Phosphorylation of eIF2 Directs ATF5 Translational Control in Response to Diverse Stress Conditions*

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Phosphorylation of eukaryotic initiation factor 2 (eIF2) is an important mechanism regulating global and gene-specific translation in response to different environmental stresses. Central to the eIF2 kinase response is the preferential translation of *ATF4* mRNA, encoding a transcriptional activator of genes involved in stress remediation. In this report, we addressed whether there are additional transcription factors whose translational expression is regulated by eIF2 kinases. We show that the expression of the basic zipper transcriptional regulator ATF5 is induced in response to many different stresses, including endoplasmic reticulum stress, arsenite exposure, and proteasome inhibition, by a mechanism requiring eIF2 phosphorylation. ATF5 is subject to translational control by the preferential association of ATF5 mRNA with large polyribosomes in response to stress. ATF5 translational control involves two upstream open reading frames (uORFs) located in the 5'-leader of the *ATF5* mRNA, a feature shared with *ATF4*. Mutational analyses of the 5'-leader of *ATF5* mRNA fused to a luciferase reporter suggest that the 5'-proximal uORF1 is positive-acting, allowing scanning ribosomes to reinitiate translation of a downstream ORF. During non-stressed conditions, when eIF2 phosphorylation is low, ribosomes reinitiate translation at the next ORF, the inhibitory uORF2. Phosphorylation of eIF2 during stress delays translation reinitiation, allowing scanning ribosomes to bypass uORF2, and instead translate the *ATF5* coding region. In addition to translational control, *ATF5* mRNA levels are significantly reduced in *ATF4*−/− mouse embryonic fibroblasts, suggesting that ATF4 contributes to basal *ATF5* transcription. These results demonstrate that eIF2 kinases direct the translational expression of multiple transcriptional regulators by a mechanism involving delayed translation reinitiation.

Phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2) is an important mechanism regulating protein synthesis in response to a diverse range of environmental stresses (1–3). Four eIF2α kinases have been described in mammals, each responding to different stress arrangements through their unique regulatory regions. For example, phosphorylation of eIF2α by PEK (also known as Perk or EIF2AK3) is induced by accumulation of malfolded proteins in the endoplasmic reticulum (ER) (4–6). Phosphorylation of eIF2α during this so-called ER stress inhibits global translation by lowering the levels of eIF2-GTP that are central for binding of initiator Met-tRNA<sub>Met</sub> to the translational machinery (1–3). Together with reduced protein synthesis, eIF2α phosphorylation increases the preferential translation of *ATF4* mRNA, encoding a basic zipper (bZIP) transcription activator that is important for directing the expression of genes involved in metabolism, the redox status of cells, and apoptosis (7–9). Decreased protein synthesis conserves energy and provides sufficient time for ATF4, and other stress-responsive transcription factors, to reconfigure gene expression that would block or ameliorate damage elicited by the underlying stress. Other members of the eIF2α kinase family include GCN2 (EIF2AK4), whose activity is enhanced by amino acid depletion, UV irradiation or proteasome inhibition; HRI (EIF2AK1), which is regulated by heme deficiency and oxidative stress; and PKR (EIF2AK2), which functions in an antiviral pathway (2, 3, 6, 10). Aberrations in these eIF2α kinase pathways are associated with a number of diseases, including diabetes, stroke, eating disorders, viral infection, anemia, and neurological disorders.

Enhanced *ATF4* expression during stress-induced eIF2α phosphorylation occurs primarily by translational control, as illustrated by increased association of *ATF4* mRNA with polysomes (11). Central to *ATF4* translational control is the 5'-leader of the *ATF4* mRNA, which encodes two uORFs that have opposing functions (8, 9). *ATF4* translation begins with the 40S ribosomal subunit bound to eIF2-GTP-Met-tRNA<sub>Met</sub> scanning from the 5'-end of the *ATF4* mRNA and initiating translation at the positive-acting uORF1. Following uORF1 translation, ribosomes are thought to retain association with *ATF4* mRNA and reinitiate translation at a downstream coding region (2, 8, 10). In non-stressed cells when eIF2α phosphorylation is low and there is abundant eIF2-GTP, ribosomes scanning downstream from uORF1 readily reinitiate translation at the next available ORF, the inhibitory uORF2. Following translation of uORF2, ribosomes are suggested to dissociate from the *ATF4* transcript, leading to lowered translation of the *ATF4* coding region. During stress conditions, elevated phosphorylation of eIF2α reduces eIF2-GTP levels, thus increasing the time required for scanning ribosomes to become competent to reinitiate translation. Following translation of uORF1, delayed reini-
tiation would allow for a portion of the ribosomes to bypass the uORF2 initiation codon, and instead translate the ATF4 coding region. The central feature in ATF4 translational control-delayed translation reinitiation in response to eIF2α phosphorylation, is shared with the mechanism that induces translation of a related bZIP transcriptional regulator GCN4 in yeast Saccharomyces cerevisiae (12).

