Induction of the mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element

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GADD153 is a CCAAT/enhancer-binding-protein-related gene that may function to control cellular growth in response to stress signals. In this study, a variety of oxidant treatments were shown to stimulate endogenous GADD153 mRNA expression and to transcriptionally activate a GADD153 promoter–reporter gene construct in transfected HeLa cells. Both commonalities and distinctions in the induction of GADD153 by H$_2$O$_2$ and the thiol-reactive compound arsenite were demonstrated. GADD153 mRNA induction by both H$_2$O$_2$ and arsenite was potentiated by GSH depletion, and completely inhibited by N-acetyl-cysteine. α-Phenanthroline and mannitol blocked GADD153 induction by H$_2$O$_2$, indicating that iron-generated hydroxyl radical mediates this induction. Concordantly, GSH peroxidase overexpression in WI38 cells attenuated GADD153 mRNA induction by H$_2$O$_2$. However, GADD153 induction by arsenite was only modestly reduced in the same cells, suggesting a lesser contribution of peroxides to gene activation by arsenite. We also demonstrated that oxidative stress participates in the induction of GADD153 by UVC (254 nm) irradiation. Finally, both promoter-deletion analysis and point mutation of the AP-1 site in an otherwise intact promoter support a significant role for AP-1 in transcriptional activation of GADD153 by UVC or oxidant treatment. Indeed, exposure of cells to oxidants or UVC stimulated binding of Fos and Jun to the GADD153 AP-1 element. Together, these results demonstrate that both free-radical generation and thiol modification can transcriptionally activate GADD153, and that AP-1 is critical to oxidative regulation of this gene. This study further supports a role for the GADD153 gene product in the cellular response to oxidant injury.

INTRODUCTION

Oxidative stress plays a causative role in a variety of human diseases, and is believed to contribute to the degenerative changes that occur with aging [1,2]. Reactive oxygen intermediates (ROI), including molecular oxygen (O$_2$), superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (HO$^·$), are ubiquitously produced in the course of aerobic metabolism and are a constant threat to cellular function. Toxins, carcinogens and environmental agents can both contribute to ROI production and compromise antioxidant defences. Further, exogenous agents can mediate oxidative injury through direct chemical interaction with cellular components, e.g. protein thiol modification by thiol-reactive agents. When the generation of ROI and other oxidizing species exceeds the cellular capacity for detoxication, oxidative stress results and damage to DNA, proteins and lipids ensues.

The mechanisms controlling the cellular response to oxidative stress have been extensively investigated at the molecular level in bacterial systems, where the OxyR and SoxR/S regulons have been shown to co-ordinately regulate the induction of genes by ROI (reviewed in ref [3]). Similarly, transcription factors have been identified that probably mediate the oxidative stress response in yeast, including yAP-1 and yAP-2 (which have homology with mammalian AP-1), and Mac-1 (reviewed in ref [4]). In mammalian systems, numerous genes have been shown to be responsive to oxidants, although a systematic mechanism for gene regulation by oxidative stress has not been elucidated.

Oxidative stress has been shown to alter the expression of mammalian antioxidant enzymes including superoxide dismutase, glutathione peroxidase (GPx), γ-glutamylcysteine synthetase, catalase, glutathione-S-transferase and quinone reductase [5-10]. Induction of haem oxygenase by oxidative stress may both function in intracellular signalling [11] and serve to protect cells from further oxidant injury. Oxidants also enhance expression and/or DNA binding of numerous transcription factors, including fos, jun, myc, erg-1, NFκB, HSF and TCF/SCF ([12,13]; reviewed in [4]). That numerous mammalian genes and regulatory proteins involved in transcription are sensitive to oxidative stress speaks for the pivotal role of ROI as likely physiological mediators of gene regulation. As such, oxidants may play a significant role in the molecular response to cellular stress.

