The Eukaryotic Initiation Factor-2 Kinase Pathway Facilitates Differential GADD45a Expression in Response to Environmental Stress*

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Phosphorylation of eukaryotic initiation factor-2 (eIF2) regulates general and gene-specific translation in response to diverse environmental stresses. Central to gene expression induced by eIF2 phosphorylation is the preferential translation of ATF4, a basic zipper transcription activator. Phosphorylation of eIF2 and its attendant induction of ATF4 can lead to different patterns of gene expression depending on the environmental stress. This is of fundamental importance because eIF2 kinases can induce the expression of genes involved in survival as well as in apoptosis. In this report, we explore the molecular basis for why there can be differential expression of GADD45a, a stress-responsive protein that regulates genome stability, apoptosis, and immune responses. We find that whereas ATF4 is required for GADD45a transcription during many different environmental stresses, GADD45a protein accumulates only during a limited number of stress arrangements. The basis for this difference between measurable GADD45a mRNA and protein lies in the observation that GADD45a protein is labile. Those stress agents that enhance ATF4-directed GADD45a transcription and impede the turnover of GADD45a protein by blocking ubiquitin/proteasome-mediated degradation elevate GADD45a protein levels. By comparison, those stress arrangements that trigger ATF4 levels and GADD45a transcription, but do not perturb the proteasome pathway, only elevate GADD45a mRNA levels. This study highlights the molecular mechanisms by which environmental stresses can differentially control central regulatory proteins targeted by the eIF2 kinase pathway.

Environmental stresses elicit programs of gene expression designed to remedy the underlying cellular disturbance or alternatively induce apoptosis. Central to these stress response pathways are regulatory proteins, including protein kinases and phosphatases, transcription factors, and ubiquitination enzymes that function to recognize cellular stresses and coordinate the expression of these target genes. One such target gene, GADD45a, was originally identified by subtractive hybridization in a search for mRNAs induced by UV irradiation (1, 2). GADD45a transcripts were subsequently found to be induced by a broad range of stress arrangements that extend beyond DNA damage. Whereas p53 is required for GADD45a transcription in response to ionizing radiation, both p53-dependent and -independent processes function to trigger GADD45a transcription in response to alkylation and oxidative stresses (2–6). GADD45a is a small acidic protein that can directly interact with two other p53-regulated proteins, p21WAF1/CIP1 and PCNA, linking GADD45a function with DNA repair and regulation of the cell cycle (2, 3, 7). Furthermore, analysis of mice deficient for GADD45a suggests a role for this stress-responsive protein in genome stability, apoptosis, and immune responses (2, 8–10).

We have been studying cellular stress responses that involve phosphorylation of the α-subunit of eukaryotic initiation factor-2 (eIF2). A family of eIF2 kinases have been characterized in mammals that are each activated by different stress arrangements (11, 12). For example, phosphorylation of eIF2α by GCN2 (EIF2AK4) is enhanced by amino acid limitation, UV irradiation, and proteasome inhibition (13–16). Phosphorylation of eIF2α inhibits general protein synthesis by reducing the levels of eIF2-GTP that are required for binding of initiator tRNA to the translation apparatus. Concomitant with lowered protein synthesis, eIF2α phosphorylation leads to preferential translation of ATF4, a basic zipper (bZIP) transcription activator important for directing transcription of stress-related genes (17–20). Reduced protein synthesis conserves energy and provides the cell time for ATF4 and other stress-responsive transcription factors to reconfigure gene expression slanted to alleviate damage elicited by the underlying stress. Other members of the eIF2 kinase family include PEK/Perk (EIF2AK3), whose activity is enhanced by endoplasmic reticulum (ER) stress (21, 22); HRI (EIF2AK1), which is regulated by heme deficiency and oxidative stress (23); and PKR (EIF2AK2), which functions in an antiviral pathway (24).

ATF4 directs expression of additional bZIP transcription factors, ATF3 and CHOP/GADD153, which contribute to transcription of genes important for cellular remediation and apoptosis (17, 25). Interestingly, CHOP/GADD153 was first identified in the differential hybridization study that first described GADD45a (1). Another stress-induced gene that was identified

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2 The abbreviations used are: eIF2, eukaryotic initiation factor-2; EMSA, electrophoretic mobility shift assay; AMF, 7-aminomethylcoumarin; NAC, N-acetylcysteine; ER, endoplasmic reticulum; ChIP, chromatin immunoprecipitation assay; CHX, cycloheximide; MEF, mouse embryore fibroblast; CREB, cAMP-response element-binding protein.
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In this earlier report, GADD34, is also expressed in response to stress via the actions of ATF4, ATF3, and CHOP. GADD34 serves as a targeting subunit for a type 1 Ser/Thr protein phosphatase, which serves to direct feedback control of the eIF2 kinase pathway by enhancing dephosphorylation of eIF2 (26–28). Perturbations of the eIF2 kinase pathway are associated with a number of medical conditions, including anemia, stroke, viral infection, eating disorders, neurological dysfunctions, and diabetes (11, 29–39).

Whereas the connections between individual eIF2 kinases and certain stress arrangements are well established, we are only beginning to understand gene expression directed by eIF2α phosphorylation and its biological significance. A complicating feature is that eIF2α phosphorylation can direct varied patterns of gene expression depending on the specific environmental stress, and these differences can contribute to cell survival or to apoptosis. For example, in response to arsenite or UV irradiation, eIF2α phosphorylation enhances resistance to the cellular insult, whereas during treatment with proteasome inhibitors, phosphorylation of eIF2α elicits a pro-apoptotic regimen (14–16, 40). In this report, we explore the molecular basis for why there can be differential expression of stress-responsive gene arrangements. The basis for this difference between measurable GADD45a mRNA and protein lies in the observation that expressed GADD45a protein is short-lived. Those stress agents that enhance ATF4 directed GADD45a transcription, and impede degradation of GADD45a protein by blocking the ubiquitin/proteasome pathway elevate GADD45a protein levels. By comparison, those stress arrangements that trigger ATF4 levels and GADD45a transcription, but do not perturb the proteasome pathway, only elevate GADD45a mRNA levels.

