly translated are associated with monosomes or disomes. We first examined the translatability of p53 mRNA in GyrB-PKR-expressing cells (Fig. 3A). Polysomal mRNA was isolated from total cellular mRNA by fractionation through 10–55% sucrose gradient. The distribution of ribosomes and mRNAs in the gradient fractions was determined by UV spectroscopy and the presence of specific mRNAs was identified by semi-quantitative RT-PCR. In each gradient, fractions 1–4 represent free mRNAs, whereas fractions 5–11 represent the ribosomal subunits (40S and 60S) and the single associated ribosome (80S or monosome). Fractions 12–20 rep-
resent mRNA associated with polyribosomes. We observed that activation of GyrB-PKR by coumermycin resulted in a significant reduction of polyribosome fractions concomitant with an increase in ribosomal subunit peaks indicative of inhibition of translation initiation. We utilized ATF4 mRNA as a control for its capacity to be better translated under conditions that induce eIF2α phosphorylation (40). We observed that ATF4 transcripts shifted toward larger polyribosome fractions upon coumermycin treatment indicative of their efficient translation (Fig. 3A, right panel, compare fractions 9–14 of control to fractions 15–20 of treated samples). The GAPDH transcripts were also used as a control for general mRNA translation. Unlike the ATF4 mRNA, a large portion of GAPDH mRNAs shifted toward monosomes indicative of the general shut-off of translation. When p53 mRNA translation was assessed, we found that the p53 transcripts remained associated with the polyribosome fractions before and after the activation of GyrB-PKR with coumermycin. These data strongly suggested that p53 mRNAs are efficiently translated under conditions of translational inhibition caused by eIF2α kinase activation.

We further verified the above observations by assessing p53 mRNA translation in HT1080 cells subjected to ER stress or treated with Sal003 (Fig. 3B). We noticed that both drugs exerted a significant inhibition of mRNA translation compared with untreated cells as indicated by the increase in ribosomal subunit peaks (compare fractions 5–11 to control) concomitant with a significant decrease of the largest class of polyribosomes (fractions 12–20). Under these conditions ATF4 mRNA translation was induced further demonstrating the dependence of this mRNA on eIF2α phosphorylation for efficient translation (Fig. 3B, compare fractions 9–14 of control to fractions 15–20 of treated samples). Contrary to ATF4, a significant fraction of GAPDH transcripts shifted toward monosomes due to translation inhibition by eIF2α phosphorylation. Interestingly, p53 transcripts exhibited a similar sedimentation pattern in both untreated and treated cells (Fig. 3B), suggesting that p53 mRNAs undergo efficient translation under conditions that induce eIF2α phosphorylation. Similar results were obtained with cells subjected to dsRNA transfection.6

Activation of eIF2α Kinases Leads to the Nucleocytoplasmic Export of p53—To gain better insight into the mechanisms of p53 destabilization, we looked at the localization of p53 in response to various forms of stress that activate the eIF2α kinases. Previous work from our group demonstrated that cytoplasmic localization of p53 is induced in cells subjected to ER stress (29, 30). In analogy to ER stress, we observed that transfection of HT1080 cells with dsRNA enhanced the cytoplasmic relocation of p53, which was prevented by treatment with MG132 (Fig. 4A). Thus, cytoplasmic localization of p53 is linked to its proteasomal degradation in response to dsRNA. These data posed the interesting question whether the eIF2α

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FIGURE 3. Translation of p53 mRNA resists eIF2α phosphorylation. A, GyrB-PKR WT cells were treated with 100 ng/ml coumermycin for 4 h and cell lysates were separated on a sucrose gradient and subjected to fractionation as described under “Experimental Procedures.” The positions of the polysomes and ribosomal subunits are indicated above each corresponding peak. Translation efficiency of TP53 mRNA (top panel), ATF4 mRNA (middle panel), and GAPDH mRNA (bottom panel) were determined by RT-PCR for each of the fractions. B, HT1080 cells were treated either with 1 μM thapsigargin (TG) for 2 h or 75 μM Sal003 for 5 h. Cell lysates were separated on a sucrose gradient and subjected to fractionation as described under “Experimental Procedures.” The positions of the polysomes and ribosomal subunits are indicated above each corresponding peak. Translation efficiency of TP53 mRNA (top panel), ATF4 mRNA (middle panel), and GAPDH mRNA (bottom panel) were determined by RT-PCR for each of the fractions. WT, wild type.