Although low in normal proliferating cells, the expression of the mammalian gene GADD153 (named from the fact that it is inducible by growth arrest and DNA damage) is dramatically increased in response to a variety of stress stimuli, including nutrient depletion, genotoxic agents and Ca$^{2+}$ ionophore [14-16]. A member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, GADD153 may control proliferation in response to cellular stress signals [17,18]. In the present study, the regulation of GADD153 by oxidative stress was investigated. The roles of HO$^·$ formation, thiol status and cellular GPx levels in mediating GADD153 induction by H$_2$O$_2$ and arsenite were assessed. Further, the contribution of oxidative stress to GADD153 induction by UVC (254 nm) irradiation was explored. Comparative analysis of the transcriptional activity of

Abbreviations used: ROI, reactive oxygen intermediates; GPx, GSH peroxidase; NAC, N-acetyl-cysteine; BSO, buthionine sulfoximine; BCS, bathocuproinedisulphonic acid; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; C/EBP, CCAAT/enhancer-binding protein; SV40, simian virus 40.

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serially deleted GADD153 promoter fragments suggested that an AP-1-binding element contributes significantly to transcriptional activation by UVC or oxidant treatment. This assertion was supported by the marked reduction in transcriptional activity engendered by point mutation of the AP-1 site in an otherwise intact promoter, and by gel mobility-shift studies demonstrating that UVC, arsenite and $H_2O_2$ treatment of cells stimulates Fos and Jun binding to an oligonucleotide encompassing the GADD153 AP-1 site.

**MATERIALS AND METHODS**

**Cell culture conditions, transfections and chemical treatments**

HeLa (human cervical carcinoma) and simian virus 40 (SV40)-transformed WI38 (human diploid lung) cells were maintained in a humidified atmosphere containing $10{\%}$ CO$_2$ in air. Cell lines were grown in Dulbecco’s modified Eagle’s medium (Gibco/BRL, Bethesda, MD, U.S.A.) supplemented with $10{\%}$ fetal bovine serum (Hyclone) and $50 \mu g/ml$ gentamicin (Gibco). Medium used for WI38 cells also contained sodium selenite ($10^{-7} M$). Transient transfections were performed with $5 \mu g$ of CsCl-purified plasmid DNA by CaPO$_4$ precipitation [19] followed by a $1.5 \text{ min}$ exposure to $15{\%}$ (v/v) glycerol in Dulbecco’s modified Eagle’s medium $4$ h after DNA addition. Relative transfection efficiencies were assessed by normalization of chloramphenicol acetyltransferase (CAT) activity to the activity of a co-transfected actin promoter–luciferase reporter construct. A luciferase assay system kit (Promega, Madison, WI, U.S.A.) was utilized to assess relative actin promoter activity among transfection groups. Transfection efficiencies were not significantly different among deletion and mutation constructs of the GADD153 promoter linked to the CAT reporter gene.

The stably transfected cell line GADD153–CAT/HeLa has been previously described [20]. The WI38 cell line overexpressing G Pax (Renard, P., Zachary, M.-D., Bougelet, C., Mirault, M. E., Haegeman, G., Remacle, J. and Raes, M., unpublished work) is a clonal isolate derived by transfection of SV40-transformed WI38 cells with a plasmid containing the constitutive promoter human cytomegalovirus linked to human G Pax. These cells have twice the G Pax specific activity of parental cells, correlated with a similar increase in G Pax protein measured by Western-blot analysis.

HgCl$_2$, phenylarsine oxide, $t$-butylhydroperoxide and cumene hydroperoxide were from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.). Phenylarsine oxide was dissolved in DMSO at 10 mM and stored at $-20{\degree}$C, and arsenite and heavy metals were stored as 100 mM solutions in water. Solutions of $H_2O_2$, $t$-butyl hydroperoxide, and cumene hydroperoxide in water ($1 \text{ mM}$ concentrations) were prepared immediately before cell treatment, as were solutions of $N$-acetyl-cysteine (NAC), buthionine sulfoximine (BSO), mannitol and bathocuproinedisulfonic acid (BCS) in medium, and $\alpha$-phenanthroline in ethanol.

