Incorporation of an Internal Ribosome Entry Site–Dependent Mechanism in Arsenic-Induced GADD45α Expression

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Abstract

We have previously shown that trivalent arsenic (arsenite, As^{3+}) is able to induce GADD45α expression in human bronchial epithelial cells through activation of c-Jun NH_2-terminal kinase and nucleolin-dependent mRNA stabilization. In the present report, we show that As^{3+} is capable of inducing translation of the GADD45α protein through a cap-independent, or rather, an internal ribosome entry site (IRES)–dependent mechanism. In growth-arrested cells, As^{3+} elevated the GADD45α protein level in a dose- and time-dependent manner which did not correlate with the GADD45α mRNA expression. Pretreatment of the cells with rapamycin, an inhibitor for the cap-dependent translation machinery through the suppression of mTOR and p70S6 kinase, failed to affect the induction of the GADD45α protein induced by As^{3+}. Sequence analysis revealed a potential IRES element in the 5′-untranslated region of the GADD45α mRNA. This IRES element in the 5′-untranslated region of the GADD45α mRNA is functional in mediating As^{3+}-induced translation of the GADD45α protein in a dicistronic reporter gene activity assay. Immunoprecipitation and proteomic studies suggest that As^{3+} impairs the assembly of the cap-dependent initiating complex for general protein translation but increases the association of human elongation factor 2 and human heterogeneous nuclear ribonucleoprotin with this complex. Thus, these results suggest that in growth-arrested cells, As^{3+} is still capable of inducing GADD45α expression through an IRES-dependent translational regulation. [Cancer Res 2007;67(13):6146–54]

Introduction

Growth arrest– and DNA damage–induced gene 45α (GADD45α) encodes a small acidic protein that is capable of interacting with a number of important intracellular signaling molecules involved in cell cycle regulation, apoptosis, and immune responses (1). A wide variety of stress inducers, including DNA-damaging reagents, UV radiation, oxidative or osmotic stress, and nutrient deprivation stimulate the expression of GADD45α mRNA and protein in a manner which may be either p53-dependent or p53-independent (2). The fact that GADD45α is preferentially localized in the nuclei suggests that the key function of this protein might be the regulation of DNA replication, DNA repair, and cell division. Indeed, the majority of the known GADD45α-interacting molecules are nuclear functional proteins, such as proliferating cell nuclear antigen (3), cyclin B/CDC2 complex (4), $p21^{cip1}$ (5), histone (6), aurora-A kinase (7), xeroderma pigmentosum complementation group G (8), and TAFII70 (9). Interaction of GADD45α with these proteins causes cell cycle arrest, repression of DNA replication or cell apoptosis, thereby providing the cells with time to repair the damaged DNA and prevent the segregation of damaged chromosomes.

Extensive efforts have been made to understand how the transcription of GADD45α is regulated in cellular responses to extracellular signals. The transcriptional induction of GADD45α by DNA damage is thought to be dependent on p53 or FoxO3a (10), which antagonizes transcriptional repressors of the GADD45α gene, such as c-myc (11) and ZBRK (12). Activation of c-Jun NH_2-terminal kinase (JNK) may play a key role in mediating p53-independent transcription of GADD45α in response to other stress signals (13). The influence of posttranscriptional events, mainly the modulation of GADD45α mRNA stability, has been recognized in recent years. In humans, the bronchial epithelial cell line, BEAS-2B, stimulation of the cells with trivalent arsenic (arsenite, As^{3+}), a general stress inducer, stabilizes GADD45α mRNA through the inducible association of nucleolin and less potently, HuR, with the GADD45α mRNA (14). Removal of the RNA destabilization protein AUF1 from the 3-untranslated region (3′-UTR) of the GADD45α mRNA was considered to be an important mechanism for the enhanced stability of the GADD45α mRNA in cells treated with genotoxin methyl methanesulfonate (15). Intriguingly, neither nucleolin nor HuR were associated with GADD45α mRNA in UV- or methyl methanesulfonate–treated HeLa cells (16). It is unknown whether the expression of GADD45α is subjected to protein translation regulation under certain circumstances.

Environmental or occupational exposure to As^{3+} is associated with a number of human diseases including nerve degeneration, skin hyperkeratinization, birth defects, hepatic cirrhosis, cardiovascular disorders, diabetes, and cancer (17). By binding to sulfhydryl groups, As^{3+} impairs the function of many proteins, especially glutathione/thioredoxin reductases and protein tyrosine phosphatases which possess vicinal thiols (18). The cumulative effects of this impairment are oxidative stress and kinase activation, including receptor type and non–receptor type tyrosine kinases, JNK, p38, p70S6 kinase (p70S6K), checkpoint kinases, and Akt. Both sustained oxidative stress and aberrant kinase activation have been linked to cell growth and malignant transformation. As^{3+} is also able to induce ubiquitination or sumoylation and proteasomal degradation of some regulatory proteins, such as CDC25C (19), AML1/MDS1/EVI1 (20), and PML (21). This property

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of As^{3+}, paradoxically, makes it an effective therapeutic agent for some types of cancers, mainly acute promyelocytic leukemia and lymphomatic malignancy (21). We previously showed that As^{3+} is a potent inducer for the expression of GADD45α through activation of JNK (13). We have also provided evidence indicating that As^{3+} could stabilize GADD45α mRNA by inducing the association of nucleolin and HuR, two mRNA stabilizing proteins, with GADD45α mRNA (14). In the present report, we investigated the regulatory role of As^{3+} on GADD45α protein translation in growth-arrested cells and showed that As^{3+} enhanced GADD45α translation in a cap-independent but internal ribosome entry site (IRES)–dependent manner.

Materials and Methods

Cell culture and Western blotting. The human bronchial epithelial cell line, BEAS-2B, was purchased from American Tissue Culture Collection. Arsenic(III) chloride (As^{3+}) and other chemicals were purchased from Sigma-Aldrich. Antibodies used in Western blotting were purchased from Santa Cruz Biotechnologies. The cell culture, treatment, and Western blotting were done as previously described (14). In some experiments, the cell lysates from the untreated or As^{3+}-treated cells were made using a TRIZOL reagent (Invitrogen) for sequential extraction of total RNA and protein according to the manufacturer's instructions.