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6 D. Baltzis and A. E. Koromilas, unpublished data.
kinases are involved in this process. To test this hypothesis, we utilized the GyrB-PKR system to examine the subcellular distribution of p53 as a result of eIF2α kinase activation. We noticed that treatment of GyrB-PKR-expressing cells with coumermycin increased the cytoplasmic localization of endogenous p53 as opposed to coumermycin-treated cells expressing the catalytically inactive GyrB-PKR K296H in which p53 remained nuclear (Fig. 4B). Treatment of cells with the CRM1 inhibitor leptomycin B resulted in the stabilization of p53 (Fig. 4C) and its nuclear retention (Fig. 4D) upon activation of GyrB-PKR with coumermycin. In addition, cytoplasmic localization of p53 was blocked by the presence of MG132 in GyrB-PKR cells treated with coumermycin (Fig. 4E). These data showed that eIF2α kinase activation is sufficient to promote both the cytoplasmic relocation and proteasomal degradation of p53.

The next step was to verify whether nuclear export of p53 requires translational control by eIF2α phosphorylation. We found that p53 remained nuclear in Sal003-treated cells (Fig. 5A) providing evidence that eIF2α phosphorylation is not sufficient for the cytoplasmic relocation of p53. We also took advantage of the generation of spontaneously immortalized MEFs containing either the wild type eIF2α allele (S/S) or an eIF2α allele bearing the S51A mutation (A/A) (42). Given that p53 in both MEF types is mutant due to immortalization, we assessed the localization of ectopically expressed GFP-p53 upon ER stress. We found that treatment with either tunicamycin or TG enhanced the cytoplasmic localization of GFP-p53 in both eIF2α S/S and eIF2α A/A MEFs (Fig. 5B) demonstrating that eIF2α phosphorylation is not a determinant of the nuclear export of p53.

The eIF2α Kinases Mediate p53 Ser<sup>315</sup> Phosphorylation and GSK-3β Nuclear Localization—We previously showed that ER stress leads to p53 phosphorylation at Ser<sup>315</sup>,...
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FIGURE 5. The nuclear export of p53 mediated by eIF2α kinases occurs independently of eIF2α phosphorylation. A, HT1080 cells were treated with 75 μM Sal003 for 5 h. Immunohistochemistry analysis was performed to detect endogenous p53 (green), and cell nuclei were detected by DAPI staining (blue). B, eIF2α A/A and eIF2α S/S MEFs were transfected with 0.5 μg of GFP-p53 WT cDNA. After 24 h, cells were left untreated or treated with either 10 μg/ml tunicamycin (TM) or 1 μM thapsigargin (TG) for 4 h. The subcellular localization of GFP-p53 (green) was determined by fluorescence microscopy. The cell nuclei were visualized by DAPI staining (blue).

The above findings prompted us to examine whether eIF2α kinases induce GSK-3β phosphorylation at either Ser9 or Tyr216 in all cells treated with conditions that activate the eIF2α kinase. These data provided evidence for a functional cross-talk between the eIF2α kinases and GSK-3β converging on p53.