To minimize variations in dose effectiveness due to cell number, WI38 cells were seeded at $1 \times 10^4$ cells/100 cm$^2$ dish and HeLa cells at $0.5 \times 10^4$ cells/100 cm$^2$ dish $48$ h before all organic peroxide experiments except for transient transfection assays. For transient transfection experiments, cells were seeded to be $50$–$75{\%}$ confluent at the time of treatment (approx. $1 \times 10^4$ HeLa cells/100 cm$^2$ dish seeded $48$ h before treatment). To compensate for increased cell number in transient transfection assays, $200 \mu M$ rather than $50 \mu M$ $H_2O_2$ was used. Because of the high antioxidant content of serum, all oxidant treatments were performed in serum-free medium or PBS (with Ca$^{2+}$ and Mg$^{2+}$). A $30$ min exposure period was utilized to control for differences in oxidant stability over time among treatments. For UVC treatment, the medium was reduced and reserved, and the cells were washed once with PBS. After irradiation at $254 \text{ nm}$, the reserved culture medium was replaced.

**RNA isolation and Northern analysis**

Total RNA was extracted from treated cells using RNA Stat-60 (Tel-Test ‘B’, Friendswood, TX, U.S.A.) according to the manufacturer’s instructions, using $1 \text{ ml}$ of Stat-60 solution per $100 \text{ cm}^2$ tissue culture dish. RNA ($10$–$20 \mu g/\text{lane}$) was size-separated in agarose/formaldehyde gels and transferred to GeneScreen Plus nylon membranes (DuPont/NEN, Boston MA, U.S.A.). GADD153 cDNA was labelled with $[\gamma-32P]dCTP$ using a random-primer labelling kit (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). Hybridization and washes were performed by the method of Church and Gilbert [21], and the hybridization signal was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). After analysis of the GADD153 signal, blots were rehybridized to a $24 \text{ bp}$ oligonucleotide ($5^{-ACCGTATCTGATCGTCTCGAACC-3'}$) complementary to $18 \text{ S}$ RNA (synthesized by Midland Certified Reagent Co, Midland, TX, U.S.A.) that had been $3'$-end-labelled with $[\gamma-32P]dATP$ by terminal deoxynucleotidyltransferase (Life Technology Laboratories, Gaithersburg, MD, U.S.A.). GADD153 signals were normalized to $18 \text{ S}$ values obtained on the same blot to control for variation in loading and transfer among samples.

**CAT assays**

CAT assays were performed as previously described [22]. Percentages of chloramphenicol acetylation were obtained over the linear range of the assay ($5$–$45{\%}$ conversion), and were normalized to protein content.

**Gel mobility-shift assays**

Control or treated HeLa cells were subjected to Dounce homogenization in $20 \text{ mM}$ Hepes, pH $7.5$, containing $1.5 \text{ mM}$ MgCl$_2$, $0.2 \text{ mM}$ EDTA, $0.4 \text{ mM}$ NaCl, $0.2 \text{ mM}$ dithiothreitol (DTT), $1 \text{ mM}$ Pefabloc SC (Boehringer-Mannheim), $20{\%}$ (v/v) glycerol and $1 \mu g/ml$ leupeptin. After centrifugation at $17000 \text{ g}$ for $30 \text{ min}$, $20 \mu g$ of protein extract was incubated with $0.5 \mu g$ of [$\gamma$-$32P]dATP-labelled $21 \text{ bp}$ oligonucleotides with either the intact GADD153 AP-1 binding sequence ($5'\text{CGATGCGA-TGACTCACTCAAT-3'}$), or a single base change of T to G within this binding element ($5'\text{CGATGCGAC-GACTCACTCAAT-3'}$). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.). Reaction buffer contained $10 \text{ mM}$ Tris/HCl, pH $7.5$, $1 \text{ mM}$ DTT, $1 \text{ mM}$ EDTA, $50 \text{ mM}$ NaCl, $5{\%}$ (v/v) glycerol and $1 \mu g$ of poly (dl-dC) as a non-specific competitor. Supershift assays were performed by the addition of antibodies against Fos or Jun (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.) following the initial binding reactions between protein extracts and oligonucleotides. Likewise, in competition experiments, $50 \times$ unlabelled oligonucleotides were added after binding of protein extracts to [$\gamma$-$32P]dATP-labelled oligonucleotides had proceeded. Samples were subjected to non-denaturing PAGE in $4{\%}$ gels, whereupon the gels were dried and protein–DNA complexes visualized by autoradiography.
RESULTS