ATF4 can form homodimers or heterodimers with other bZIP transcription factors, and elevated ATF4 synthesis directly contributes to increased binding of this transcription activator to the promoters of targeted genes (4, 6, 13, 14). Among these genes is CHOP/GADD153, a bZIP transcriptional regulator that facilitates apoptosis during stress conditions (5, 6, 15, 16). ATF4 and CHOP contribute to the transcriptional expression of GADD34, encoding a targeting subunit for a type 1 Ser/Thr protein phosphatase that dephosphorylates eIF2α (5, 16). Therefore, the ATF4/GADD34 path is important for directing feedback control of the eIF2α kinase pathway, allowing for translation of stress-related mRNAs induced via ATF4. Microarray studies utilizing PEK/PERK−/− and ATF4−/− mouse embryo fibroblast (MEF) cells reported that of the genes requiring eIF2α phosphorylation for their induction in response to ER stress, about half required ATF4 function (7). These results suggest that there may be additional transcription factors that are important for directing the eIF2α kinase pathway and are subject to translational control.

We reasoned that, given the central role for bZIP transcription activators in the eIF2α kinase response, there may be additional bZIP proteins that are subject to translational control in response to eIF2α phosphorylation. One candidate is ATF5, a bZIP transcriptional regulator that is encoded by an mRNA that contains two uORFs with analogous proximity to that described in the ATF4 transcript (13, 17). ATF5 mRNA is expressed in many different tissues, with the highest levels present in liver (17). ATF5 is suggested to play a role in cell survival, and reduced ATF5 function by siRNA approaches was shown to lead to selective destruction of certain cultured cancer cells, such as glioblastomas (18). Because ATF5 is suggested to be highly expressed in neuroprogenitor cells, and is diminished in postmitotic neurons, appropriate expression of ATF5 is proposed to be critical for neural differentiation (18–21). In this study we find that expression of ATF5 protein is induced in response to eIF2α phosphorylation during a range of different stress conditions. Increased ATF5 expression occurs by a translational mechanism involving ribosomal reinitiation and uORFs located in the 5′-leader of the ATF5 mRNA. Superimposed with this translational control, we find that ATF5 mRNA levels are significantly reduced in ATF4−/− MEF cells, suggesting a role for ATF4-directed transcription of ATF5. Together, these results suggest that eIF2α phosphorylation directs ATF5 expression, and this transcription factor is integral to the eIF2α kinase response.

**EXPERIMENTAL PROCEDURES**

**Expression of Recombinant ATF5 and Antibody Production**—Human ATF5 cDNA was inserted between the BamHI and XhoI restriction sites of plasmid pET28, yielding p834 that encodes an N-terminal polyhistidine-tagged version of full-length ATF5 expressed from an inducible T7 promoter. This plasmid was introduced and expressed in Escherichia coli strain BL21(DE3) (F ompT rpsL− containing lysogen DE3), and bacterial cells were grown at 37 °C with shaking in Luria-Bertani medium supplemented with 100 μg/ml ampicillin until an A_{600} of between 0.4 and 0.6. 1 mM isopropyl β-D-thiogalactoside was added to the cultures, and after further incubation at 37 °C for 6 h, cells were collected by centrifugation. The cell pellet was suspended in Buffer A solution containing 20 mM Tris (pH 7.9), 500 mM NaCl, and 10% glycerol containing 10 mM imidazole and lysed using a French press. Proteins in the soluble and insoluble portions of the lysates were separated by SDS-PAGE and visualized by staining with Coomassie R-250. The majority of recombinant ATF5 protein was found to be in the insoluble fraction. The soluble lysate portion was applied to nickel-nitrilotriacetic acid-agarose (Qiagen) equilibrated with Buffer A and incubated at 4 °C. The agarose was washed with Buffer A solution containing 50 mM imidazole, and the ATF5 protein was eluted with buffer A solution containing 200 mM imidazole. The purified recombinant ATF5 protein was M r ~ 35,000 and was specifically recognized by antibody recognizing the polyhistidine tag.

To prepare ATF5-specific antibody, insoluble lysate containing recombinant ATF5 was separated by SDS-PAGE, and the ATF5 protein was sliced from the polyacrylamide gel and injected into rabbits. ATF5 polyclonal antibody was affinity-purified using recombinant ATF5 protein. As described further below in the immunoblot analyses, the ATF5-specific antibody recognized purified recombinant ATF5 protein and ATF5 in mouse and human cell lines that was induced by different stress conditions.

**Cell Culture and Stress Conditions**—MEF cells that were derived from S/S (wild-type eIF2α) and A/A (mutant eIF2α-Ser51A) mice were previously described (22, 23). PEK−/− and GCN2−/− MEF cells, and their wild-type counterparts, were reported previously (24). MEF cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1 mM non-essential amino acids, 100 units/ml penicillin, 10% fetal bovine serum, and 100 μg/ml streptomycin. ER stress was elicited in MEF cells by the addition of either 0.1 μM or 1 μM thapsigargin to the medium, followed by incubation for up to 6 h, as indicated. Alternatively, 20 μM arsenite or 1 μM of the proteasome inhibitor, MG132, was added to the culture medium, and the MEF cells were cultured for up to 6 h, as indicated. To block transcription, 10 μM actinomycin D was added to MEF cells, as indicated. Human HepG2 hepatoma cells were cultured as described (25) and treated with either 20 μM arsenite or 1 μM thapsigargin.