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which is required for its nuclear export and degradation (29, 30). Considering the enhanced nuclear export of p53 by eIF2α kinases, we hypothesized a role of Ser315 phosphorylation in this process. Due to the unavailability of antibodies that specifically recognize Ser315 of p53,6 we employed the GyrB-PKR system to examine the localization of GFP-p53 WT and GFP-p53 bearing either the S315A mutation or the phosphomimetic S315D mutation (Fig. 6A). We observed that GFP-p53 S315A was nuclear in untreated cells and remained nuclear in GyrB-PKR cells treated with coumermycin. On the other hand, GFP-p53 WT was nuclear in untreated cells and became both nuclear and cytoplasmic in response to coumermycin treatment (Fig. 6A). Contrary to this, GFP-p53 S315D exhibited both nuclear and cytoplasmic localization in untreated cells, which was not altered after treatment with coumermycin (Fig. 6A). These findings provided evidence that Ser315 phosphorylation facilitates the nuclear export of p53 caused by eIF2α kinase activation.

For cells subjected to ER stress, we showed that Ser315 phosphorylation of p53 is mediated by the nuclear translocation and activation of GSK-3β (29, 30). In analogy to ER stress, we found that transfection of cells with dsRNA induces the nuclear localization of GSK-3β in HT1080 cells (Fig. 6B). This event is not dependent on eIF2α phosphorylation because treatment of cells with Sal003 did not induce the nuclear localization of GSK-3β (Fig. 6B). When the GyrB-PKR system was used, we found that activation of GyrB-PKR by coumermycin was sufficient to induce the nuclear localization of GSK-3β (Fig. 6C). Induction of nuclear localization of GSK-3β was not possible in coumermycin-treated cells expressing GyrB-PKR K296H, demonstrating that this event requires the catalytic activity of eIF2α kinases. These data provided evidence for a functional cross-talk between the eIF2α kinases and GSK-3β converging on p53.

6 O. Pluquet, A. I. Papadakis, and A. E. Koromilas, unpublished data.

FIGURE 4. The activation of eIF2α kinase induces the nuclear export of p53. A, HT1080 cells were treated (dsRNA) or transfected (lipo + dsRNA) with 10 μg/ml dsRNA for 4 h. Immunohistochemistry analysis was performed to detect endogenous p53 (green), and cell nuclei were detected by DAPI staining (blue). B, GyrB-PKR WT and K296H cells were treated with coumermycin (100 ng/ml) for 4 h. Immunohistochemistry analysis was performed to detect endogenous p53 (green), and cell nuclei were detected by DAPI staining (blue). C, GyrB-PKR WT cells were pre-treated with 3 ng/ml leptomycin B (LMB) for 2 h (lanes 3–4 and 6–7) prior to treatment with 100 ng/ml coumermycin (Coum) for 3 or 6 h (lanes 2, 4, 5, and 7). Protein extracts were subjected to immunoblot analysis with anti-p53 (DO-1) (top panel) and anti-phosphoserine 51-eIF2α (bottom panel) antibodies. D, GyrB-PKR WT cells were pre-treated with 3 ng/ml leptomycin B for 2 h prior to treatment with 100 ng/ml coumermycin (Coum) for 6 h. Immunohistochemistry analysis was performed to detect endogenous p53 (green), and cell nuclei were detected by DAPI staining (blue). E, GyrB-PKR WT cells were pre-treated with 10 μM MG132 for 30 min prior to treatment with 100 ng/ml coumermycin for 4 h. Immunohistochemistry analysis was performed to detect endogenous p53 (green), and cell nuclei were detected by DAPI staining (blue). WT, wild type.
extracts by measuring the phosphorylation of a CREB peptide containing an optimal (i.e. primed) GSK-3 phosphorylation site in kinase assays in vitro. We found that phosphorylation of the optimal substrate was induced after its incubation with protein extracts from HT1080 cells subjected to dsRNA transfection or ER stress with TG (Fig. 7A) indicating that both treatments activate GSK-3. We also detected an induction of CREB peptide phosphorylation in protein extracts from dsRNA-transfected NIH 3T3 cells, which was impaired by the presence of 1-azakenpaullone, an inhibitor of GSK-3 (Fig. 7B). However, induction of the GSK-3 substrate phosphorylation was not observed in dsRNA-transfected NIH 3T3 cells in which PKR was eliminated by shRNA (Fig. 7B), suggesting that GSK-3 activation upon this treatment requires PKR. When we looked at PERK, we noticed an induction of peptide phosphorylation in protein extracts from PERK+/+ MEFs after TG treatment, which was compromised by the pharmacological inhibition of GSK-3 (Fig. 7C). TG treatment, however, did not induce the phosphorylation of the peptide in extracts from PERK−/− MEFs providing evidence that PERK is upstream of GSK-3 in cells subjected to ER stress (Fig. 7C). The in vitro phosphorylation assays were further verified in vivo by looking at the phosphorylation of endogenous glycogen synthase (GS), which is the best characterized substrate of GSK-3, with phosphospecific antibodies. We observed that a higher amount of GS was phosphorylated at Ser641/Ser645 in PERK+/+ MEFs than in PERK−/− MEFs after TG treatment. GS phosphorylation was proportional to PERK activation as judged by the eIF2α phosphorylation levels in TG-treated cells (Fig. 7D, compare the levels of phosphorylated proteins in first and fourth panels).