Oxidant induction of GADD153

A variety of oxidants were shown to induce GADD153 mRNA and activate a GADD153 promoter–CAT reporter gene construct in stably transfected HeLa Cells (Table 1). Arsenicals and heavy metals caused a 20–50-fold induction, and free-radical-generating compounds consistently induced GADD153 expression 10–20-fold over control levels. The potency of heavy metals as inducers of GADD153 corresponded to the reactivity of these agents with GSH [7], suggesting that thiol interaction functions in gene induction by these agents. Oxidant treatment in serum-free medium or PBS showed GADD153 inductions at significantly lower doses than previously reported for direct addition to growth medium [20], probably because the high serum content of growth medium decreased the effective oxidant dose. Study of GADD153 induction was thus undertaken within the physiologically relevant range of oxidant dosages. Of the agents tested, arsenite and H$_2$O$_2$ were selected for more detailed investigation of oxidative induction of GADD153.

Kinetic analysis of the increase in GADD153 mRNA stimulated by H$_2$O$_2$ in HeLa cells indicated that maximal mRNA levels were reached 2 h after treatment (Figure 1A). The induction of GADD153 mRNA appears to be biphasic with a second increase occurring at 8 h. The induction of GADD153 mRNA by H$_2$O$_2$ was dose-dependent (Figure 1B); 50 µM H$_2$O$_2$ consistently mediated a 5–10-fold increase in the mRNA of this gene, with interexperiment variability in fold induction arising from minor changes in the basal level of GADD153 gene expression. The induction of GADD153 by H$_2$O$_2$ typically declined at doses above 200 µM, presumably because of excessive toxicity.

Arsenite likewise resulted in a time-dependent increase in GADD153 mRNA in HeLa cells (Figure 2A) with a similar pattern to that occurring with H$_2$O$_2$. The first peak of induction occurred at 4 h, and a second increase was evident at 8 h. However, the relative magnitude of the second induction was significantly greater than that seen with H$_2$O$_2$. The dose–response relationship for the GADD153 mRNA increase stimulated by arsenite is shown in Figure 2(B). A dose of 200 µM arsenite consistently mediated at least a 20-fold induction of GADD153 mRNA, with inter-experiment variability in fold induction arising from minor changes in the basal level of GADD153 gene expression.

Both arsenite and H$_2$O$_2$ enhanced the expression of GADD153–CAT in stably transfected HeLa cells (Figure 3). The dose–response relationships for activation of the GADD153–CAT reporter gene construct were consistent with those resulting in induction of GADD153 mRNA. These results indicate a transcriptional mechanism for GADD153 gene activation by both arsenite and H$_2$O$_2$.