**Preparation of Protein Lysates and Immunoblot Analyses**—MEF and human HepG2 cells cultured in stressed or non-stressed conditions were washed two times with chilled phosphate-buffered solution, and lysed in a solution containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 100 mM NaF, 17.5 mM β-glycerophosphate, 10% glycerol supplemented with protease inhibitors (100 μM of phenylmethylsulfonyl fluoride, 0.15 μM aprotinin, 1 μM leupeptin, and 1 μM of peptatin) and sonication for 30 s. Cell lysates were clarified by centrifugation, and protein content was determined by the Bio-Rad protein quantitation kit for detergent lysis following...
the manufacturer’s instructions. Equal amounts of each protein sample were separated by SDS-PAGE, and proteins were then transferred to nitrocellulose filters. Polypeptide markers of known molecular weights (Bio-Rad) were included to determine the size of proteins identified in the immunoblot analysis. Transferred filters were then incubated in TBS-T solution containing 20 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.2% Tween 20 supplemented with 4% nonfat milk, followed by incubation with TBS-T solution with antibody that specifically recognized the indicated proteins. ATF5 antibody was prepared against recombinant human ATF5 protein, as described above. ATF4 antibody was prepared against an affinity-purified rabbit polyclonal antibody prepared against purified polyhistidine-tagged human ATF4. CHOP (sc-7351) antibody was obtained from Santa Cruz Biotechnology, and β-actin monoclonal antibody (A5441) was purchased from Sigma. Polyclonal antibody that specifically recognized phosphorylated eIF2α at Ser-51 was purchased from BioSource (44–728G). Monoclonal antibodies that recognizes either phosphorylated or nonphosphorylated forms of eIF2α was provided by Dr. Scot Kimball (Pennsylvania State University, College of Medicine, Hershey, PA). Filters were then washed three times in TBS-T, and the protein-antibody complexes were visualized using horseradish peroxidase-labeled secondary antibody and chemiluminescent substrate. Autoradiograms shown in the figures are representative of three independent experiments.

RNA Isolation and Analyses—Northern analyses were carried out as previously described (26). Total cellular RNA was isolated from S/S and A/A MEF cells treated with 1 µM thapsigargin, 20 µM sodium arsenite, or no stress, for the indicated number of hours using the TRIzol reagent (Invitrogen) following the manufacturer’s instructions. 10 µg of total RNA from each sample preparation was separated by electrophoresis using a 1.2% agarose gel and visualized by using ethidium bromide staining and UV light. RNA was transferred onto nylon filters and hybridized to 32P-labeled-DNA probes specific for the indicated genes. Filters were washed using high stringency conditions and visualized by autoradiography. Levels of ATF5-luciferase mRNA expressed in S/S and A/A MEF cells transfected with the ATF5-Luc fusion constructs were treated with 0.1 µM thapsigargin for 6 h, or no stress. A 32P-labeled probe complementary to the luciferase reporter gene was used in a Northern blot analysis to measure ATF5-Luc transcripts.

Plasmid Constructions and Luciferase Assays—PCR was used to generate a HindIII-PagI fragment DNA encoding the full-length ATF5 mRNA leader and ATF5 initiation codon, which was inserted between HindIII and NcoI restriction sites in a derivative of plasmid pG5L. The resulting plasmid contains the 5′-portion of the ATF5 coding sequence fused to the luciferase reporter gene downstream of a minimal TK promoter. The ATG initiation codons in each of the uORFs in the ATF5 mRNA were mutated individually or in combination to AGG using the site-directed mutagenesis kit (Stratagene), following the manufacturer’s instructions. All mutations were sequenced to ensure that there were only the desired changes. Plasmid transfections were performed using the S/S and A/A MEF cells grown to 40% confluency and the FuGENE 6 transfection reagent (Roche Applied Science). Co-transfections were carried out in triplicates using wild type or mutant versions of the ATF5-Luc fusion plasmids and a Renilla luciferase plasmid serving as an internal control (Promega, Madison, WI). 24 h after transfection, MEF cells were treated with 0.1 µM thapsigargin for 6 h, or with no ER stress. Dual luciferase assays were carried out as described by the Promega instruction manual. Values are a measure of a ratio of firefly versus Renilla luciferase units (relative light units) and represent the mean values of three independent transfections. Results are presented as means ± S.E. that were derived from three independent experiments. The Student’s t test was used to determine the statistical significance.