**Experimental Procedures**

**Cell Culture and Stress Conditions**—ATF4⁺/⁺, ATF3⁻/⁻, and p53⁻/⁻ mouse embryo fibroblast (MEF) cells, and their wild-type counterparts were previously described (25, 41). MEF cells contain a wild-type version of eIF2α and p53, whereas the S/S counterparts were previously described (25, 41). MEF cells express a mutant form of eIF2α and p53 and GADD45α transcription, but do not perturb the proteasome pathway. ATF4 is required for GADD45α transcription in response to many different environmental stresses, GADD45α protein accumulates in response to a limited number of stress arrangements. The basis for this difference between measurable GADD45α mRNA and protein lies in the observation that expressed GADD45α protein is short-lived. Those stress agents that enhance ATF4 directed GADD45α transcription, and impede degradation of GADD45α protein by blocking the ubiquitin/proteasome pathway elevate GADD45α protein levels. By comparison, those stress arrangements that trigger ATF4 levels and GADD45α transcription, but do not perturb the proteasome pathway, only elevate GADD45α mRNA levels.

Equal amounts of each protein sample were separated by electrophoresis using an SDS-polyacrylamide gel. Separated proteins were then transferred to nitrocellulose filters, and molecular weights were measured by using low and high range polypeptide markers (Bio-Rad). Protein-bound filters were incubated in TBS-T solution containing 20 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 pepstatin). Following sonication for 30 s, the cell lysates were clarified by centrifugation. Protein content in the lysate preparations was determined by the Bio-Rad protein quantitation kit for detergent lysis according to the manufacturer’s directions.

Equal amounts of each protein sample were separated by electrophoresis using an SDS-polyacrylamide gel. Separated proteins were then transferred to nitrocellulose filters, and molecular weights were measured by using low and high range polypeptide markers (Bio-Rad). Protein-bound filters were 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. Filters were then incubated in the TBS-T solution with antibody that specifically recognized the indicated proteins. ATF3 (sc-188), ATF4 (sc-200), CHOP (sc-7351), GADD45α (sc-792 and sc-797), and total p53 (sc-6243) antibodies were obtained from Santa Cruz Biotechnology and β-actin monoclonal antibody (A5441) was purchased from Sigma. Monoclonal antibody that specifically recognized phosphorylated eIF2α at Ser-51 was purchased from BioSource (44-728G). Monoclonal antibodies that specifically recognized p21 (OP79) was obtained from Oncogene Research Products, and antibodies that detected p53 phosphorylated at Ser-15 (9284) or ATF2 phosphorylated at Thr-71 (9220) were purchased from Cell Signaling. Monoclonal antibody that recognizes either phosphorylated or nonphosphorylated forms of eIF2α was provided by Dr. Scott Kimball (Pennsylvania State University, College of Medicine, Hershey, PA). After incubating the filters with the antibody preparations, the filters were washed three times in TBS-T, and the protein-antibody complexes were visualized using horseradish peroxidase-labeled secondary antibody and chemiluminescent substrate. Quantitation of visualized bands was carried out by densitometry. Autoradiograms shown in the figures are representative of three independent experiments.

**RNA Isolation and Northern Analyses**—Northern analyses were carried out as previously described (43). Total cellular RNA was isolated from MEF cells treated with the indicated stress condition using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions. 20 μg of total RNA from each sample preparation were separated by electrophoresis using a 1.4% agarose, 6% formaldehyde gel and visualized using ethidium bromide staining and ultraviolet light. RNA was transferred onto GeneScreen Plus filters (PerkinElmer Life Sciences), hybridized to 32P-labeled DNA probes specific for the indicated mRNAs, and filters were washed under high stringency conditions and visualized by autoradiography.
Electrophoretic Mobility Shift Assays (EMSA) and ChIP Assays—Nuclear extracts were prepared from ATF4\(^{-/-}\), ATF3\(^{-/-}\), and CHOP\(^{-/-}\) MEF cells and their wild-type counterparts, which were treated with 20 \(\mu\)M arsenite for up to 6 h as previously described (43). Cells were then resuspended in 1 ml of cold hypotonic RSB solution (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 3 mM MgCl\(_2\)) supplemented with 0.5% Nonidet P-40 and protease inhibitors. Cell lysis was carried out using a Dounce homogenizer, and following centrifugation, the nuclei were resuspended in two packed nuclear volumes of a Dounce homogenizer, and following centrifugation, the nuclei and protease inhibitors. Assays were prepared, and GADD45a was immunoprecipitated, and the samples were analyzed by PCR to establish the linearity of the assay. PCR was carried out using 1:100 diluted input DNA and 5 \(\mu\)l of the 100-\(\mu\)l DNA preparation using two DNA oligonucleotide primers that spanned the ATF/CREB-related element in the GADD45a promoter region. The 5'-primer was 5'-CAGTGTACTAATGTTAGGGTTGATA-3' and the 3'-primer was 5'-ACAATACAGTAACTGGCAGCT-3', resulting in a 245-bp PCR DNA product. PCR products were separated by electrophoresis using a 2% agarose gel that contained ethidium bromide to visualize the DNA. Electronic images of the PCR DNA were illustrated as an inverted image.