Reverse transcription-PCR. The expression levels of GADD45α and GAPDH mRNAs in the cells were monitored by RT-PCR (reverse transcription-PCR) using the AccessQuick RT-PCR system (Promega) with a temperature scale of 45°C for 50 min for reverse transcription, and 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. The RT-PCR was carried out using the primers: GADD45α (5′-GGAGAGCAGAAGACCGAAA-3′, 5′-TCACTGGAACCCATTGATC-3′); GAPDH (5′-CTGAACGGGAAGCTCACTGGCATGGCCTTC-3′, 5′-CATGAGGTCCACCACCCTGTTGCTGTAGCC-3′).

Dicistronic reporter gene activity assay. The dicistronic reporter construct pRL-HCV-FL was a kind gift from Dr. Richard E. Lloyd at Baylor College of Medicine. This construct contains a *Renilla* luciferase reporter gene at the upstream 5′-UTR of the hepatitis C virus (*HCV*) gene, and a *Firefly* luciferase reporter gene (FL) downstream (22). The 5′-UTR of the HCV in the vector was replaced with the fragments derived from the 5′-UTR of the GADD45α mRNA. These 5′-UTR fragments were amplified by RT-PCR with the following primers: UTR1-328 (5′-TGATGCGGCCGCTCCAGTGGCTGGTAGGCAGT-3′, 5′-TCGATCCGCGGCTGCTCTCCAGCCGAGAAT-3′); UTR2-223 (5′-TGATGCGGCCGCGTGGCTGGTAGGCAGTGG-3′, 5′-TCGATCCGCGGGCCTGCTTTCTGCACTCACT-3′); UTR200-318 (5′-TGATGCGGCCGCTGTGAGTGAGTGCAGAAAGCA-3′, 5′-TCGATCCGCGGGCCGAGAATTCCTCCAAAGT-3′). The *Not*I and *Sac*II sites that were artificially added to the primers for cloning purposes were underlined and double-underlined, respectively. The promoterless dicistronic reporter vector, pRL-FL(-P), and the GADD45α promoter and intron 3 reporter vectors were gifts from Dr. Jian-Ting Zhang at the Indiana University Cancer Center and Dr. Daniel Haber at Massachusetts General Hospital Cancer Center, respectively. Each plasmid DNA was transfected into the cells with LipofectAMINE 2000 (Invitrogen) in the serum-free medium for 24 h. The transfection efficiency for each plasmid was determined by cotransfection of the pcDNA-GFP followed by flow cytometry analysis that showed a similar transfection rate of these vectors. The cells were then incubated with the regular cell culture medium containing 5% fetal bovine serum for an additional 24 h followed by dual-luciferase reporter gene activity assay.

Protein peptide analysis. Proteins recovered from immunoprecipitation using anti-eIF4G antibody were separated by SDS-PAGE. The protein bands were visualized by silver staining. The excised protein gel bands were subjected to in-gel digestion by adding 50 μL of 50 mmol/L ammonium bicarbonate containing 10 ng/μL of modified trypsin, and then incubated at 37°C overnight. Tryptic peptides were extracted with 100 μL of extraction buffer (0.1% trifluoroacetic acid and 50% acetonitrile; pH 2.5), desalted, and analyzed by online reversed phase nano-LC-MS/MS on a Finnigan LTQ mass spectrometer using a 1-h gradient data-dependent MS/MS collection.

Results

As^{3+} induces GADD45α translation in arrested cells. We have previously reported that exposure of the BEAS-2B cells to As^{3+} resulted in a dose- and time-dependent increase in both GADD45α mRNA and/or protein when the cells were cultured in a logarithmically growing phase (13, 14, 23, 24). We also frequently observed a variable degree of the GADD45α mRNA and protein induction by As^{3+} among the cells under different growing conditions. To determine the source of such variations, we deliberately cultured the cells at two well-defined conditions: logarithmically growing condition and the arrested condition in which the cells reached confluence with a high density (Fig. 1*A*). To minimize variations in sampling of total RNA and protein from the cells with or without As^{3+} treatment, we used the same cells to isolate total RNA and protein sequentially by TRIZOL reagent. The levels of GADD45α mRNA and protein were determined by RT-PCR and Western blotting, respectively. As previously reported, exposure of the BEAS-2B cells in a growing condition to 20 μmol/L of As^{3+} resulted in a time-dependent increase in GADD45α mRNA and protein (Fig. 1*B, lanes 1–6*). Both mRNA and protein of the GADD45α were undetectable in the growing cells without As^{3+} treatment (Fig. 1, *lane 1*). The peak induction of GADD45α protein lagged behind the induction of GADD45α mRNA by As^{3+} in these cells (compare RT-PCR results with the Western blots in Fig. 1*B*). To our surprise, a considerable level of GADD45α mRNA was present in the arrested cells without As^{3+} treatment (Fig. 1*B, lane 7*). Despite the expression of the GADD45α mRNA in these arrested cells without As^{3+} treatment, the GADD45α protein was undetectable. Treatment of these arrested cells with As^{3+} did not increase but rather decreased GADD45α mRNA expression. In contrast, a time-dependent induction of GADD45α protein was observed in these cells (Fig. 1*B, lanes 9–12*) similar to that of growing cells (Fig. 1*B, lanes 3–6*). Therefore, these data suggest that expression of the GADD45α mRNA itself is not sufficient for the expression of the GADD45α protein, and As^{3+} up-regulates protein translation, but not mRNA transcription of the GADD45α in arrested cells. It has been generally viewed that the activity of the classic protein translational machinery, cap-dependent translation, is repressed in the cells under growth arrest or stress conditions (25). The longer the exposure of the cells to As^{3+}, the more pronounced is the cellular stress induced. Thus, the time-dependent decrease of GADD45α mRNA and the increase of GADD45α protein in the arrested cells in response to As^{3+} is possibly a result of cellular switch from cap-dependent to IRES-dependent protein translation (discussed later).