Transcriptional Start Site of ATF5 Transcripts—The cDNAs corresponding to the 5′-ends of the ATF5-Luc transcripts expressed in S/S MEF cells treated with 0.1 µM thapsigargin, or no stress, were amplified using a RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE kit, Ambion) following the manufacturer’s instructions. Alternatively, there was amplification of cDNAs corresponding to the 5′-ends of ATF5 transcripts prepared from human HepG2 hepatoma cells treated with this ER stress condition, or no stress. Briefly, 10 µg of total RNA was treated with calf intestinal phosphatase, resulting in the removal of free 5′-phosphates from RNAs other than mRNAs containing intact 5′-cap structures. The RNA preparations were then treated with tobacco acid pyrophosphatase to remove the cap structure, leaving a 5′-monophosphate that was ligated using T4 RNA ligase to a 45-base RNA adapter oligonucleotide that was supplied in the kit. A random-primed reverse transcription (RT) reaction and nested PCR were then carried out to amplify the 5′-end of endogenous ATF5 transcripts, as well as transfected thymidine kinase-minimal promoter driven ATF5-Luc mRNAs. The primers corresponding to the 5′-RACE adapter sequence were provided by the manufacturer. The sequences of the two nested antisense primers specific to endogenous ATF5 mRNA were the outer primer 5′- TTCCCCATAGTCTACAGGCACTCCC-3′ and inner primer 5′- CATGGGCTGTAGCACAGGTGCT-3′. The outer primer used for amplifying the 5′-ends of ATF5-Luc transcript was 5′-CACCTCTTCCAGGCGATAGAA-3′, which was combined with the same inner primer described earlier. A portion of the amplified DNA products were analyzed by agarose gel electrophoresis, and the DNA was visualized by ethidium bromide staining and UV irradiation. The major DNA band was excised from the gel and sequenced (Fig. 1). The transcriptional start site was determined as the first nucleotide residue that was 3′- to the adapter sequence that was ligated to 5′- of the cDNA.

Polysome Analysis of ATF5 Translational Control—S/S cells were cultured in Dulbecco’s modified Eagle’s medium, as highlighted above, in the presence of 1.0 µM thapsigargin, or to no stress, for 6 h. 10 µg/ml cycloheximide was added to the medium prior to collection and analysis. Cells were washed in cold phosphate-buffered saline solution supplemented with 10 µg/ml cycloheximide, and then lysed with ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 100 mM NaCl, 0.4% Nonidet P-40, and 10 µg/ml cycloheximide. The extracts were passed through a 23-gauge needle for proper lysis of cells, incubated for 10 min on ice, and insoluble material was collected by microcentrifugation at 10,000 rpm for 10 min at
4 °C. The resulting supernatant was then applied onto a 15–45% sucrose gradient containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, and cycloheximide, and centrifuged for 2 h at 40,000 rpm in a Beckman SW-41Ti rotor. Following centrifugation, the gradients were fractionated, and the absorbance of cytosolic RNA at 254 nm was recorded by an in-line UV monitor. Total RNA was isolated from a portion of each fraction using TRIzol reagent as described by the manufacturer’s instructions (Invitrogen). The mRNAs from fractions collected were amplified using RT-PCR kit (Invitrogen) and the following primers: ATF4 forward (5’-TCACGAAATCCAGCAGCAGTGG-3’), ATF4 reverse (5’-CAAGCCATCATCA-TAAGCCG-3’), ATF5 forward (5’-CTAACCCTCCATTCCACTTTCC-3’), ATF5 reverse (5’-TCGTAGACTGCTTCACCTAGGTTG-3’), β-actin forward (5’-TCTTTTGAGCTCCTGTTGCG-3’), and β-actin reverse (5’-TGGAAGCTACGTA-CATGGCTGGG-3’). For RT-PCR analysis of cytosolic mRNA levels, equal volumes were reverse transcribed using oligo(dT), then 50 ng of cDNA from each fraction was amplified with Bullseye R-Taq (MIDSCI). PCR was carried out for 25, 27, 30, 33, and 35 cycles to determine the linear range of amplification. In this study, 25 cycles were used for PCR for ATF4, and 27 cycles for ATF5 and β-actin. Densitometry was performed using the software provided with Quantity One imaging system (Bio-Rad).

RESULTS

Phosphorylation of eIF2α Is Required for Increased ATF5 Protein Levels in Response to Diverse Stress Conditions—The ATF5 mRNA has two uORFs that are conserved among many different vertebrates, including human, mouse, rat, cow, and frogs (Fig. 1). The 5’-proximal uORF1 encodes a polypeptide that is only three amino acid residues in length, Met-Ala-Leu, that is conserved among the different ATF5 orthologs. The downstream uORF2 encodes a polypeptide ranging from 59 residues in length in human and cow, to 53 residues in the frog ATF5 mRNA. In each example, the uORF2 overlaps, out of frame, with the ATF5 coding region (Fig. 1).

Given the importance of uORFs in ATF4 translational control in response to eIF2α phosphorylation, we addressed whether the levels of ATF5 protein were increased in response to environmental stresses by a mechanism requiring eIF2α phosphorylation. Wild-type MEF cells, designated S/S, and a mutant version containing alanine substituted for the serine-51 phosphorylation site in eIF2α, termed A/A, were exposed to three different stress conditions known to activate eIF2α phosphorylation and its downstream target, ATF4. The first stress arrangement involved treatment of these MEF cells with thapsigargin, a well-characterized ER stress agent that specifically activates the eIF2α kinase PEK (6, 10). The second was oxidative stress that was elicited by arsenite exposure. Arsenite appears to activate multiple eIF2α kinases, because deletion of any one eIF2α kinase gene in MEF cells does not block phosphorylation of eIF2α. The third stress involved treatment with MG132, a potent inhibitor of proteasome function that preferentially activates GCN2 phosphorylation of eIF2α in MEF cells (27). Each of these three stress conditions activates the eIF2α kinase pathway in S/S cells, with enhanced eIF2α phosphorylation and increased ATF4 protein levels within 1–3 h of treatment (Fig. 2, A–C). As expected, in the A/A cells there was no measurable phosphorylation of eIF2α and minimal ATF4 protein. Importantly, there were increased ATF5 protein levels in S/S cells in response to each of the stress treatments. Upon thapsigargin exposure, ATF5 expression was induced after 1 h, with high levels of this bZIP transcriptional activator within 3 h of the onset of ER stress (Fig. 2A). Arsenite and MG132 treatments also showed robust increases in ATF5 expression, although high levels of ATF5 protein were detected only after 6 h of the stress treatments (Fig. 2, B and C). No ATF5 protein was detected in the A/A MEF cells, which are devoid of eIF2α phosphorylation, in response to each of the three stress conditions.