Ubiquitin/Proteasome Assays—Wild-type S/S MEF cells were exposed to 20 \(\mu\)M arsenite, 1 \(\mu\)M MG132, 1 \(\mu\)M thapsigargin, or leucine starvation for up to 6 h. Proteasome activity in the cell lysates was measured using the proteasome activity assay kit (Chemicon International Inc) following the manufacturer's instructions. This assay involves detection of the fluorophore 7-amino-4-methylcoumarin (AMC) following cleavage from a peptide substrate LLVY-AMC. Free AMC fluorescence was measured using a 380/460 nm filter set in a fluorometer in concert with an AMC fluorogenic standard curve. Relative fluorescence units (RFUs) were normalized for that determined in cells devoid of stress. Results are presented as means \(\pm\) S.E. derived from three independent experiments, and the Student's \(t\) test was used to determine the statistical significance.

GADD45a Half-life Measurements—Wild-type S/S MEF cells were seeded at 2 \(\times\) 10\(^5\) cells per 60-mm dish and grown to 50% confluency in Dulbecco's modified Eagle's medium as described above. Cells were then exposed to 20 \(\mu\)M arsenite or 1 \(\mu\)M thapsigargin for 5 h, or to no-stress treatment. Prior to harvesting, cells were incubated in 4 ml of trans-labeling medium without methionine or cysteine supplemented with 10% dialyzed fetal bovine serum. The MEF cells were then labeled with 500 \(\mu\)Ci of [\(^{35}\)S]Met/Cys express labeling mix (ICN Biomedicals, Irvine, CA) for 30 min. Cells were washed twice with ice-cold phosphate-buffered solution containing non-radiolabeled methionine and cysteine. Cells were collected, lysed, and clarified, and GADD45a was immunoprecipitated using GADD45a-specific antibody. The immune complex was separated by SDS-PAGE and visualized by autoradiography.

Pulse-chase experiments were carried out by radiolabeling cells 30 min as described above, followed by washing with non-radiolabeled medium, and incubation in medium containing excess non-radiolabeled methionine and cysteine for 10 and 80 min. GADD45a was immunoprecipitated, and the levels of radiolabeled GADD45a were measured by SDS-PAGE and autoradiography. Immunoblot analyses measuring total levels of actin were carried out in parallel to be certain that similar levels of total protein were analyzed in each lysate preparation. Uptake of [\(^{35}\)S]Met/Cys was similar between the different cultured cell preparations.

Another measure of GADD45a turnover involved exposure of wild-type MEF cells to 20 \(\mu\)M arsenite for 5 h, or no-stress. Stressed cells were then treated with 10 \(\mu\)g/ml cycloheximide and cultured between 10 and 80 min, as indicated. Cell lysates were prepared, and GADD45a immunoblot analysis was carried out as described above.

RESULTS

Arsenite Exposure Induces eIF2\(\alpha\) Kinase Stress Response—Arsenite exposure has been reported to be a potent inducer of eIF2\(\alpha\) phosphorylation (17, 40, 44, 45). Exposure of wild-type MEF cells to as little as 5 \(\mu\)M sodium arsenite induced eIF2\(\alpha\)
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phosphorylation and its attendant ATF4 translation (Fig. 1A). Increased eIF2α phosphorylation and ATF4 expression occurred in the MEF cells following 1 h of exposure to 20 μM sodium arsenite. We confirmed a prior report that oxidative stress is an important underlying reason for induced eIF2α phosphorylation and ATF4 translation in response to arsenite exposure, as prior treatment with the antioxidant N-acetylcysteine (NAC) significantly lowered the levels of ATF4 and eIF2α phosphorylation (Fig. 1B) (45). As expected in A/A cells, containing eIF2α with Ala substituted for Ser-51, there was no phosphorylation of eIF2α or ATF4 expression during arsenite treatment (Fig. 1C). These results are consistent with earlier studies showing that ATF4 translation is dependent on eIF2α phosphorylation in response to diverse stress conditions, including arsenite exposure (17, 18, 25, 45).

ATF4 directs the expression of additional transcriptional regulators, such as CHOP and ATF3, as well as increase the levels of GADD34, which is important for feedback control of the eIF2 kinase pathway (26). This is illustrated in wild-type MEF cells treated with the ER stress agent thapsigargin, which leads to elevated expression of each of these target genes following 3 h of this stress condition (Fig. 1, C and D). By comparison, there was minimal expression of CHOP, ATF3, and GADD34 in the thapsigargin-treated A/A cells, which are devoid of translational control. CHOP, ATF3, and GADD34 levels were also enhanced in response to arsenite treatment of wild-type S/S MEF cells (Fig. 1C). Interestingly, whereas there was no CHOP expression in the A/A cells subjected to arsenite, ATF3 levels were similarly elevated between A/A and wild-type cells, and GADD34 levels were partially induced in the absence of eIF2α phosphorylation (Fig. 1C). There was also a partial induction of ATF3 and GADD34 in ATF4−/− MEF cells subjected to arsenite, supporting the idea that other transcriptional regulators can functionally replace ATF4 in response to certain stress conditions (Fig. 1D). Interestingly, CHOP expression was only partially diminished in the ATF4−/− cells, suggesting that eIF2α phosphorylation may directly expression of an additional transcription factor that enhances CHOP expression during arsenite exposure, but not during the ER stress condition. Furthermore, phosphorylated ATF2 levels were partially delayed in response to A/A cells treated with arsenite, suggesting some modest links between eIF2 and MAP kinase pathways (Fig. 1C). These patterns of gene expression support the idea that there can be variations in the requirement for eIF2α phosphorylation during different cellular stresses. Under some stress conditions, such as ER stress, eIF2α phosphorylation is largely obligate for enhanced gene expression, whereas under other stress conditions, such as arsenite exposure, eIF2α kinase function appears to be at least partially dispensable. In the latter example, other stress-induced transcription factors may functionally replace ATF4 in the process of gene expression.