Table 1 Oxidant inducers of GADD153–CAT and GADD153 mRNA

HeLa and GADD153–CAT/HeLa cells were treated for 30 min with the indicated agents in serum-free medium (A) or PBS (B). CAT activity was measured 24 h later in GADD153–CAT/HeLa stable transfectants. RNA was isolated from HeLa cells 4 (A) or 2 (B) h after treatment, and analysed as described in the Materials and methods section. The range of induction of GADD153–CAT and GADD153 mRNA is relative to time-matched untreated controls.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Concentration</th>
<th>Fold induction</th>
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<tbody>
<tr>
<td>(A) Thiol-reactive reagents</td>
<td></td>
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</tr>
<tr>
<td>Phenylarsine oxide</td>
<td>1–5 nM</td>
<td>20–25</td>
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<tr>
<td>Sodium arsenite</td>
<td>100–400 µM</td>
<td>25–50</td>
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<tr>
<td>Mercury chloride</td>
<td>10–30 µM</td>
<td>10–20</td>
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<td>Cadmium chloride</td>
<td>100–400 µM</td>
<td>25–40</td>
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<tr>
<td>Zinc chloride</td>
<td>0.75–1 mM</td>
<td>25–50</td>
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<tr>
<td>(B) Free-radical generators</td>
<td></td>
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</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>10–100 µM</td>
<td>5–20</td>
</tr>
<tr>
<td>t-Butyl hydroperoxide</td>
<td>10–100 µM</td>
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<tr>
<td>Cumene hydroperoxide</td>
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Figure 1 Kinetics and dose–response relationship for induction of GADD153 by H$_2$O$_2$

HeLa cells were treated with H$_2$O$_2$ in PBS for 30 min, and harvested for RNA isolation after the indicated incubation times. Northern-blot analysis was performed as described in the Materials and methods section. Values are expressed as fold induction over time-matched controls, and represent the means of at least two independent experiments. (A) Time course of GADD153 induction with 20 µM H$_2$O$_2$. (B) dose–response analysis at 2 h. Inset, signal for GADD153 and 18 S on representative RNA blot.

Figure 2 Kinetics and dose–response relationship for induction of GADD153 by arsenite

HeLa cells were treated with arsenite for 30 min in serum-free medium and harvested for RNA isolation after the indicated incubation times. Northern-blot analysis was performed as described in the Materials and methods section. Values are expressed as fold induction over time-matched controls, and represent the means of at least two independent experiments. (A) Time course of GADD153 induction with 200 µM arsenite; (B) dose–response analysis at 4 h. Inset, signal for GADD153 and 18 S on representative RNA blot.
Role of cellular thiol status in GADD153 gene activation by arsenite and H$_2$O$_2$

Depletion of cellular GSH by BSO pretreatment markedly potentiated GADD153 induction by arsenite and H$_2$O$_2$ (Figure 4). At identical doses, BSO-pretreated HeLa cells showed an approx. 2-fold induction of GADD153 mRNA by both arsenite and H$_2$O$_2$ relative to that seen in the absence of BSO. In contrast, NAC pretreatment, which elevates cellular GSH levels, completely prevented GADD153 mRNA induction by either arsenite or H$_2$O$_2$ (Figure 5). NAC completely blocked induction by 200 $\mu$M arsenite, and reduced the 60-fold induction by 400 $\mu$M arsenite by 96% (left panel). Likewise, NAC diminished the 6-fold induction mediated by 50 $\mu$M H$_2$O$_2$ by 85% (right panel). In the absence of NAC, 100 $\mu$M H$_2$O$_2$ induced significant toxicity, and was thus beyond the dose of maximal GADD153 induction; however, NAC treatment resulted in significant protection against the toxicity induced by 100 $\mu$M H$_2$O$_2$ and prevented GADD153 induction (right panel).
Role of intracellular ROI in GADD153 gene activation by arsenite and H$_2$O$_2$

Induction of GADD153 by H$_2$O$_2$ was blocked by the iron chelator o-phenanthroline, whereas the copper chelator BCS was without effect (Figure 6, right panel). H$_2$O$_2$-stimulated GADD153 induction was also diminished in the presence of mannitol, an HO$^-$ scavenger. Taken together, these results suggest that iron-dependent HO$^-$ formation mediates GADD153 gene activation by H$_2$O$_2$. In contrast, neither metal chelators nor mannitol inhibited GADD153 mRNA induction by arsenite (Figure 6, left panel). However, more direct assessment of the role of ROI in GADD153 induction by constitutive overexpression of GPx suggested a partial contribution of peroxide moieties to GADD153 induction by arsenite. In cells overexpressing GPx, GADD153 activation by arsenite was reduced approx. 35% (Figure 7, left panel). GADD153 induction by H$_2$O$_2$ was 2-fold lower in GPx-overexpressing cells (right panel), indicating a greater degree of ROI involvement in gene activation by H$_2$O$_2$ than arsenite.