These central observations were extended to other MEF cells and to human HepG2 hepatoma cells that have been well studied for regulation of the eIF2α kinase/ATF4 pathway (14, 24, 28). PEK⁻/⁻ MEF cells, and its wild-type counterpart, were exposed to thapsigargin for up to 6 h. ATF5 expression was increased in the PEK⁻/⁻ cells after 3 h of ER stress, coincident with elevated eIF2α phosphorylation and increased levels of ATF4, and its target gene CHOP (Fig. 2D). By contrast in the PEK-deficient cells, there was lowered ATF5 expression, along with reduced eIF2α phosphorylation and downstream targets ATF4 and CHOP. In response to MG132, loss of GCN2 blocked
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**FIGURE 2. Phosphorylation of eIF2α is required for increased levels of ATF5 protein in response to diverse stress conditions.** Wild-type S/S MEF cells, and A/A cells containing an alanine residue substituted for the phosphorylated serine-51 in eIF2α, were treated with 1 μM thapsigargin (A, TG), 20 μM arsenite (B, ARS), or 1 μM MG132 (C, MG) for 1, 3, or 6 h, or to no stress (0 h), as indicated. Alternatively, PKR−/− and GCN2−/− MEF cells, and their wild-type counterparts, were treated with 1 μM thapsigargin (D, TG), or 1 μM MG132 (E, MG) for 3 or 6 h, or to no stress (0 h), as listed. Protein lysates were prepared from the cultured cells, and the levels of ATF5, ATF4, CHOP, phosphorylated eIF2α, total levels of eIF2α, and β-actin were measured by immunoblot analysis using antibody specific to each protein. In F, a similar immunoblot analysis was carried out using lysates prepared from human HepG2 hepatoma cells treated with either arsenite (ARS) or thapsigargin for up to 6 h, or to no stress (0 h). Each panel is representative of three independent experiments.

Both eIF2α phosphorylation and induced ATF5 expression, supporting the essential role of the eIF2α kinase pathway for increased ATF5 levels in response to proteasome inhibition (Fig. 2E). Finally, human HepG2 hepatoma cells have been reported to display a robust eIF2α kinase stress response during different stress conditions (14, 25). We treated the HepG2 cells with thapsigargin or arsenite for up to 6 h and found that ATF5 levels were increased in a time frame similar to that of ATF4 in response to both stress conditions (Fig. 2F). Levels of the ATF4 target, CHOP, were increased later, after about 6 h of each stress. These results indicate that ATF5 expression is induced by a diverse range of environmental stresses by a mechanism requiring eIF2α phosphorylation. Increased ATF5 expression during the different stress arrangements can occur in both humans and mice and in different cell types.

Phosphorylation of eIF2α and ATF4 Are Required for High Levels of ATF5 mRNA—The eIF2α kinase pathway can increase both translational and transcriptional expression in response to cellular stress. To determine whether ATF5 mRNA levels change in response to stress, we carried out Northern analyses using RNA prepared from S/S and A/A MEF cells treated with thapsigargin or arsenite. There were increases in ATF4 mRNA levels in S/S cells treated with thapsigargin or arsenite for up to 6 h (Figs. 3, A and B). No ATF4 mRNA was detected in ATF4−/− MEF cells that were treated with either stress agent, confirming the identity of the transcripts in the Northern analyses. In these studies, ATF4 mRNA levels were reduced, albeit measurable, in the A/A cells treated with thapsigargin or arsenite (Fig. 3, A and B). The lowered levels of ATF4 transcripts in the MEF cells devoid of eIF2α phosphorylation could result from decreased transcription or increased decay of the ATF4 mRNA that was inefficiently translated. These experiments suggest that regulation of ATF4 expression during stress can involve both the well characterized translational control and changes in mRNA levels.

ATF4 binds to the CHOP promoter, increasing CHOP transcription in response to different cellular stresses (14, 29, 30). Consistent with this premise, our Northern analysis showed that CHOP mRNA levels are significantly increased in response to treatment with either thapsigargin or arsenite (Fig. 3). Minimal CHOP expression was detected in the stressed ATF4−/− cells, or the A/A cells, which expressed low levels of ATF4 protein.