**ATF4**

**ATF4 Kinase Pathway Directs GADD45α Expression during Arsenite Stress—GADD45α is induced in response to a range of stress conditions, and we wished to address whether this key stress-regulatory protein requires eIF2α phosphorylation for its expression during arsenite stress. There was enhanced expression of GADD45α in wild-type MEF cells exposed to 10 μM sodium arsenite, with full expression following exposure to 20 μM sodium arsenite (Fig. 1A). These concentrations were higher than that required for eIF2α phosphorylation and ATF4 expression, which were elicited at concentrations as low as 5 μM arsenite. Furthermore, full GADD45α expression occurred following 6 h of exposure to 20 μM arsenite. Phosphorylation of eIF2α facilitated expression of GADD45α, as there was a significant reduction in the levels of this key regulatory protein in A/A cells treated with arsenite (Fig. 1C). There was minimal detectable GADD45α in ATF4−/− MEF cells subjected to arsenite stress, suggesting a role for this transcription factor in its induced expression (Fig. 1D). Furthermore, pretreatment with NAC blocked GADD45α expression in response to arsenite stress (Fig. 1E). Interestingly, GADD45α was not expressed in wild-type MEF cells in response to ER stress, suggesting that eIF2α phosphorylation alone is not sufficient for its protein expression. Expression of GADD45α in response to diverse stress conditions will be addressed further below.

DNA-damaging conditions have been reported to result in the nuclear localization of GADD45α, where it can participate in the regulation of DNA repair and the cell cycle (46, 47). We prepared nuclear and cytoplasmic fractions from ATF4+/+ and ATF4−/− MEF cells subjected to arsenite and found that indeed GADD45α is predominantly localized to the nucleus (Fig. 1E). Minimal GADD45α was detected in either fraction derived from ATF4−/− cells. For controls we showed that eIF2 kinase-directed transcription factors, CHOP and ATF4, were also located in the nucleus. Furthermore, phosphorylated ATF2, which is induced by the MAP kinase pathways, is present in the nucleus of arsenite-stress MEF cells independent of ATF4 function. Tubulin was present only in the cytoplasmic fraction as expected.

We transiently expressed an ATF4 cDNA into the ATF4−/− MEF cells to determine if expression of the transcription activator could restore GADD45α expression in response to arsenite exposure. In parallel, we also transiently expressed ATF3 into the ATF4−/− MEF cells to discern the role of this down-

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**FIGURE 1. Arsenite induces eIF2α phosphorylation and ATF4 expression.** A, wild-type S/S MEF cells were treated with 20 μM arsenite for up to 6 h or to no cellular stress (0 h), as indicated. Alternatively, wild-type S/S MEF cells were treated with between 0.01 and 40 μM arsenite for 5 h, as indicated. Cellular lysates were prepared, and the levels of phosphorylated and total eIF2α, ATF4, ATF3, CHOP, GADD45α, and actin were measured by immunoblot analysis. Equal amounts of the protein preparations were analyzed in each lane. The dashed lines were included between the time and dosage lanes to assist in aligning the immunoblot panels. B, wild-type S/S MEF cells were treated with 20 μM arsenite (Ars) for up to 6 h, as indicated. Alternatively the S/S cells were pretreated with N-acetylcysteine prior to the arsenite stress (Ars + NAC). Immunoblot analyses were carried out to measure phosphorylated eIF2α, ATF4, CHOP, GADD45α, and actin. C, wild-type S/S MEF cells and A/A cells, which contain a mutant version of eIF2α with an Ala substituted for the phosphorylated residue Ser-51, were treated with 1 μM thapsigargin (Tg) or 20 μM arsenite (Ars) for up to 6 h. Immunoblot analyses were carried out to measure phosphorylated eIF2α and ATF2, and the levels of the indicated proteins. The arrow indicates the GADD45α protein, which was distinct from an unrelated, larger molecular weight, protein that was recognized by this antibody preparation. D, ATF4+/+ and ATF4−/− MEF cells were treated with thapsigargin (Tg) or arsenite (Ars) for up to 6 h, and immunoblot analyses were carried out as described in A and B. E, ATF4+/+ and ATF4−/− MEF cells were treated with arsenite for up to 6 h and nuclear (Nuc) and cytoplasmic (Cyt) portions of cellular lysates were prepared and analyzed by immunoblot for the indicated proteins.
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![Diagram showing expression levels of ATF4, ATF3, GADD45a, and Actin in MEF cells treated with arsenite stress.](image)

**FIGURE 2.** ATF4 is required for increased GADD45a mRNA in response to arsenite stress. A, lanes 1–3, ATF4−/− MEF cells were treated with 20 μM arsenite for 3 or 6 h, or to no cellular stress (0 h). Lysates were prepared from the cultured cells, and the levels of ATF4, ATF3, GADD45a, and Actin were measured by immunoblot analysis. Alternatively, ATF4+/+ MEF cells were transiently transfected with expression vector (None, lanes 4–6), or plasmids encoding ATF3 (lanes 7–9), ATF4 (lanes 10–12), or both ATF3 and ATF4 (lanes 13–15). Lysates were prepared, and the levels of the indicated proteins were measured by immunoblot analyses. B, wild-type MEF cells were treated with 20 μM arsenite (Ars) for up to 6 h, and cellular lysates were analyzed by immunoblot analysis to measure the levels of ATF4, ATF3, CHOP, GADD45a, and Actin. Alternatively, arsenite was combined with cycloheximide (CHX) or actinomycin D (AD), and immunoblot analyses were carried out to measure the levels of the indicated proteins. C, ATF4+/+ and ATF4−/− MEF cells were treated with arsenite for up to 6 h, and the levels of the indicated mRNAs were measured by Northern analyses. D, wild-type MEF cells were treated with arsenite (Ars) or leucine starvation (-Leu) for up to 6 h, as indicated. The levels of ATF4, ATF3, GADD45a, and actin transcripts were measured by Northern analyses.