Involvement of oxidative stress in GADD153 induction by UVC

Modulation of cellular thiol status also moderately influenced the ability of UVC to induce GADD153 (Figure 8). The induction of GADD153 mRNA by UVC in HeLa cells was somewhat enhanced by depletion of intracellular thiols with BSO, and partially blocked by NAC (left). The effects of BSO and NAC were confirmed in HeLa/GADD153–CAT stable transfectants (right). Although pronounced, the sensitivity of UVC-induced GADD153 expression to cellular thiol status was not as dramatic as that for H$_2$O$_2$- or arsenite-induced expression. NAC had completely abolished both H$_2$O$_2$ and arsenite induction of GADD153 (Figure 5); in contrast, NAC decreased UVC induction of GADD153–CAT by 45% and 75% for UVC doses of 12 and 16 J/m$^2$ respectively (Figure 8). Potentiation of the UVC response by BSO (50–60% increase) was also less than that seen with arsenite and H$_2$O$_2$ (compare Figures 4 and 8). Further, UVC-induced GADD153 mRNA expression was shown to be reduced 50% by o-phenanthroline pretreatment and 40% by mannitol pretreatment (results not shown). These results indicate that, although oxidative stress contributes to the induction of GADD153 by UVC, HO$^-$ is not the sole mediator of the response, as appears to be the case with H$_2$O$_2$ (Figure 6). Because UVC does not result in a significant induction of GADD153 mRNA in WI38 cells, a comparison of the ability of GPx overexpression to modulate induction of GADD153 mRNA by UVC vis à vis arsenite and H$_2$O$_2$ could not be undertaken.

Common promoter elements function in the induction of GADD153 by arsenite and H$_2$O$_2$

The GADD153 promoter contains numerous regulatory elements that are likely to function in controlling the expression of this gene in response to cellular stress [23] (Figure 9A). Serial deletion of the GADD153 promoter was undertaken to highlight regions responsive to oxidative stimuli (Figure 9B). The magnitude of promoter activation in response to arsenite, H$_2$O$_2$ or UVC was diminished by decreasing the promoter size from −778/+21 to −483/+21, indicating removal of positive regulatory elements; however, basal activity increased nearly 2-fold with loss of DNA sequences in this region. Interestingly, the magnitude and pattern of expression that results on further deletion from −483 to −36 was nearly identical for arsenite and H$_2$O$_2$. Deletion of the sequences from −483 to −250 resulted in an increased responsiveness to arsenite and H$_2$O$_2$, suggesting that this region contains negative regulatory elements for these agents. In contrast, deletion of these same sequences resulted in a diminished response to UVC. These findings support the view that distinct regulatory elements are involved in mediating GADD153 gene activation in response to UVC and the oxidative agents arsenite and H$_2$O$_2$. Nonetheless, further deletion of promoter from −250 to −225, a 25 bp region that contains an AP-1-binding element, resulted in a similar 2-fold reduction in the remaining activity for all three agents, indicating commonalities among cellular signals leading to gene activation by UVC, arsenite and H$_2$O$_2$.