Induction of GADD45α protein by As^{3+} is through a cap-independent mechanism. More than 90% of the cellular proteins are translated in eukaryotes in a manner that depends on the m^7GpppN 5′-cap structure of mRNA, which recruits cap-binding protein eIF4E along with the ribosomal preinitiation complex (26). A successful assembly of the translational initiation complex requires the activation of mTOR kinase, which phosphorylates 4E-BP and p70S6 kinase (p70S6K). Thus, functional interruption of mTOR will inhibit cap-dependent protein translation. To determine whether As^{3+} affects the cap-dependent translational machinery for the expression of GADD45α protein in arrested cells, we pretreated the cells with 60 or 300 nmol/L of rapamycin (a specific inhibitor of mTOR kinase) overnight, followed by exposure of the cells to 20 μmol/L of As^{3+} for an additional 6 h.

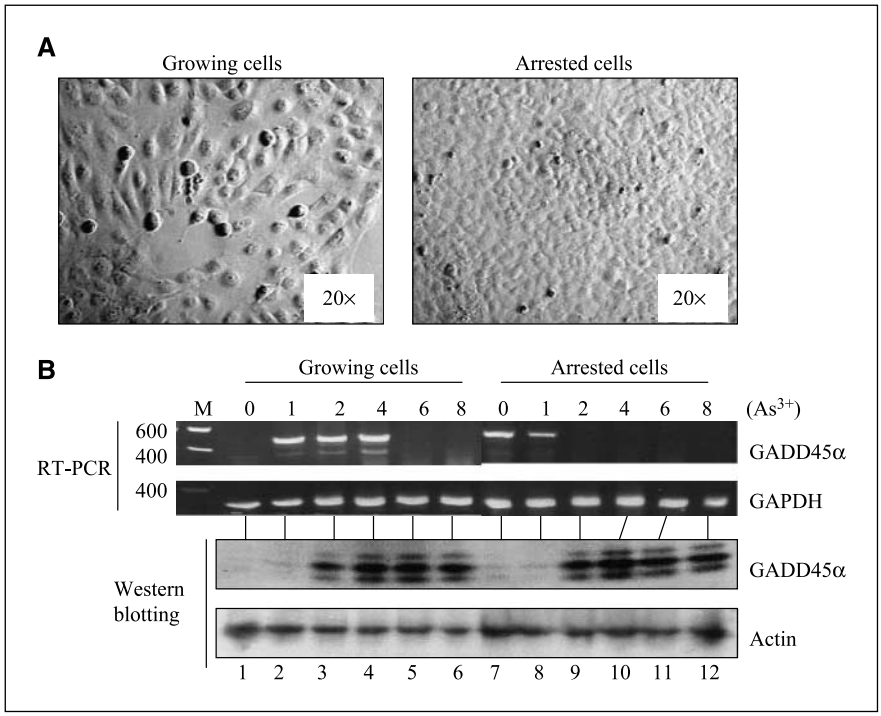

Figure 1. As^{3+} induces GADD45α mRNA and protein to different degrees in growing cells and arrested cells. *A,* cell morphology of the BEAS-2B cells in a logarithmic growth phase (*left*) and in arrested phase (*right*). *B,* GADD45α mRNA and protein in the growing cells and arrested cells treated with 20 μmol/L of As^{3+} for the indicated times were determined by RT-PCR (*top*) and Western blotting (*bottom*), respectively. *Left,* sizes of DNA markers (*M*).

Both concentrations of rapamycin exhibited potent inhibition of the activation of p70S6K as evidenced by the disappearance of the phosphorylated form of the p70S6K in a Western blotting experiment using an antibody specific for the phosphorylated p70S6K on serine389 (Fig. 2*A, lanes 7–10, middle*). However, rapamycin showed no inhibition on the expression of the GADD45α protein induced by As^{3+} (Fig. 2*A, lanes 9* and *10, top*). To determine the effect of the newly synthesized mRNA on As^{3+}-induced GADD45α protein expression, we also preincubated the cells with 4 or 8 μmol/L of actinomycin D overnight to diminish mRNA synthesis. As expected, both concentrations of actinomycin D showed no effect on the activation of p70S6K, but abolished induction of the GADD45α protein by As^{3+}, indicating that the newly synthesized GADD45α mRNA is required for protein synthesis (Fig. 2*A, lanes 5* and *6*). Based on these results, we concluded that As^{3+} induces GADD45α protein translation through a cap-independent mechanism requiring GADD45α mRNA synthesis.

To investigate whether this cap-independent mechanism is only specific for cellular response to As^{3+} or is a general response for other forms of stress signal–induced GADD45α expression, we next stimulated the cells with taxol or UV radiation. The induction of GADD45α by taxol, a microtubule-stabilizing drug, and UV had been previously shown to be dependent on p53- or JNK-mediated transcription of the GADD45α gene in some cancer cell lines (27–29). Both taxol (2 or 10 μmol/L) and UV (2 or 4 kJ/m^2) induced an appreciable elevation of the GADD45α protein in a roughly dose-dependent manner (Fig. 2*B*). The dosages of taxol and UV were predetermined based on the minimal cytotoxic response of the tested cells. Pretreatment of the cells with 100 nmol/L of rapamycin showed no remarkable effect on taxol- or UV-induced GADD45α protein, despite the fact that this concentration of rapamycin abrogated p70S6K activation completely (Fig. 2*B*). Thus, these data suggest that a cap-independent translational mechanism of the GADD45α protein is involved not only in cellular response to As^{3+} but to other stress signals from microtubule interference (taxol) and DNA damage (UV) as well. Blockage of the cap-dependent translational machinery by rapamycin has no effect on the induction of the GADD45α protein using these inducers.