The function of GSK-3 downstream of eIF2α kinases was further verified by looking at p53 degrada-
Protein extracts were subjected to immunoblot analysis with anti-phosphoserine 641/645 glycogen synthase. Experiments were performed in triplicate. The activation of eIF2α kinases mediates p53 Ser315 phosphorylation and GSK-3β nuclear localization. A, GyrB-PKR WT cells were transiently transfected with 0.5 μg of GFP-p53 WT cDNA or the indicated GFP-p53 mutant cDNAs (S315A or S315D). After 24 h, cells were left untreated or treated with 100 ng/ml coumermycin for 4 h. Protein extracts were used to assess the kinase activity of endogenous GSK-3β using a synthetic phospho-CREB substrate peptide. B, NIH 3T3 cells stably transfected with shRNA against PKR were transfected with 10 μg/ml dsRNA for 4 h in the presence or absence of 1 μM of the GSK-3 inhibitor, 1-azakenpaullone (Aza). Protein extracts were used to assess the kinase activity of endogenous GSK-3β using a synthetic phospho-CREB substrate peptide. C, immortalized PERK+/− and PERK−/− MEFs were treated with 1 μM TG for 4 h in the presence or absence of 1 μM of the GSK-3 inhibitor, 1-azakenpaullone. Protein extracts were used to assess the kinase activity of endogenous GSK-3β using a synthetic phospho-CREB substrate peptide. D, immortalized PERK+/− and PERK−/− MEFs were treated with 1 μM TG for the indicated periods of time. Protein extracts were subjected to immunoblot analysis with anti-phosphoserine 641/645 glycogen synthase (top panel), anti-glycogen synthase (second panel), anti-tubulin (third panel), anti-phosphoserine 51-eIF2α (fourth panel), and anti-eIF2α (fifth panel) antibodies. The ratio between the phospho-glycogen synthase levels (GS-P) versus the total glycogen synthase levels (GS) was quantified and normalized to tubulin levels using Scion Image 4.0 software.

Control of p53 by eIF2α Kinases

We have identified a novel signaling pathway mediated by the eIF2α kinases PERK and PKR. That is, activation of the eIF2α kinases negatively regulates p53 by enhancing the nuclear export and proteasome-dependent degradation of the tumor suppressor (Fig. 9). The molecular mechanism of p53 inactivation is explained by the ability of eIF2α kinases to induce GSK-3β. Previous work from our laboratory established an important role for GSK-3β in nuclear export and degradation of p53 in ER stressed cells (30). That is, degradation of p53 by ER stress requires the nuclear localization and activation of GSK-3β, which in turn mediates the phosphorylation of p53 at Ser315 and Ser376 (29). Activation of GSK-3β is required for the nuclear export and degradation of p53 through the Mdm2 pathway (30). Consistent with these findings, we have showed that the eIF2α kinases are capable of promoting the nuclear localization of GSK-3β concomitantly.
Control of p53 by eIF2α Kinases