Expression of ATF4 and ATF3 was carried out by using the constitutive CMV promoter, and the ATF4 cDNA was devoid of 5′-regulatory sequences important for translational control, which should enhance expression independent of stress conditions. Whereas ATF3 was successfully expressed in these MEF cells, it was difficult to transiently express high levels of ATF4 (Fig. 2A). However, if ATF3 was co-expressed with ATF4, ATF4 expression was rescued in the ATF4−/− cells. We are uncertain why only co-expression was successful for elevated ATF4 expression. ATF4 can heterodimerize with other bZIP proteins, and ATF3 may directly or indirectly contribute to ATF4 synthesis and/or stabilization (48–51). With the co-expression strategy for rescuing ATF4 expression in the ATF4−/− MEF cells, there was increased GADD45a levels in response to arsenite stress.

Phosphorylation of eIF2α can trigger translational and transcriptional expression of stress-related genes. To delineate between these two induction processes, we added either actinomycin D, a potent transcriptional inhibitor, or cycloheximide, which blocks translation elongation, to the arsenite stressed cells (Fig. 2B). Consistent with the idea that translational control is central to ATF4 expression in response to eIF2α phosphorylation, we found that addition of cycloheximide blocked ATF4 expression during arsenite exposure. Actinomycin D had only a partial effect, supporting the idea that there is also a modest induction of ATF4 mRNA levels during arsenite stress. By contrast, expression of ATF3, CHOP, and GADD45a were each significantly diminished by actinomycin D, as well as by cycloheximide. These results suggest that transcriptional expression is important for GADD45a during arsenite stress.

To address whether ATF4 is required for enhanced GADD45a mRNA levels during arsenite stress, we carried out a Northern blot analysis using RNA prepared from ATF4+/+ and ATF4−/− MEF cells treated with 20 μM sodium arsenite for up to 6 h. Whereas CHOP and GADD45a transcript levels were markedly increased in the ATF4+/+ MEF cells, there was minimal expression in the ATF4−/− cells (Fig. 2C). By contrast HSP70 transcripts were enhanced in response to arsenite stress independent of ATF4 function. Furthermore, we carried out an analogous Northern blot analysis using ATF3−/− MEF cells and found only a modest reduction in GADD45a mRNA levels compared with wild-type cells (Fig. 2D). In addition to arsenite stress, GADD45a mRNA was increased in response to amino acid starvation, a condition that also enhances eIF2α phosphorylation. The role of eIF2α kinases in GADD45a expression in response to a broader spectrum of stress conditions will be further discussed below. Together, these results suggest that ATF4 can direct the transcription of GADD45a.

**Interactions between the eIF2 Kinase Pathway and p53—**GADD45α transcription has been reported to have both p53-dependent and -independent components (2–6). We measured the levels of p53 and its phosphorylation status at Ser-15 in ATF4+/+ and ATF4−/− MEF cells in response to arsenite exposure (Fig. 3A). Loss of ATF4 did not lower the amounts of p53, but did delay its phosphorylation, with phosphorylated p53 detected following 1 h of arsenite stress in wild-type MEF cells, compared with 3 h in the ATF4−/− cells. We also analyzed p53+/+ and p53−/− MEF cells during arsenite stress (Fig. 3B).
Consistent with the important role for p53 for transcriptional expression of Cdk inhibitory protein p21, we found no induction of p21 levels in p53−/− cells following arsenite exposure. By comparison, GADD45α expression was not significantly changed with the loss of p53 transcription factor. ATF4 expression showed a reduction and delay in the p53−/− MEF cells, further supporting the idea that there is some cross regulation between portions of the eIF2 kinase pathway and p53. There were no differences in the induced levels of ATF3 between p53+/+ and p53−/− cells. Together, these results suggest that altered p53 function is not an underlying contributor to the induction of GADD45α expression by the eIF2 kinase pathway.

**ATF4 Is Required for GADD45α Transcription in Response to Diverse Stress Conditions**—ATF4 is required for elevated GADD45α mRNA levels in response in MEF cells exposed to arsenite. An ATF/CREB-related binding element, TGAAGTCA, is located in the GADD45α promoter region, and a radiolabeled DNA fragment containing this element showed enhanced binding in the EMSA using nuclear lysate prepared from wild-type MEF cells subjected to arsenite (Fig. 4A). EMSA using lysates prepared from ATF4−/− cells treated with arsenite for 6 h showed a significant reduction in binding to this GADD45α promoter element, further supporting a role for this transcription factor in the regulation of GADD45α expression. No changes were detected in the control Oct-1 binding between these nuclear lysate preparations (data not shown). Arsenite-induced binding to the GADD45α promoter element was reduced when a related ATF/CREB DNA element was added to the binding mixture. By comparison, there was no competition for binding to the GADD45α promoter element when a DNA

**FIGURE 3.** ATF4 induces GADD45α expression independent of p53. ATF4+/+ and ATF4−/− (A) or p53+/+ and p53−/− (B) MEF cells were treated with arsenite for 3 or 6 h, or to no cellular stress (0 h). Immunoblot analyses were carried out to measure the levels of phosphorylated p53 or the indicated protein levels. Equal amounts of the protein preparations were analyzed in each lane. The arrow indicates the GADD45α protein, which was delineated from an larger, unrelated, protein that bound to this antibody preparation.