GADD45α mRNA contains an IRES. Less than 10% of cellular proteins were translated in a cap-independent but IRES-dependent mechanism (30). Because rapamycin was unable to inhibit As^{3+}- as well as UV- or taxol-induced GADD45α protein translation in the arrested cells, we believe that an IRES-dependent mechanism must be involved in this case. The IRES-dependent pathway translates proteins when the cells are in growth-arrested, apoptotic, mitotic, or extreme stress conditions in which the cap-dependent translational machinery is stalled. In apoptotic and mitotic cells, this translational mechanism is considered to be important for the expression of some functional proteins for the completion of apoptotic and mitotic processes, despite a substantial reduction in the rate of general protein synthesis (31). The mRNAs subjected to cap-independent but IRES-dependent translation usually contain IRES element(s) in the 5′-UTR. To determine whether the human GADD45α mRNA contains a potential IRES element responsible for the As^{3+}-induced GADD45α translation, we first analyzed the 5′-UTR region of the GADD45α mRNA using an online version of UTRScan program.[4] After inputting the 295nt 5′-UTR along with an additional 25nt of the open reading frame, a potential IRES element from 209 to 300 nt was identified (Fig. 3*A*). The sequence was derived from Genbank NM_001924.

To verify the IRES activity of the 5′-UTR from the GADD45α mRNA, we next inserted the 5′-UTR and its derivatives into the pRL-FL dicistronic vector that contains the *Renilla* luciferase (RL) reporter gene and the *Firefly* luciferase reporter gene (Fig. 3*B*). The expression of the RL is cap-dependent, whereas the expression of the FL is dependent on the presence of the

[4] http://www.ba.itb.cnr.it/BIG/UTRScan/

IRES element between these two reporter genes in this dicistronic vector (22). The IRES activity was determined by the ratio of FL versus RL. After transient transfection of the BEAS-2B cells with these vectors for 48 h, the cells were treated with 20 μmol/L of As^{3+} for an additional 12 h. Figure 3*C* showed relative basal (control) and As^{3+}-induced IRES activities in the cells transfected with the indicated dicistronic vectors. The pRF vector that did not contain any IRES elements was used as a control vector in this experiment. As depicted in this figure, the cells transfected with vectors containing either HCV IRES or GADD45α IRES showed a notable increase in As^{3+}-induced IRES activity relative to the cells transfected with the pRF vector. A marginal increase of the IRES-like activity was observed in the cells transfected with the pRF vector and treated with As^{3+}, which was very likely a result of ribosomal read-through due to shortening of the space between the two cistrons by removal of the IRES element (data not shown). As^{3+} is able to increase the IRES activity of the whole 5′-UTR (UTR1-328) and the UTR200-318 of the GADD45α mRNA in which an IRES element was identified by the UTRScan program. Interestingly, the UTR2-223 region also exhibited As^{3+}-induced IRES activity, indicating the possible presence of a cryptic IRES element in this region, or that the region from 200 to 223 is the key element for the IRES activity in the GADD45α mRNA. The induction of IRES activity in the 5′-UTR region from GADD45α mRNA using As^{3+} is also dose-dependent. An induction plateau occurred at the As^{3+} dose range from 5 to 20 μmol/L (Fig. 3*D*).

The ability of As^{3+} to stimulate IRES activity was further confirmed in the cells transfected with pRL-HCV-FL dicistronic vector, which contains a well-documented HCV IRES element. In a single dose test, As^{3+} induced a 2- to 3-fold increase of the HCV IRES activity (Fig. 3*C*). A dose-dependent increase in the HCV IRES activity was observed when the cells were treated with As^{3+} at a concentration ranging from 0 to 30 μmol/L (Fig. 3*D*). The elevated FL/RL ratio obtained in the presence of As^{3+} was not a result of reduced RL translation because As^{3+} did not inhibit the RL activity (Fig. 3*E*). Taken together, all of these data clearly suggest that As^{3+} is capable of inducing IRES-dependent protein translation.

The IRES activity of the GADD45α 5′-UTR is not a result of cryptic promoter activity. It has been reported that some so-called IRES activities were actually the result of a cryptic promoter element in the 5′-UTR of the given mRNAs (32). To differentiate between the IRES activity and the cryptic promoter activity of the 5′-UTR derived from the GADD45α mRNA, we first constructed monocistronic vectors by inserting the 5′-UTR of ?show [nonbspace]>the GADD45α mRNA to the promoterless pGL3 basic vector (Fig. 4*A*). We also used the pGL3-based GADD45α promoter reporter vector as a positive control. As previously reported (14), As^{3+} induced GADD45α promoter activity marginally (Fig. 4*B*). UTR2-223 showed a notable increase in the luciferase activity