with an induction of its activity. It is very likely that the a isoform of GSK-3 also contributes to the down-regulation of p53 by eIF2α kinases. However, confirmation of GSK-3α function has been hindered by the lack of GSK-3α−/− cells and its incomplete inactivation by RNA interference.7 Previous data established a role of protein synthesis in the regulation of p53 localization possibly through the action of short-lived protein(s) that can tether p53 in the cytoplasm (45). Nevertheless, our data clearly show that the translational properties of the eIF2α kinases are dispensable for nuclear export and destabilization of p53. The eIF2α kinases may mediate post-translational modifications of GSK-3β that are essential for its activity. We have performed in vitro PKR kinase assays showing that GSK-3β is not a physical substrate of PKR, suggesting that the activation of GSK-3β is not a direct mechanism mediated by PKR activation.7

The PI3K-Akt pathway has been reported to inhibit the transcriptional activity of p53, and reduce the pro-apoptotic functions of p53. Activation of Akt enhances the ubiquitination-promoting function of Hdm2 by phosphorylation of Ser186 (46). We recently reported that conditional activation of PKR acts upstream of PI3K and in turn induces the activation of the Akt/ PKB pathway (47). However, when cells were pre-treated with the PI3K inhibitor, LY294002, or transfected with a dominant negative form of Akt (hemagglutinin-tagged Akt K179M) prior to eIF2α kinase activation, p53 protein levels were still down-regulated.7 This suggests that the PI3K-Akt pathway does not regulate p53 degradation in response to eIF2α kinase activation.

Therefore the regulation of GSK-3β by eIF2α kinases may utilize indirect mechanisms and involve, for example, protein(s) that control GSK-3β activity and/or localization. In this regard, the GSK-3-binding protein is an inhibitor of GSK-3-which was first identified in Xenopus embryos (48). GSK-3-binding protein is homologous to the mammalian T cell proto-oncogene FRAT1 (frequently rearranged in advanced T cell lymphomas 1) (49). GSK-3-binding protein/FRAT inhibits GSK-3 activity toward -catenin by preventing Axin from binding to GSK-3 (50). Genetic inactivation of FRAT1 and its homologues FRAT2 and FRAT3 in mice failed to verify the proposed stimulatory effects on canonical Wnt signaling (51). This indicated
Consistent with our findings, recent work implicated NOXA in ER stress-mediated apoptosis independently of p53 (55). The cause of these differences is not clear but it could be explained, at least partly, by the regulation of GSK-3. Cells may not have the same capacity to induce GSK-3β and degrade p53 in response to ER stress. The PERK-GSK-3β pathway may be compromised in some tumor cells and this could be exerted by modifications of the duration and strength of PERK activation and/or inhibition of GSK-3β activation.

The cytoprotective role of PERK has been best documented by its ability to repress protein synthesis, an adaptation that takes place by lowering the load of the client proteins that ER has to process (22). Our findings show a new cytoprotective role of PERK has been best documented by its ability to repress protein synthesis, an adaptation that takes place by lowering the load of the client proteins that ER has to process (22). Our findings show a new cytoprotective role of PERK. When cells are exposed to ER stress, virus infection, or dsRNA, the eIF2α kinases activate different signaling pathways, mainly the one involving the inhibition of protein synthesis upon eIF2α phosphorylation of Ser51. The eIF2α kinases also control the nuclear localization and activation of GSK-3β leading to the induction of p53 phosphorylation at Ser51 and Ser376 (29). These phosphorylation events enhance the ubiquitination of p53 by Mdm2, and the cytoplasmic translocation of the tumor suppressor. As a result, p53 degradation occurs in the cytoplasm. WT, wild type.

FIGURE 9. Schematic model of eIF2α kinase-mediated p53 degradation. When cells are exposed to ER stress, virus infection, or dsRNA, the eIF2α kinases activate different signaling pathways, mainly the one involving the inhibition of protein synthesis upon eIF2α phosphorylation of Ser51. The eIF2α kinases also control the nuclear localization and activation of GSK-3β leading to the induction of p53 phosphorylation at Ser51 and Ser376 (29). These phosphorylation events enhance the ubiquitination of p53 by Mdm2, and the cytoplasmic translocation of the tumor suppressor. As a result, p53 degradation occurs in the cytoplasm. WT, wild type.