**FIGURE 4.** ATF4 binds to a GADD45α promoter element in response to arsenite stress. A, nuclear lysates were prepared from ATF4+/+ and ATF4−/− MEF cells exposed to 20 μM arsenite for 3 or 6 h, or to no cellular stress (0 h). Equal amounts of nuclear lysate were used in each EMSA mixture containing radiolabeled DNA with an ATF/CREB-related binding element derived from the GADD45α promoter. Binding mixtures were analyzed by gel electrophoresis and imaged by autoradiography. Bound DNA is indicated by the arrow, and radiolabeled DNA at the bottom of panels is unbound probe. B, to determine the specificity for the ATF-related binding site, non-radiolabeled DNA containing the ATF/CREB DNA binding site, or the NF-κB site URE binding site, were added to EMSA binding mixtures containing nuclear lysates prepared from ATF4+/− MEF cells treated with arsenite for 6 h. Competition indicates that non-radiolabeled competitor DNA was added at a 1×, 10×, or 100× molar excess. Free probe (F) indicates only radiolabeled NF-κB DNA fragments without nuclear lysate. C, ATF4+/+ and ATF4−/− MEF cells were treated with 20 μM arsenite or 1 μM MG132 for 6 h, or no-stress (0). Chromatin prepared from the MEF cells was subjected to immunoprecipitation using antibody specific to ATF4 as described under “Experimental Procedures.” PCR products derived from the GADD45α promoter region were separated by agarose gel electrophoresis. The panel is an inverted image of the stained gel and is representative of two independent experiments. As an input control, PCR was carried out using chromatin preparations from ATF4+/+ MEF cells, and input controls prepared from ATF4−/− MEF cells were similar to wild-type cells (data not shown). The ratio of immunoprecipitated DNA to input (×107) in the ATF4+/− MEF cells was as follows: arsenite stress, 1.9; MG132 stress, 2.5; and no-stress, 0.7. Minimal PCR products were detected in the immunoprecipitations derived from the ATF4−/− cell preparations.

**FIGURE 3.** ATF4 induces GADD45α expression independent of p53. ATF4+/+ and ATF4−/− (A) or p53+/+ and p53−/− (B) MEF cells were treated with arsenite for 3 or 6 h, or to no cellular stress (0 h). Immunoblot analyses were carried out to measure the levels of phosphorylated p53 or the indicated protein levels. Equal amounts of the protein preparations were analyzed in each lane. The arrow indicates the GADD45α protein, which was delineated from an larger, unrelated, protein that bound to this antibody preparation.

**FIGURE 4.** ATF4 binds to a GADD45α promoter element in response to arsenite stress. A, nuclear lysates were prepared from ATF4+/+ and ATF4−/− MEF cells exposed to 20 μM arsenite for 3 or 6 h, or to no cellular stress (0 h). Equal amounts of nuclear lysate were used in each EMSA mixture containing radiolabeled DNA with an ATF/CREB-related binding element derived from the GADD45α promoter. Binding mixtures were analyzed by gel electrophoresis and imaged by autoradiography. Bound DNA is indicated by the arrow, and radiolabeled DNA at the bottom of panels is unbound probe. B, to determine the specificity for the ATF-related binding site, non-radiolabeled DNA containing the ATF/CREB DNA binding site, or the NF-κB site URE binding site, were added to EMSA binding mixtures containing nuclear lysates prepared from ATF4+/− MEF cells treated with arsenite for 6 h. Competition indicates that non-radiolabeled competitor DNA was added at a 1×, 10×, or 100× molar excess. Free probe (F) indicates only radiolabeled NF-κB DNA fragments without nuclear lysate. C, ATF4+/+ and ATF4−/− MEF cells were treated with 20 μM arsenite or 1 μM MG132 for 6 h, or no-stress (0). Chromatin prepared from the MEF cells was subjected to immunoprecipitation using antibody specific to ATF4 as described under “Experimental Procedures.” PCR products derived from the GADD45α promoter region were separated by agarose gel electrophoresis. The panel is an inverted image of the stained gel and is representative of two independent experiments. As an input control, PCR was carried out using chromatin preparations from ATF4+/+ MEF cells, and input controls prepared from ATF4−/− MEF cells were similar to wild-type cells (data not shown). The ratio of immunoprecipitated DNA to input (×107) in the ATF4+/− MEF cells was as follows: arsenite stress, 1.9; MG132 stress, 2.5; and no-stress, 0.7. Minimal PCR products were detected in the immunoprecipitations derived from the ATF4−/− cell preparations. DNA size markers (M) are listed in base pairs.
fragment containing an unrelated NF-κB binding site was added to the EMSA (Fig. 4B). Nuclear lysates prepared from ATF3 \(^{-/-}\) or CHOP \(^{-/-}\) MEF cells similarly treated with arsenite did not show reduced binding to the GADD45a promoter element in the EMSA (data not shown). Together, these results suggest that ATF4 binds to the GADD45a promoter, inducing its transcription in response to arsenite stress.