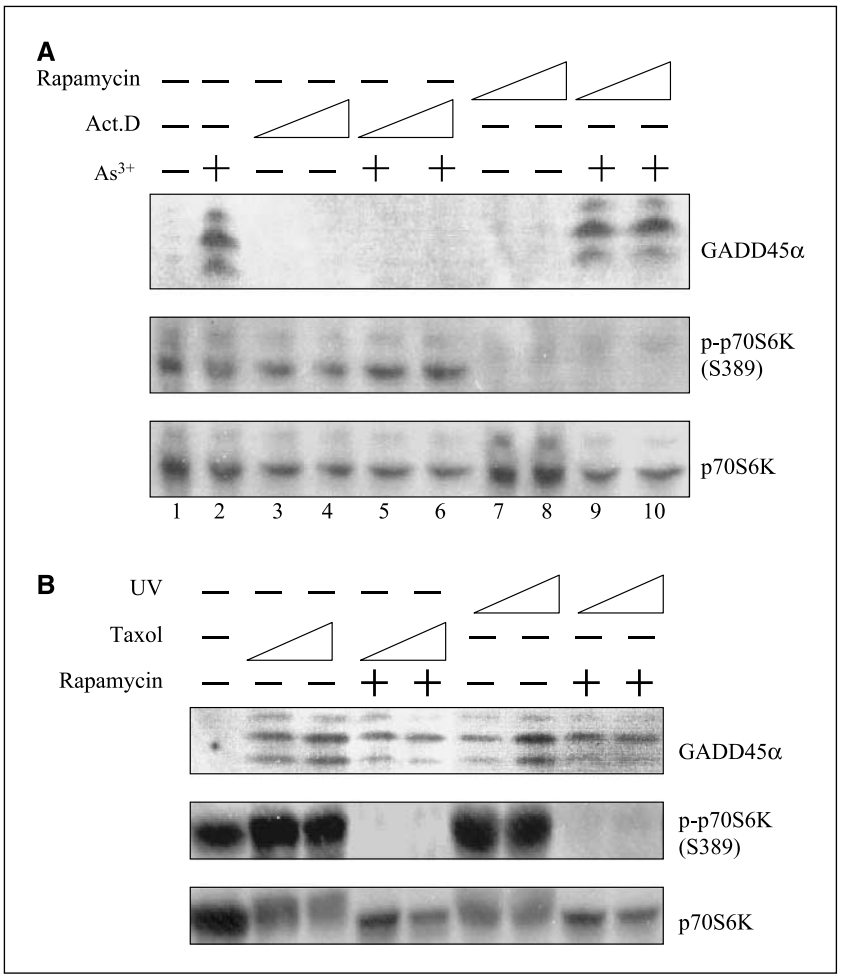

Figure 2. As^{3+} induced translation of the GADD45α protein is cap-independent. *A,* BEAS-2B cells were pretreated overnight with actinomycin D (*Act. D,* 4 or 8 μmol/L), or rapamycin (60 or 300 nmol/L) and then treated with 20 μmol/L of As^{3+} for an additional 6 h. The expression of the GADD45α protein, phosphor-p70S6K and total p70S6K were determined on the same membrane by sequential Western blotting using the indicated antibodies. *B,* the BEAS-2B cells were pretreated overnight with 100 nmol/L of rapamycin and then treated with taxol (2 or 10 μmol/L) for 6 h, or UV radiation (2 or 4 kJ/m^2). The levels of GADD45α, phosphor-p70S6K, and total p70S6K were determined as in (*A*).

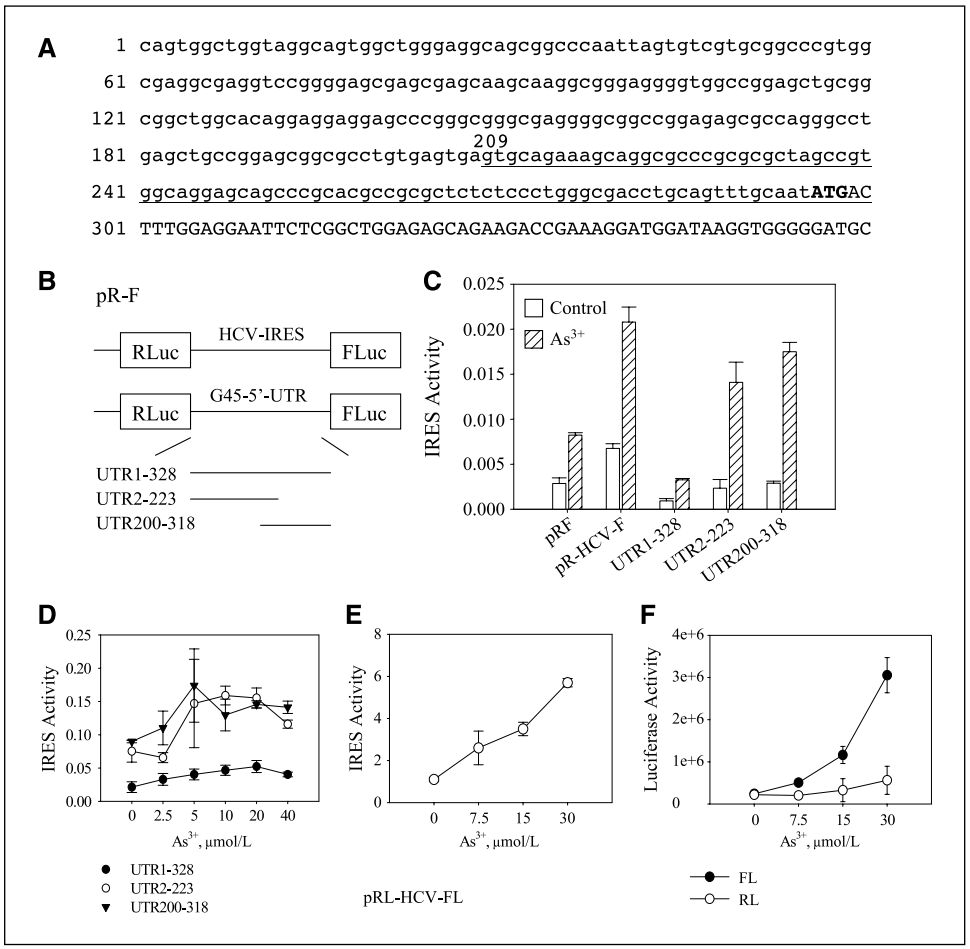

Figure 3. The 5′-UTR of the GADD45α mRNA contains an IRES element. *A,* the 5′-UTR of the GADD45a mRNA was analyzed by the UTRScan program. http://www.ba.itb.cnr.it/BIG/UTRScan/ *Underlined sequence,* the computer-identified IRES element. The ATG translation start code was highlighted. *B,* schematic illustration of the dicistronic reporter vectors used in IRES activity assay. *C,* IRES activity was determined in the BEAS-2B cells transfected with the indicated dicistronic vectors for 48 h and then cultured for an additional 12 h in the absence or presence of 20 μmol/L of As^{3+}. *D,* dose-dependent induction of the GADD45α IRES activity by As^{3+} in the cells transfected with the indicated dicistronic vectors harboring the indicated 5′-UTR region from the GADD45α mRNA. *E,* dose-dependent induction of the HCV IRES activity by As^{3+} in the cells transfected with the dicistronic vector containing an established IRES element from HCV promoter. *F,* the increased ratio of FL versus RL is not due to inhibition of the *Renilla* luciferase translation by As^{3+}. *Points,* means of four repeats; *bars,* SD.

relative to other 5′-UTR constructs in the cells without any treatment, indicative of the cryptic promoter activity in this region. However, this cryptic promoter activity was not induced, but was instead inhibited by As^{3+}. There was no detectable cryptic promoter activity in UTR200-318, which showed the highest IRES activity induced by As^{3+} as indicated in Fig. 3*C*, in either the control cells or in the As^{3+}-treated cells (Fig. 4*B*). We also tested the potential cryptic promoter activity of the 5′-UTR derived from GADD45α mRNA in a promoterless pRL-FL dicistronic vector. Again, although UTR1-328 and UTR2-223 showed cryptic promoter activity in the control cells, this activity was not induced, but rather, was inhibited by As^{3+} (data not shown). Similar to the monocistronic reporter vector, the UTR200-318 showed no basal or inducible cryptic promoter activity in this promoterless dicistronic reporter system (data not shown).