The levels of ATF5 mRNA were virtually unchanged in S/S MEF cells in response to thapsigargin treatment. By comparison, there was an increase in the amount of ATF5 mRNA following 3 h of arsenite stress, although ATF5 transcripts were readily measurable in non-stressed condition. Interestingly, there was a significant decrease in the amount of ATF5 mRNA in the ATF4−/− and A/A cells, including lowered transcript
levels in the basal conditions, and no detectable increase upon stress treatment. These results suggest that the eIF2α kinase pathway, specifically the transcriptional activator ATF4, is responsible for elevating ATF5 mRNA levels in both the basal and stressed conditions. As will be highlighted further under “Discussion,” some eIF2α kinase target genes are regulated by both translational and transcriptional control mechanisms. ATF5 is a candidate for such an arrangement given the important role of ATF4 in increased ATF5 mRNA levels, and the fact that ATF5 protein is measurable in the S/S MEF cells only following environmental stress, despite the availability of ATF5 mRNA.

Expression of ATF5 Is Regulated by Post-transcriptional Control Mechanisms—We next wished to address the importance of post-transcriptional regulation in the induction of ATF5 expression in response to stress. Comparison of ATF5 protein levels between ATF4+/+ and ATF4−/− MEF cells subjected to arsenite stress confirmed that induced ATF5 expression required ATF4 (Fig. 4A). Although ATF5 protein was fully increased following 3 h of treatment of arsenite exposure, there was minimal ATF5 protein in the stressed ATF4−/− cells. This requirement for ATF4 was comparable to that found for CHOP, which has been shown to require ATF4 transcriptional activation in response to several different stress conditions, including arsenite treatment (Fig. 3, A and B) (7, 11, 30, 31).

To begin to delineate the role of transcriptional regulation from post-transcriptional modes of control, we pretreated the wild-type MEF cells with actinomycin D, a known inhibitor of transcription, prior to arsenite stress. ATF5 protein levels were increased in response to the combined actinomycin D and arsenite treatment, supporting the idea that post-transcriptional mechanisms significantly contribute to induced ATF5 expression in response to environmental stress (Fig. 4B). It is noted that ATF5 levels were reduced in the combined actinomycin D and arsenite treatment compared with arsenite alone, suggesting that transcriptional mechanisms are a contributor to ATF5 expression. Interestingly, an additional higher molecular weight version of the ATF5 protein was readily detected in the combined treatment preparation, suggesting that ATF5 may be subject to post-transcriptional modification(s), such as protein phosphorylation (Fig. 4B). This higher molecular weight form of ATF5 was also found in cells treated with arsenite alone, although not as prevalent as when cells were first pretreated with actinomycin D. Finally, there was minimal ATF5 protein in the MEF cells exposed to only actinomycin D, indicating that this treatment regimen alone did not lead to enhanced ATF5 expression (Fig. 4B).
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uORF1 and uORF2 Differentially Regulate Translation of ATF5 mRNA—To address the role of translational control in ATF5 expression in response to stress, we constructed a luciferase reporter system that included the ATF5 mRNA leader sequence and initiation codon that were inserted upstream of a luciferase reporter. DNA size markers listed to the left are indicated in base pairs. Bottom panel, the sequence of the S′-leader of the ATF5 mRNA, with boxes indicating the uORF1 and uORF2 sequences upstream of the ATF5-Luc coding region. Note the uORF2 sequence overlaps, out of frame, with the ATF5-Luc reporter sequence. A HindIII restriction site was engineered into the ATF5 DNA. The major transcription start site of the ATF5 gene, as determined by sequencing of S′-leader products, is indicated by an arrow. The initiation codons in uORF1 and uORF2 were substituted to AGG, as indicated below the sequences.

The initiation site in the ATF5-luciferase reporter or endogenous transcript. ATF5-luciferase was measured in the S′-leader of the ATF5-Luc reporter that were treated with 0.1 μM thapsigargin (TG) or no stress. As a control, S′-RACE was also carried out using RNA preparations from HepG2 treated with the ER stress agent, or no stress condition. ATF5 indicates S′-RACE products prepared from the endogenous ATF5 mRNA, and ATF5-Luc indicates products derived from the ATF5-reporter. DNA size markers listed to the left are indicated in base pairs. Bottom panel, the sequence of the S′-leader of the ATF5 mRNA, with boxes indicating the uORF1 and uORF2 sequences upstream of the ATF5-Luc coding region. Note the uORF2 sequence overlaps, out of frame, with the ATF5-Luc reporter sequence. A HindIII restriction site was engineered into the ATF5 DNA. The major transcription start site of the ATF5 gene, as determined by sequencing of S′-leader products, is indicated by an arrow. The initiation codons in uORF1 and uORF2 were substituted to AGG, as indicated below the sequences.