To address whether ATF4 directly binds to the GADD45a promoter region in vivo, we carried out a ChIP analysis using ATF4-specific antibody. Cross-linked chromatin was isolated from ATF4 \(^{+/+}\) and ATF4 \(^{-/-}\) MEF cells exposed to arsenite or to no-stress, and the immunoprecipitated DNA was analyzed by PCR. There was an increase in the amount of ATF4 binding to the promoter region in response to arsenite treatment (Fig. 4C). No PCR product was visualized using chromatin preparations from the ATF4 \(^{-/-}\) MEF cells, demonstrating the specificity of the immunoprecipitations. These results support the idea that ATF4 directly binds to the GADD45a promoter region, facilitating increased GADD45a transcription in response to arsenite stress.

We next addressed whether GADD45a mRNA levels were elevated in response to a spectrum of stress conditions previously shown to enhance eIF2α phosphorylation. In addition to arsenite treatment, the ATF4 \(^{+/+}\) and ATF4 \(^{-/-}\) MEF cells were exposed to leucine starvation, the proteasome inhibitor MG132, or thapsigargin (Fig. 5, A and B). GADD45a mRNA levels were significantly increased in response to each of these four stress conditions, and its induction was dependent on ATF4. For experimental controls, we also measured ATF4 and its target genes and HSP70 mRNA levels (17, 18, 25, 45). Consistent with our prior report, ATF3 mRNA levels were significantly diminished in ATF4 \(^{-/-}\) MEF treated with nutrition or ER stress (Fig. 5B) (25). By comparison, ATF4 was largely dispensable for ATF3 transcription during arsenite or MG132 stress, emphasizing the idea that ATF4 is required for ATF3 expression only during a portion of the stress conditions inducing eIF2α phosphorylation (16). The levels of HSP70 mRNA were enhanced by only arsenite and MG132 treatments and, as expected, this induction was independent of ATF4. Finally, as described above for arsenite stress, there was increased ATF4 binding to the GADD45a promoter region in response to MG132 treatment as measured by the ChIP analysis (Fig. 4C). These results indicate that ATF4 is obligate for GADD45a transcription in response to a range of different environmental stresses.

Arsenite Stress Blocks the Ubiquitin/Proteasome Pathway and Stabilizes GADD45a—Earlier we observed no increase in GADD45a protein levels in response to ER stress, and there was also no elevation in GADD45a protein levels in MEF cells subjected to leucine starvation (Figs. 1, B and C and 6A). Interestingly, in response to proteasome inhibition induced by exposure of the MEF cells to MG132, GADD45a protein levels were significantly enhanced (Fig. 6B). As noted earlier for arsenite stress, expression of GADD45a was largely dependent on eIF2α phosphorylation, as there were appreciable protein levels in the A/A MEF cells only following 6 h of MG132 treatment. Loss of ATF4 blocked expression of GADD45a. These results suggest that there is an increase in GADD45a transcription during ER stress and nutrient deprivation, but GADD45a translation is impaired or the expressed proteins are unstable.

The fact that MG132 treatment led to accumulation of both GADD45a mRNA and proteins, while ER and nutritional stresses did not, suggests that GADD45a protein may be labile. Given the linkage between GADD45a accumulation during arsenite and proteasome inhibition, we wished to determine whether arsenite also blocked ubiquitin-mediated degradation of proteins. We carried out this analysis using two approaches.
eIF2 Kinase Pathway Induces GADD45a

First, we measured protein ubiquitination by immunoblot analysis using antibody specific to ubiquitin. There was a significant elevation in ubiquitinated proteins following 6 h of treatment of MEF cells with MG132 or arsenite, whereas the levels of ubiquitinated protein in cells subjected to ER or nutritional stress were similar to the untreated preparations (Fig. 6A). Furthermore, in vitro measurements of proteasomal activity using a fluorescently labeled substrate and lysates prepared from cells treated with one of each of the stress conditions indicated that arsenite is a potent inhibitor of ubiquitin-mediated degradation (Fig. 7). There was a 20% reduction in cells treated with 20 μM arsenite for 3 h, and 53% lowered proteasome activity following 6 h of this stress condition. As expected treatment of these MEF cells with MG132 led to less than 5% activity within 3 h. By contrast, leucine starvation or ER stress did not significantly change proteasome activity. These results suggest that a central reason for accumulation of GADD45α during arsenite stress or proteasome inhibition is stabilization of this key regulatory protein combined with its ATF4-directed transcription. In response to those stresses that do not thwart ubiquitin-mediated degradation, our results suggest that ATF4 directs GADD45α transcription, but GADD45α protein is labile and is not appreciably measured.

To measure the half-life of GADD45α protein, we treated wild-type MEF cells with arsenite for 5 h, followed by the addition of cycloheximide to block further protein synthesis. Cells were then cultured between 10 and 80 min, and then GADD45α, and other eIF2 kinase-targeted gene products, including ATF4, CHOP, and ATF3, were measured by immunoblot analysis (Fig. 7). The half-life of GADD45α induced during arsenite stress was ~20 min. By comparison, ATF3 was stably expressed with minimal reductions in protein levels during the 80-min time course. Interestingly, ATF4 and CHOP were also short-lived, with half-lives of ~25 min and ~30 min, respectively. We next compared the synthesis and turnover of GADD45α protein in MEF cells treated with arsenite or ER stress. Following the stress condition, cells were incubated with [35S]Met/Cys, and GADD45α was specifically immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography. Synthesis of
GADD45a protein in response to arsenite stress was modestly higher than that measured during thapsigargin treatment, similar to that measured for GADD45a mRNA (Figs. 6D, lanes 2 versus 8, and 5A). Additionally, we carried out a pulse-chase analysis to measure the turnover of GADD45a protein. The half-life of GADD45a in cells treated with arsenite was ~25 min, modestly higher than that measured via the cycloheximide-based method. GADD45a protein was more labile during ER stress, with a half-life of less than 10 min in the thapsigargin-treated cells (Fig. 6D). These results suggest that GADD45a is preferentially stabilized during arsenite stress, as compared with ER stress, and that this stabilization is a primary reason for accumulation of GADD45a protein during stress conditions that reduce ubiquitin/proteasome-mediated degradation.