Role of AP-1 in oxidative induction of GADD153

Several experimental approaches were utilized to explore the role of the AP-1 site in regulating the transcriptional activity of
GADD153 in response to oxidative stress. Gel mobility-shift assays were employed to determine whether oxidative stress stimulates binding of cellular factors to an oligonucleotide containing the GADD153 AP-1 sequence. H$_2$O$_2$ and arsenite treatment resulted in a time-dependent increase in binding activity to the oligonucleotide containing the GADD153 AP-1 site (Figure 10A). UVC treatment similarly stimulated GADD153 AP-1 DNA-binding activity. By contrast, GADD153mutAP-1, a 21 bp oligonucleotide containing a single base pair mutation within the AP-1 consensus sequence (TGACTCA changed to gGACTCA), did not bind proteins from arsenite-, H$_2$O$_2$-, or UVC-treated cells (Figure 10B). Further, whereas unlabelled GADD153 AP-1 oligonucleotide was an effective competitor for radiolabelled DNA in the DNA–protein complex, the GADD153mutAP-1 oligonucleotide was not (Figure 10C). Finally, addition of Fos or Jun antibody after binding of GADD153 AP-1 DNA to protein from treated cells caused a shift in the mobility of the resultant complexes to a more slowly migrating species (Figure 10C). This supershift indicates the presence of Fos and Jun in complexes of the GADD153 AP-1 oligonucleotide and proteins from oxidant-treated cells.

Two additional experiments directly demonstrated the functional activity of the AP-1-binding element in regulating GADD153 transcription. First, a full-length promoter construct was generated which contains the same T to G mutated GADD153 AP-1 site shown to lack protein-binding activity in gel mobility-shift assays above (−778/+21mutAP-1). In the absence of treatment, the point mutation did not affect promoter activity. However, the induction ratio of CAT activity for arsenite, H$_2$O$_2$ or UVC was reduced by approx. 50 % when the T to G point mutation was introduced in the AP-1 site of an otherwise intact GADD153 promoter fragment (Figure 11). Secondly, the functional capacity of the AP-1 sequence was directly demonstrated using a construct containing a trimer of this site linked to a minimal GADD153 promoter construct (−36/+21 fragment). The enhanced expression of the 3 × AP-1 construct displayed in transiently transfected cells treated with arsenite, H$_2$O$_2$ or UVC confirmed the transcriptional activity of this particular promoter element (Figure 11).

**DISCUSSION**

GADD153 gene expression is known to be ubiquitously enhanced in response to a diversity of stressful stimuli. This report provides a comprehensive examination of the regulation of GADD153 by oxidative stress, with particular focus on the cellular factors and promoter elements that contribute to transcriptional activation after oxidant injury. Although both thiol-reactive agents and free-radical-generating compounds transcriptionally activate GADD153, more detailed analysis revealed some differences in the gene inductive signal for the two classes of oxidants. The magnitude of GADD153 induction by the thiol-reactive reagent arsenite was 2–5-fold greater than that seen with the free-radical-generating compound H$_2$O$_2$ (Figures 1 and 2). Similar kinetics of induction were found for both H$_2$O$_2$ and arsenite, characterized by a biphasic pattern with the second peak evident 8 h after treatment. The first peak of induction, however, occurred at 2 h after treatment with H$_2$O$_2$, and 4 h after exposure to arsenite.
indicating the presence of Fos and Jun proteins. Potentiated GADD153-specific binding; AP-1, protein extracts were performed as described in the Materials and methods section. NS, Non-(Ab). HeLa cells were treated as indicated, and gel mobility-shift analyses using the resultant arsenite- (2 h) or UVC- (4 h) treated cells supershift in the presence of Fos and Jun antibodies.

Gel mobility-shift analysis using 21 bp oligonucleotides having either (A) the intact GADD153 AP-1-binding sequence (5'-CGATGCAGACTCACTCAT-3') or (B) a single base change of T to G within this binding element (5'-CGATGCAGACTCACTCAAT-3') (GADD153mutAP-1). (C) Binding to the intact GADD153 AP-1 site is not subject to competition with GADD153mutAP-1 (50:1); the complexes of GADD153 AP-1 oligonucleotide and proteins from H2O2 (2 h), arsenite (2 h) or UVC (4 h) treated cells supershift in the presence of Fos and Jun antibodies (Ab). HeLa cells were treated as indicated, and gel mobility-shift analyses using the resultant protein extracts were performed as described in the Materials and methods section. NS, Nonspecific binding; AP-1, GADD153 AP-1 binding; arrow, supershifted DNA-binding complexes indicating the presence of Fos and Jun proteins.