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tive and negative signals that converge on p53. This might have important ramifications to chemotherapeutic approaches aimed at restoring the p53 levels and increasing its tumor suppressor function by inhibiting GSK-3. The role of GSK-3 in hypoxia has just started to emerge as indicated by recent reports documenting the activation of GSK-3 in mouse brain under hypoxic conditions (66) and the phosphorylation of tumor suppressor VHL by GSK-3 (67).

From our data, it is clear that the eIF2α kinases mediate negative effects on the stabilization of p53. It is of interest, however, that activation of eIF2α kinases also takes place in signaling pathways that stabilize p53, such as DNA damage. For example, previous work from our laboratory showed that PKR is involved in the phosphorylation and p53-mediated gene transcription in response to the chemotherapeutic drug adriamycin or γ-irradiation (68). Furthermore, ultraviolet (UV) light, which stabilizes p53, leads to eIF2α phosphorylation through the activation of PERK (69) and general control non-derepressible-2 (70). Therefore, the ability of eIF2α kinases to regulate p53 is stress-type dependent and is likely to involve post-translational modifications that affect the activation of the eIF2α kinases and/or activation of downstream effectors such as GSK-3β. Whatever the effects of eIF2α kinase activation on p53 may be, our data show that these effects cannot be exerted at the translational level. Using polysome profile analysis, we demonstrate the efficient translation of p53 transcripts under conditions that translation initiation is severely blocked as a result of eIF2α phosphorylation. Furthermore, we show that down-regulation of p53 is not possible in cells treated with Sal003, which prevents the dephosphorylation of eIF2α, demonstrating that eIF2α phosphorylation alone is insufficient to down-regulate the synthesis of p53. The ability of mouse and human p53 transcripts to bypass the translational inhibitory effects of eIF2α phosphorylation indicates the presence of a unique mechanism(s) of translation initiation in both species. Recent studies, provided evidence for the function of an internal ribosome entry site within the human p53 mRNA (71, 72). Given that specific viral and cellular internal ribosome entry sites can mediate efficient translation under conditions of increased eIF2α phosphorylation (73), the presence of the internal ribosome entry site could explain, at least in part, the efficient translation of p53 mRNA under the activation of eIF2α kinases.

Down-regulation of p53 by dsRNA provides a link between PKR and p53 in virus infection. Because dsRNA is an interme-
diately product of virus replication, its ability to inactivate p53 is not in line with its well documented anti-viral function (74, 75). However, the dsRNA pathway is one of the many pathways induced in infected cells (76), whose coordinated action determines the apoptotic potential of p53. In agreement with its anti-viral activity (74, 75), we found that tumor cells can undergo p53-dependent apoptosis only after infection with specific viruses (77). These data indicated that tumors cells may have evolved distinct pathways to activate p53 in a manner that is dependent on their origin and/or the virus type. In light of our findings here, it is possible that in virus-infected cells, the p53 levels are controlled by GSK-3. Although all viruses have the capacity to activate the eIF2α kinases, specific viruses only are capable of down-regulating p53 (78). For example, infection of HT1080 cells with vesicular stomatitis virus leads to eIF2α phosphorylation without causing the down-regulation of p53 (96). On the other hand, poliovirus infection activates GSK-3 as documented by the increased phosphorylation of GS at the GSK-3 site, whereas vesicular stomatitis virus infection did not (supplemental Fig. S2). This suggests that eIF2α can be used as a measure to determine the levels and activation potential of p53 in virus-infected cells as a result of eIF2α kinase activation. Therefore control of GSK-3 by pharmacological inhibitors may prove of immense significance for the efficacy of therapies aimed at the destruction of tumors containing wild type p53 with oncolytic viruses (41, 44).

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