**DISCUSSION**

Phosphorylation of eIF2α elicited during different environmental stresses can induce distinct patterns of protein expression. This report focused on GADD45a, a small protein implicated in the regulation of the cell cycle, DNA repair and genome stability, innate immunity, and apoptosis (2, 8–10). Transcription of GADD45a is induced in response to different genotoxic and non-genotoxic stress conditions. Whereas p53 is required for the enhanced expression of GADD45a following ionizing radiation, induction of GADD45a levels is regulated by both p53-dependent and -independent mechanisms in response to other conditions linked with DNA damage (1–3, 52). We determined that ATF4 and the eIF2 kinase pathway facilitate the p53-independent GADD45a transcription. Deletion of ATF4 in MEF cells blocked GADD45a transcription in response to treatment with arsenite, MG132, thapsigargin, or leucine deprivation (Figs. 2C and 5). Our studies are consistent with the idea that ATF4 directly binds to the GADD45a promoter, contributing to enhanced transcription.

Despite induced GADD45a mRNA levels in response to each of the four stress conditions surveyed, only two stress arrangements, exposure to arsenite or MG132, led to measurable amounts of GADD45a protein, as judged by immunoblot analysis. We reasoned that inhibition of the ubiquitin/proteasome pathway may result in stabilization of GADD45a protein. MG132 is a potent inhibitor of proteasome function, and earlier reports suggested that arsenite can impede the ubiquitin-dependent proteolytic pathway (53, 54). We found that there was a 2-fold reduction in proteasome activity in MEF cells treated with 20 μM arsenite for 6 h (Fig. 7). By comparison during ER stress; a condition that did not diminish proteasome activity, there was minimal induction of GADD45a protein that was only detectable by [35S]Met/Cys labeling. Direct measurements of GADD45a half-life support the idea that GADD45a has reduced turnover in response to arsenite exposure (~25 min) compared with ER stress (<10 min) (Fig. 6D). Therefore, the combined action of ATF4-directed GADD45a transcription and stabilization of short-lived GADD45a can lead to elevated GADD45a protein levels during arsenite stress.

*The eIF2 Kinase Pathway Induces Differential Gene Expression*—Phosphorylation of eIF2α leads to the preferential translation of ATF4, and increased transcription of its target genes, CHOP and ATF3. These transcription factors can combine together, or dimerize with additional bZIP proteins, to direct stress-responsive programs of gene expression. Another contributor to the regulation of the levels of the bZIP transcription factors is their individual rates of protein turnover. In response to arsenite stress, we found that the half-lives of ATF4 and CHOP are short, ~25 and ~30 min, respectively (Fig. 6C). Therefore, ATF4 and CHOP transcriptional functions are tightly linked to their synthesis during the cellular stress response. In the case of ATF4, proteasome-mediated degradation, which is facilitated by the SCF^{HIF1α} ubiquitin ligase, is important for modulation of its activity (55, 56). By comparison, ATF3 does not appear to be subject to such rapid turnover and therefore is available for a longer duration for regulation of transcription (Fig. 6C).

The eIF2 Kinase Pathway Induces Both Pro-survival and Pro-apoptotic Pathways—ATF4 is important for cellular survival in response to oxidative stress (17). In fact, addition of reducing agents to the culture medium has been reported to enhance the survival of ATF4-deficient cells. Oxidative stress appears to be an important underlying reason for the induction of eIF2α phosphorylation by arsenite, as pretreatment with NAC diminished induced translation expression of ATF4 and its target genes GADD45a and CHOP. It is curious that despite these pro-survival properties, ATF4 can also direct the transcription of pro-apoptotic CHOP and ATF3. Deletion of CHOP and ATF3 each contribute to reduced programmed cell death in response to arsenite stress as judged by annexin V detection. Whereas exposure to 20 μM arsenite for 16 h leads to 48% ± 5 apoptosis in wild-type MEF cells, deletion of ATF3 or CHOP leads to a significant reduction in apoptosis (ATF3^{−/−} 22% ± 4 apoptotic cells; and CHOP^{−/−} 36% ± 3 apoptotic cells). By comparison, loss of ATF4 in MEF cells similarly treated with arsenite enhanced apoptosis (75% ± 5 apoptotic cells) compared with wild-type. ATF4 induces GADD45a transcription, with significantly elevated GADD45a mRNA and protein between 3 and 6 h of arsenite exposure. This induction of GADD45a expression may be an important contributor to the pro-survival function of ATF4. Using GADD45a-deficient hematopoietic cells, it was reported that the GADD45a function increases cell survival during genotoxic stress, including UV irradiation and certain anticancer drugs (9).

Arsenic, an Environmental Contaminant and Anticancer Therapy—Exposure to arsenic is associated with a spectrum of diseases, including cardiovascular disease, developmental abnormalities, neurological disorders, fibrosis of the liver and lung, hematological disorders, and cancers (58, 59). Arsenic can also function as an anticancer agent in the treatment of acute promyelocytic leukemia and is being studied for its therapeutic potential in other cancers, including colonic, breast, and pancreatic (60–62). Much effort has gone into understanding the stress response pathways that recognize arsenic stress and their biological consequences. This study supports the idea that eIF2α phosphorylation and ATF4 are major players in the cellular response to arsenite stress, and that arsenite can impede proteasome-mediated protein degradation, which are central to the regulation of stress response pathways.