Depletion of cellular GSH by BSO pretreatment significantly potentiated GADD153 induction by both H2O2 and arsenite (Figure 4). Conversely, NAC pretreatment, which is known to elevate GSH levels [24], completely abolished GADD153 induction by these oxidants (Figure 5). That either BSO or NAC treatment alone can mildly stimulate GADD153 expression indicates that oxidation or reduction of critical thiol moieties may mediate GADD153 induction. The ability of the reducing agent DTT to stimulate GADD153 induction further supports this assertion [25]. However, the relatively small magnitude of GADD153 induction stimulated by BSO alone indicates that H2O2 and arsenite do not function solely through GSH depletion. Instead, BSO and NAC are likely to influence opposingly the accessibility of intracellular targets for oxidative modification of H2O2 and arsenite.

The cellular effects of H2O2 are thought to be largely mediated through the metal-catalysed production of HO•, which will react indiscriminately with the entire spectrum of biomolecules, including proteins, lipids and DNA (reviewed in ref [26]). The inhibition of H2O2-induced GADD153 expression by α-phenanthroline and mannitol indicates that iron-mediated conversion of H2O2 to HO• plays a significant role in gene activation by H2O2 (Figure 6). That GPx overexpression also diminishes the induction of GADD153 by H2O2 strengthens this conclusion. Whereas GPx acts to detoxify H2O2 directly, peroxide moieties generated in the course of HO• reactions with cellular components will also serve as a substrate for this enzyme. Thus GPx may not only serve to diminish the effective dose of H2O2, but it may also act on oxidatively damaged lipids or proteins.

The role of ROI in the induction of GADD153 by arsenite was less pronounced. Neither metal iron chelators nor the HO• scavenger, mannitol, inhibited GADD153 induction by arsenite (Figure 6), and GPx overexpression only modestly influenced GADD153 mRNA induction by arsenite (Figure 7). These data are in keeping with the assertion that arsenite can stimulate the intracellular production of oxidative stress, and that peroxide-moiety generation arising as a consequence of the ensuing reactions of ROI with lipids may function in gene activation by arsenite [27]. However, because arsenite has considerable preferential reactivity with vicinal dithiol moieties, its primary mechanism of action is likely to be through the direct oxidation of critical cellular thiols [28–31].

Oxidative stress has also been proposed to contribute, in part, to the cellular effects of UVC [32,33]. Indeed, induction of GADD153 by UVC displayed moderate sensitivity to intracellular thiol status modification by BSO and NAC (Figure 8). Similarly, induction of GADD153 could be partially inhibited by...
Deletion analysis of the GADD153 promoter region suggested that multiple elements within an 800 bp region are likely to contribute to regulation of GADD153 activity in response to oxidant stress. Activation by arsenite and H₂O₂ relied on certain elements common to those involved in mediating the response to UVC, but also others that are distinct. In particular, the region spanning nucleotides –250 and –225 was equally important for GADD153 induction by UVC, H₂O₂ and arsenite. Additional experiments identified an AP-1-binding element within this region as an important regulator of GADD153 expression. A single base-pair change in the AP-1-binding sequence abolished the enhancement in transcriptional activity in response to oxidative treatment (Figure 11). Similar sequences have been shown to play a role in the induction of other genes by UVC and oxidative stress. In addition, AP-1 may participate in the mammalian response to GADD153 transcriptional activation of GADD153 gene expression, as an important regulator of GADD153 expression, and that this induction is mediated, at least in part, by an AP-1 site in the AP-1 promoter. These data suggest that GADD153 may participate in the mammalian response to oxidant injury. Although the precise function served by GADD153 has not been clarified, as a transcription factor it may be involved in mediating growth arrest in response to cellular stress, allowing critical repair processes to proceed before cell cycling [17,18]. As such, this gene may play a pivotal role in survival subsequent to oxidant injury.

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