As^{3+} impairs the assembly of the cap-dependent preinitiation complex. Accumulating evidence suggests that the rate of protein synthesis is reduced in cells under growth arrest or stress conditions (33). To obtain further insight into the translational regulation of As^{3+} on protein synthesis, coimmunoprecipitation was then done using antibodies against eIF4G, a general component of the cap-binding preinitiation complex for protein translation. Although the As^{3+}-treated cells showed a similar level of total eIF4E to the control cells (Fig. 5*A, input*), an appreciable decrease in the interaction between eIF4G and eIF4E was noted in the cells treated overnight with 20 μmol/L of As^{3+} (Fig. 5*A, top*). It is well-known that the association of eIF4E with eIF4G is essential for the further assembly of the translational initiation complex (26). Perturbation in the interaction between eIF4G and eIF4E by As^{3+}, therefore, must influence the general translation of the cellular proteins. Indeed, analysis of the total protein synthesis by running the protein samples in SDS-PAGE followed by Coomassie blue staining showed a reduction of protein synthesis in As^{3+}-treated cells (Fig. 5*B, filled bars, right*). As^{3+} induced a mild increase of the proteins with a molecular weight >200 kDa and <30 kDa (Fig. 5*B, open bars, right*), which possibly resulted from cap-independent, but rather, IRES-dependent translation.

To determine whether As^{3+} affects the association of other proteins with the translational initiation complex, we analyzed the protein complexes from eIF4G immunoprecipitation in SDS-PAGE and silver staining. Although the majority of protein bands are equally presented between the immunoprecipitated complexes from the control cells and the cells treated with 20 μmol/L of As^{3+} overnight, changes in a few bands in the As^{3+}-treated cells did occur (Fig. 5*C*). Bands 1 and 2 were increased, whereas bands 3 and 4 were decreased by As^{3+} treatment. To identify these changed bands, the gel slices were excised (Fig. 5*C, asterisk*) and subjected to in-gel digestion by trypsin to generate peptides with different lengths based on the distribution of tryptic cleavage sites (arginine and lysine). The digested products were analyzed by reversed phase nano-LC-MS/MS on a Finnigan LTQ mass

spectrometer. This analysis identified 8 peptides from various regions of the human elongation factor 2 (eEF-2) for band 1, 6 peptides of human heterogeneous nuclear ribonucleoprotin M (hnRNP M) for band 2, 10 peptides of protein disulfide-isomerase A3 (PDIA3) along with 46 peptides of vimentin for band 3, and 7 peptides of eIF4A-I for band 4. Figure 5*D* shows the representative fragmentation spectrum for bands 1 to 4 from Fig. 5*C*. The eEF-2 is a key factor for efficient peptide elongation during protein translation (34). The increased association of eEF-2 with eIF4G in the As^{3+}-treated cells might be a reflection of reciprocal functional antagonism or coordination between cap-dependent and IRES-dependent protein translations through sharing or competing common cofactors. Some hnRNP family members had been previously implicated in direct binding to the IRES elements (31, 35). Thus, the increased association of hnRNP M with translational initiation complex by As^{3+} is suggestive of As^{3+} regulation of IRES-dependent translation. The role of PDIA3 might be mainly to order proper folding of the newly translated proteins. A decrease in PDIA3 association with the initiation complex by As^{3+}, thus, would likely result in improper protein folding. The most important finding in this analysis is the nearly complete depletion of eIF4A from the translational initiation complex by As^{3+}. eIF4A is an essential subunit of the eIF4F complex for cap-dependent initiation, which possesses RNA helicase activity to unwind secondary structures within the 5′-UTR of mRNA (36). Taken together, these data show that As^{3+} treatment favors IRES-dependent translation by impairing the assembly of the cap-dependent translational complex.

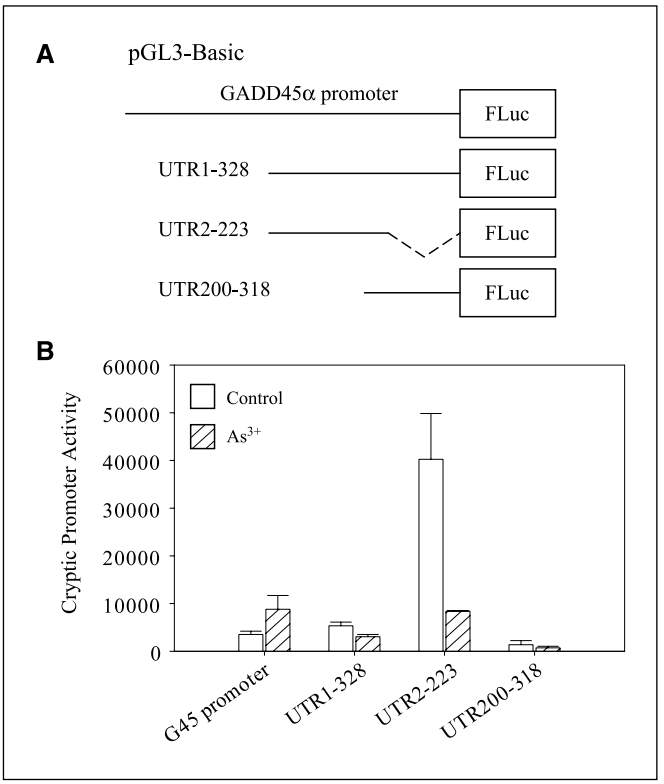

Figure 4. As^{3+}-induced IRES activity does not result from a cryptic promoter in the 5′-UTR of the GADD45α mRNA. *A,* schematic illustration of the monocistronic luciferase reporter vectors. The indicated regions of the GADD45α 5′-UTR were inserted into the promoterless pGL3 basic vector. The GADD45α promoter was used as a positive control. *B,* luciferase activity was determined in the cells transfected with these vectors in (*A*) and cultured in the absence or presence of 20 μmol/L of As^{3+} for 12 h (*right*). *Columns,* means of four repeats; *bars,* SD.