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FIGURE 7. The levels of wild-type and mutant versions of the ATF5-Luc reporter mRNA are similar in the MEF cells. Total RNA was prepared from S/S and A/A MEF cells transfected with the wild-type or mutant versions of the ATF5-luciferase (ATF5-Luc) reporter plasmids, and Northern blots were carried out to measure the mRNA levels for the ATF5-Luc reporter and β-actin. The MEF cells were treated with 0.1 μM thapsigargin (+) or no stress agent (–), as indicated. Wild-type indicates that both uORF1 and uORF2 were present in the S′-leader of the reporter mRNA. ΔuORF1 and ΔuORF2 indicate that the reporter transcript contains a mutation in the initiation codon of the specified uORF, rendering this reading frame nonfunctional for translational control. The combined ΔuORF1 and ΔuORF2 highlights that there were mutations in initiation codons of both reading frames. The top panel illustrates the Northern analysis of RNA prepared from transfected S/S cells. The bottom panel was derived from A/A cells, and for comparison purposes RNA from S/S cells expressing the wild-type reporter from S/S cells was included in this panel. In this lower panel, the A/A and S/S Northern lanes were derived from the same Northern blot experiment.

Luciferase were also seen when the uORF1 and uORF2 mutations were combined into the ATF5-luciferase reporter (Fig. 6). Northern analyses of the different mutant versions of ATF5-luciferase mRNA in the S/S and A/A cells indicated that changes in transcript levels were not a significant contributor to the differences in ATF5-luciferase expression (Fig. 7). These results indicate that uORF2 functions as an inhibitory element in ATF5 translational control. Only after uORF2 is removed is uORF1 dispensable for ATF5 expression (Fig. 6). We conclude that uORF1 and uORF2 have opposing functions in the regulation of ATF5 translation, with uORF1 enabling ribosomes to overcome the inhibitory affects of uORF2.

ATF5 mRNA Is Preferentially Translated in Response to Stress—The ATF5-luciferase reporter assays indicated that ATF5 mRNA is preferentially translated in response to stress. To directly address this idea, we measured the efficiency of mRNA association with translating ribosomes by polysome profile analyses. In this technique, sucrose gradient centrifugation is used to separate free ribosomal subunits and monosomes from polyribosomes (32–34). Transcripts that are efficiently translated are bound to multiple ribosomes, or large polysomes, whereas those mRNAs that are weakly translated localize to monosomes or disomes. In the non-stressed S/S cells, actin mRNA was bound to large polyribosomes (Fig. 8, fractions 12–14). By comparison ATF4 mRNA, which is poorly translated in the absence of stress, was associated with fewer ribosomes (Fig. 8, fractions 7 and 8).

Upon ER stress, total protein synthesis is reduced due to PEB phosphorylation of eIF2α. The resulting lowered eIF2-GTP levels leads to reduced polyribosomes and accumulation of free ribosomal subunits in the polysome profile (Fig. 8, top panels). This stress arrangement led to a shift in the ATF4 mRNA to the larger polysome fractions (Fig. 8, fractions 7–11). By comparison, there was some lowering in the number of ribosomes associated with actin mRNA. Our analysis of ATF5 mRNA in the polysome profiles revealed a pattern that paralleled that described for ATF4. In the non-stressed condition, when protein synthesis is plentiful, the ATF5 mRNA was associated with fewer ribosomes, as compared with the large polysomes associated with actin mRNA. In response to ER stress, ATF5 mRNA was readily detected in the larger polysome fractions, consistent with the idea that ATF5 mRNA was bound to multiple ribosomes and was more efficiently translated. These results further support the idea that translational control is an important underlying reason for increased ATF5 expression during environmental stress.

DISCUSSION

Phosphorylation of eIF2α induced in response to diverse environmental stress conditions elicits translational and transcriptional regulatory mechanisms to direct the expression of genes that alleviate cell damage, or alternatively induce apoptosis. In this report, we showed that eIF2α phosphorylation is required for enhanced expression of the bZIP transcriptional regulator ATF5 in response to each of three different stress conditions: ER stress, arsenite exposure, and proteasome inhibition (Fig. 2). Increased expression of ATF5 in response to stress involves two mechanisms. First, ATF5 is subject to translational control. There was minimal expression of ATF5 protein in non-stressed conditions, despite the availability of ATF5...
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mRNA (Figs. 2 and 3). Only in response to stress conditions, and enhanced eIF2α phosphorylation, was there a significant increase in ATF5 protein levels (Fig. 2). In fact, increased levels of ATF5 during stress occurred even in the presence of actinomycin D, indicating that transcription is not obligatory for the enhanced ATF5 expression during stress (Fig. 4B). ATF5 mRNA was preferentially associated with large polysomes in response to ER stress, directly supporting the idea that there is increased translation of ATF5 mRNA. Assays of an ATF5-luciferase reporter suggested that the 5′-leader of the ATF5 mRNA directs ATF5 translational control. The underlying mechanism involves a positive-acting uORF1 that allows reinitiating ribosomes to bypass an inhibitory uORF2 (Figs. 5 and 6).

The key features of ATF5 translational control share those described for ATF4. The regulatory mechanism begins with translation of uORF1, which allows for retention of ribosomes and reinitiation at a downstream ORF. In non-stressed conditions, eIF2-GTP is readily available, allowing for scanning ribosomes to rapidly reinitiate translation at the next ORF, uORF2. Following translation of the inhibitory uORF2, terminating ribosomes would be positioned downstream of the start codon of the ATF5 coding region, and thus incapable of ATF5 expression. Furthermore, upon translation of uORF2, terminating ribosomes may dissociate from the ATF5 mRNA. Therefore, translation of the inhibitory uORF2 would lead to lowered synthesis of the ATF5 transcription regulator. This central feature of the model, in which uORF2 plays an inhibitory role in ATF5 translational control, is also suggested in a report by Watatani et al. (35) that followed the original submission of this manuscript.