Discussion

The findings presented here provide the first evidence that As^{3+} is able to induce translation of the GADD45α protein in an IRES-dependent manner in the cells under growth-arrested conditions. The 5′-UTR of the GADD45α mRNA contains an IRES element, the activity of which can be induced by As^{3+} as evidenced in a dicistronic reporter gene activity. Although the 5′-terminal 200 nt 5′-UTR of the GADD45α mRNA possibly possesses a cryptic promoter activity under basal conditions, As^{3+} was unable to induce this cryptic promoter activity in an analysis using either a monocistronic vector or a promoterless dicistronic vector. The IRES-dependent induction of the GADD45α protein was further supported by the fact that As^{3+} impairs the assembly of the cap-dependent translational initiation complex by decreasing the association of eIF4G with both eIF4E and eIF4A. This impairment causes a reduction of the global protein translation. As^{3+} has previously been shown to be capable of regulating transcription and stabilization of the GADD45α mRNA. The effect of As^{3+} on IRES-dependent translation of the GADD45α protein suggested an additional layer of regulation for the expression and function of the GADD45α protein in cellular responses to As^{3+}.

A wide spectrum of extracellular signals could induce rapid elevation of GADD45α (1). Remarkable progress has been made during the past decade on the regulation and functional aspects of GADD45α protein in cellular responses to DNA damage and a variety of stress inducers. However, the answer to how the expression of the GADD45α is regulated when the cells are under extremely stressed conditions in which the major transcriptional and translational machineries are severely impaired, is still elusive. It was assumed that cells can use an alternative approach to synthesize proteins with the remaining trace amount mRNAs when the canonical cap-dependent protein translation process is compromised either by dephosphorylation of 4E-BP1 or cleavage of eIF4G (37, 38). When the cells are in a state of mitosis, quiescence, differentiation, or hypoxia, the phosphorylated free 4E-BP1 is dephosphorylated, possibly by protein phosphatase 2A, leading to association with and inhibition of eIF4E. Consequently, cap-dependent protein translation is drastically reduced (25, 39). A hallmark of cell apoptosis is the activation of initiating and executing caspases. It has been shown that caspases are highly capable of cleaving cap-dependent translational initiation factors such as eIF4B, eIF3, eIF2a, eIF4GI, and eIF4GII, leading to global repression of protein translation (33). However, a subset of mRNAs, <10% of total mRNAs, manages to escape this translational depression and maintains translation through a cap-independent, or rather, IRES-dependent translation. Considering the fact that GADD45α was induced mostly by the signals leading to cell cycle arrest or apoptosis, it is very likely that the expression of GADD45α protein is achieved via IRES-mediated translation.

The IRES-dependent translation was initially discovered in the viral gene translation of picornaviruses in host cells (40). The mRNAs of these viruses are naturally uncapped at their 5′-end. More importantly, many of these viruses produce proteases to proteolytically deplete cap-binding complex to shut off protein translation of the host cells completely during their infection. However, the viral proteins are still efficiently translated in the