In response to stress, the reduced levels of eIF2-GTP that occur during eIF2α phosphorylation delay reinitiation, allowing for scanning ribosomes to bypass the uORF2 initiation codon. Ribosomes scanning the interval between the initiation codon of uORF2 and the ATF5 coding region would reacquire eIF2-GTP/Met-tRNAiMet and begin translation at the ATF5 coding region. Elevated levels of ATF5 protein would then be available to regulate transcription of targeted genes.

A second mechanism contributing to ATF5 expression involves accumulation of ATF5 mRNA. Although, in wild-type MEF cells, there was minimal increase in ATF5 transcript levels following up to 6 h of ER stress, there was some elevation in ATF5 mRNA levels during arsenite treatment. This increase in mRNA levels during this oxidative stress condition may contribute to the increased synthesis of ATF5 protein. Interestingly, in A/A MEF cells or in ATF4-deficient cells there was a significant lowering in ATF5 mRNA (Fig. 3, A and B). This suggests that ATF4, another target of eIF2α phosphorylation, contributes directly or indirectly to increased ATF5 transcription. Importantly, the requirement of ATF4 for accumulation of ATF5 mRNA is visible even during non-stressed conditions, suggesting that there is a basal level of ATF4 protein that directs elevated ATF5 mRNA levels. ATF4-c−/− cells were reported to be sensitive to oxidative stress, requiring reducing agents in the medium (7). This observation suggests that ATF4 can have critical functions in cells even in the absence of treatment with stress agents. In this case, there may be physiological stresses that trigger modest, transient increases in ATF4 levels. These results suggest that translational control and ATF4 regulation of ATF5 mRNA function together to enhance ATF5 expression in response to different stress conditions.

These key features of combined translational and transcriptional mechanisms have also been described for GCN4 orthologs in fungi, such as Candida albicans, Neurospora crassa, and Aspergillus nidulans (36–39). In each of these cases, increased synthesis of mRNA was suggested to be important for insuring maximal expression of these GCN4-related transcriptional activators. Transcriptional expression of these GCN4 orthologs is suggested to be coupled to translational control mechanisms involving uORFs and translation reinitiation that is delayed by eIF2α phosphorylation. However, it is noteworthy that, although a single “master regulator” directs the eIF2α kinase pathway in these fungi (40), this report shows that mammals have multiple transcriptional regulators subject to translational control in response to eIF2α phosphorylation.

Prior studies on ATF5 have largely focused on ATF5 mRNA expression, the role of ATF5 in neural differentiation, and the potential role of ATF5 in cancer progression (17–21). Our findings that ATF5 expression is induced by stress by mechanisms requiring eIF2α phosphorylation may be integral to each of these research topics. Concerning ATF5 expression, two versions of ATF5 mRNA, derived from alternative splicing, were reported to be expressed in humans and mice (17). The major form studied in this manuscript, referred to as ATF5a mRNA, is expressed during early mouse development and in adult tissues, with highest levels in liver and certain neural tissues (17). Given our findings that ATF5 is subject to translational control, ATF5 transcript levels are not necessarily an accurate measure of ATF5 protein and activity. A second version of ATF5 mRNA, designated ATF5b, is present at much lower levels, and was reported to be restricted to early mouse development (17). Interestingly, ATF5b shares an identical ATF5 coding region with ATF5a, differing only in the 5′-leader region of the mRNA. Three uORFs are present in the ATF5b transcript, each differing from that described for the ATF5a version. Like the ATF5a transcript, the 3′-proximal uORF in ATF5β is longest, encoding a 63-amino acid residue polypeptide that overlaps out-of-frame with the ATF5 coding region. It is inviting to speculate that this alternative 5′-leader configuration in the ATF5β mRNA may change important features of translational control as compared with the ATF5a transcript. For example, the 5′-leader of ATF5β mRNA may alter the efficiency of ribosome scanning and change the timing of translation reinitiation, thus altering the sensitivity of translational control to eIF2α phosphorylation.

We are only beginning to understand the biological functions of ATF5. Recent studies suggest that ATF5 is highly expressed in neural progenitor cells, and lowered ATF5 mRNA expression is a prerequisite for differentiation into neurons and glia (18–21). Illustrating this point, overexpression of ATF5 in rat PC12 cells repressed neurite outgrowth in response to NGF, while expression of a dominant-negative version of ATF5 accelerated neuritogenesis (20). In keeping with the idea that ATF5 represses differentiation of neural progenitor cells and facilitates proliferation, it was observed that ATF5 is expressed in a number of different human glioblastomas (18). In fact, the pres-
ence of ATF5 mRNA has been suggested to be a good prognostic marker in histopathologic examinations (41). The finding that ATF5 expression is integrated into the eIF2α kinase response suggests that this pathway may be a factor in neural differentiation and cancer, and that stress-induced ATF5 may alter these processes.

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