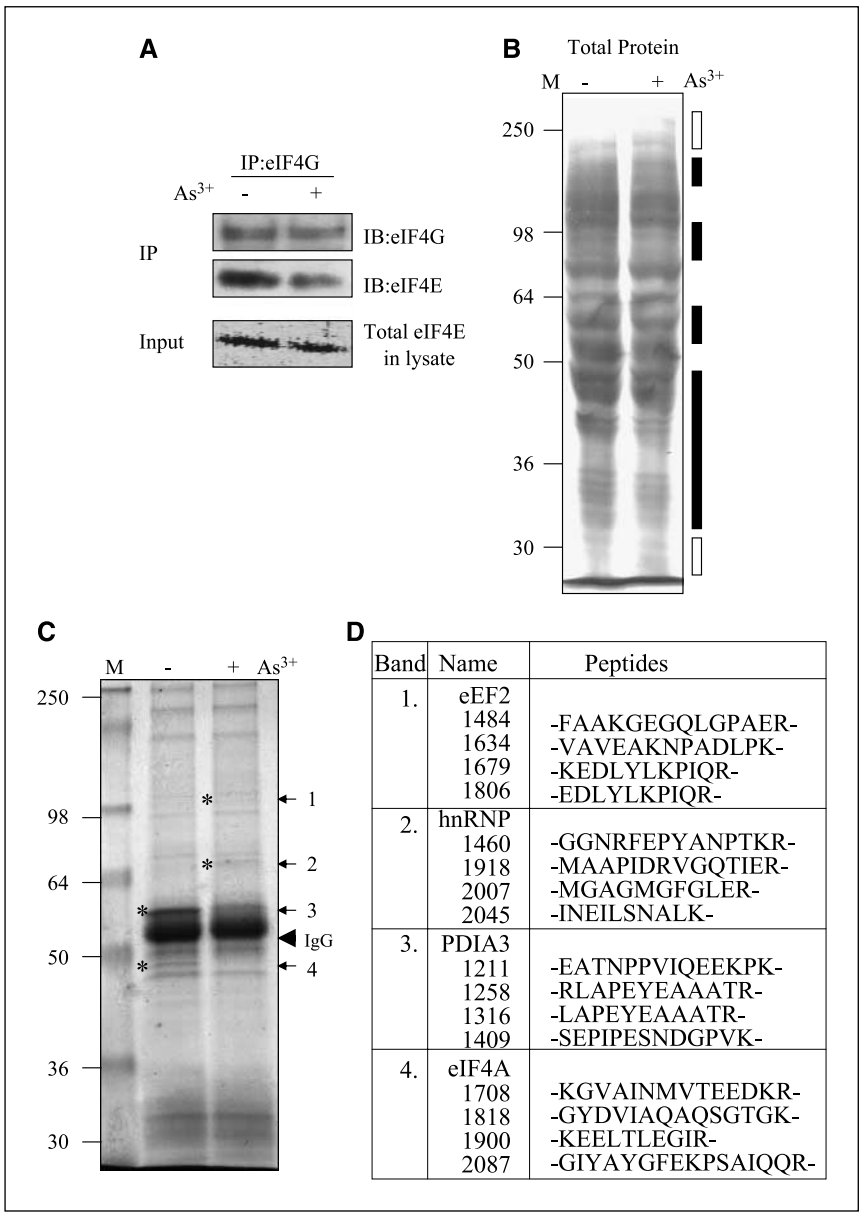

Figure 5. As^{3+} impairs the assembly of the cap-dependent translational initiation complex. *A,* BEAS-2B cells cultured in the absence or presence of 20 μmol/L of As^{3+} for 12 h and then subjected to immunoprecipitation using antibody against eIF4G. The immunoprecipitated complexes were separated on 14% SDS-PAGE gel and were sequentially immunoblotted with antibodies for eIF4G and eIF4E. Total cell lysates were used as input to determine the level of eIF4E. *B,* total cellular proteins were separated on 14% SDS-PAGE gel and stained by Coomassie blue. *Left,* molecular weights (*M*, kDa). *Right,* open and filled bars indicate increased and decreased expression, respectively, of proteins induced by 20 μmol/L of As^{3+} for 12 h. *C,* the complexes from eIF4G immunoprecipitation of the control and As^{3+}-treated cells were separated on 14% SDS-PAGE gel and monitored by silver staining. *Left,* molecular weights (*M*, kDa). *Right,* number of protein bands that were affected by As^{3+} treatment. *, positions of the protein bands that were excised for in-gel digestion followed by reversed phase nano-LC-MS/MS analysis. *D,* representative peptide fragmentation spectrum for bands 1 to 4 from (*C*).

infected cells. Further studies led to the discovery of the viral IRES element in the 5′-UTR of viral mRNA which mediates viral protein translation by recruiting IRES binding and/or recognition ribosomal proteins and other RNA-binding proteins from the infected host cells (41). Since then, a growing list of mammalian cell mRNAs have been found to possess an IRES element, especially for those containing a relatively long 5′-UTR and function as important regulators for cell growth and stress responses (30). These mRNAs include c-myc (42), p53 (43), vascular endothelial growth factor (44), fibroblast growth factor 2 (45), platelet-derived growth factor B/c-sis (46), hypoxia-inducible factor-1 (47), death-associated protein 5 (33), apoptotic protease activating factor (48), X-linked inhibitor of apoptosis (31), and others. The IRES-dependent translation of the growth factors confers a survival advantage of the cells under hypoxia or stressed conditions, such as solid tumor cells undergoing chemotherapy. On the other hand, cells use the IRES-dependent translational machinery to express proteins functioning in apoptosis or cell cycle arrest to eliminate genetically damaged cells, or prevent accumulation of genomic damage. GADD45α has been implicated in cell cycle regulation and DNA damage repair. Thus, the IRES-dependent translation of the GADD45α protein might be theoretically beneficial for protecting normal cells from malignant transformation under extreme stress conditions. The final outcome of the IRES-dependent expression of the GADD45α protein in either normal cells or tumor cells, however, might be largely dependent on the integrity of other apoptotic and DNA repair machineries which act in concert with GADD45α. In addition, the fate of a cell following GADD45α induction is also dependent on whether the expressed GADD45α can be sufficient to override the tumorigenic signals that are induced simultaneously or asynchronously by the treatments.

Because IRES-dependent protein translation occurred mostly in the cells under circumstances of genotoxic stress, apoptosis, nutrient starvation, mitosis, or growth arrest, it is conceivable

that manipulating the IRES pathway would alter the growth or survival status of the cell. Evidence supporting this notion is mainly from studies that show mutations of IRES elements or functional impairment of IRES binding proteins, the so-called IRES trans-acting factors in cancers or diseases associated with cancer susceptibility (49, 50). In cell lines derived from patients with neoplasia multiple myeloma, a single C to U transition in the IRES region of c-myc mRNA was found to be responsible for the enhanced IRES activity and the consequent increased expression of c-myc protein (49). The increase of c-myc protein obviously provides the cells with a growth advantage and elevated oncogenic potentials. On the other hand, a defect in the IRES machinery due to a genetic deficiency in a gene encoding pseudouridine synthase, dyskerin, has been linked to cancer predisposition, a result of decreased IRES-mediated expression of antiapoptotic protein, p27 (50). Currently, IRES-mediated protein translation has been implicated in the expression of both prosurvival and antisurvival factors. An interesting question to be asked is, therefore, whether different IRES machineries present in given cells mediate the expressions of proteins with unique functions in context with the type and duration of extracellular signals.

In summary, our data support the model that GADD45α protein translation is up-regulated by As^{3+}, as well as UV radiation or taxol treatment, through the IRES element identified in the 5′-UTR of the GADD45α mRNA. Treatment of the cells with As^{3+}, and possibly other stress inducers, compromises the function of the cap-dependent translational machinery, and thereafter favors IRES-dependent protein translation. The IRES-dependent translation of the GADD45α protein may not be limited to the cells in severe stress conditions, such as growth arrest in this study, but also in the cells with mild environmental insults. The regulation of the GADD45α protein on transcriptional, mRNA stability, and translational levels might be important to ensure that cells produce sufficient amounts of the GADD45α protein to reduce the possibility of improper chromosomal segregation and cell division.

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