The Pathway Regulating GADD153 Induction in Response to DNA Damage Is Independent of Protein Kinase C and Tyrosine Kinases1

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Abstract

Treatment of cells with agents that damage DNA leads to the induction of numerous genes. Recent studies aimed at understanding the events preceding the transcriptional activation of some of these DNA damage-inducible genes in mammalian cells have demonstrated that various extranuclear protein kinases are involved in the signaling cascades. The mammalian GADD153 gene, a member of the CCAAT enhancer-binding protein family of transcription factors, is highly induced by a variety of DNA-damaging agents as well as by certain growth arrest conditions and oxidative stresses. We have examined the effects of numerous protein kinase and phosphatase inhibitors on the DNA damage-induced expression of GADD153, to identify the signal transduction components involved in its transcriptional regulation. In contrast to the transcriptional activation of c-jun and collagenase in response to DNA damage, GADD153 induction involves neither protein kinase C nor tyrosine kinases but does appear to require an unidentified serine-threonine kinase. Elevation of intracellular glutathione levels by treatment with N-acetylcysteine did not affect the methyl methanesulfonate-induced expression of the GADD153 gene, although it did diminish cadmium chloride-induced expression. These findings suggest that oxidative stress and DNA damage regulate GADD153 transcription through different pathways. Based on our findings and those of others with respect to other DNA damage-inducible genes, we propose a model depicting the complex pathways which appear to be involved in the regulation of mammalian genes in response to genotoxic stress and in which the DNA damage-induced expression of GADD153 represents a unique pathway independent of either protein kinase C or tyrosine kinase.

Introduction

DNA-damaging agents not only are important causative factors in the carcinogenic process but, paradoxically, also are among the most effective chemotherapeutic agents used for the treatment of malignancies. Thus, there is an obvious importance to understanding the cellular and molecular responses to these genotoxins. Exposure of cells to genotoxic agents results in a variety of biological responses including the rapid induction of numerous gene products, at least some of which function to protect the cell and/or repair the incurred damage. While the activation of gene expression in mammalian cells following DNA-damaging treatments is regulated primarily at the level of transcription (1, 2), the mechanisms of signal transduction involved in mediating this gene induction are not well understood.

There is considerable evidence that one or more protein kinases play a role in the activation of gene expression in response to genotoxic stress. Most DNA damage-inducible genes are also responsive to treatment with phorbol esters, which are potent activators of PKC1 (1, 2). In fact, the transcriptional activation of several genes following treatment with either DNA-damaging agents or the phorbol ester TPA has been shown to converge to the same regulatory elements [e.g., the AP-1 sites of the c-jun and collagenase genes (3, 4) and the serum response element of the c-fos gene (3, 5)]. For these reasons, PKC has often been implicated as a participant in the signal transduction pathway mediating the responses to both mitogenic stimulation and DNA damage. In particular, Hallahan et al. (6, 7) have reported that the inductions of the immediate early genes EGR1, c-jun, and tumor necrosis factor α are regulated through PKC-dependent pathways following exposure of cells to ionizing radiation.

Recently, Devary et al. (8) reported that membrane tyrosine kinases activated by UV radiation elicit a cascade of signaling events which eventually activates AP-1 binding activity to induce transcription of c-jun mRNA and possibly other AP-1-regulated genes. One merging signal between the UV and phorbol ester pathways has been identified as Raf-1 kinase, which acts on MAP kinase kinase to activate MAP kinase and, ultimately, induces the activation of AP-1 binding activity via phosphorylation of preexisting c-jun transcription factors (8–10). While Devary et al. (8) have suggested that UV-induced oxidative stress may be the initiating signal into this pathway, other groups have hypothesized a reverse signaling pathway originating in the nucleus with the recognition of DNA damage itself, followed by passage of "signals" through the cytoplasmic protein kinase cascade and eventual return to the nucleus to elicit changes in transcription factors and gene expression (9). As implied above with respect to the regulation of c-jun expression, it is possible that, even for a given gene, multiple distinct pathways may exist to activate its expression, depending on the particular genotoxic agent (6, 8–10).

Our studies have focused on identifying the signal transduction pathways leading to the induction of GADD153, a growth arrest- and DNA damage-inducible member of the CCAAT enhancer-binding protein family of transcriptional activators (11, 12). While GADD153 expression is induced by a wide variety of DNA-damaging agents, it is not responsive to X-irradiation or treatment with X-ray-mimetic agents (13), nor is it responsive to phorbol ester treatments in most cell types, despite the presence of a consensus AP-1 site within its promoter region (14, 15). To better define the pathways involved in activating GADD153 expression in response to DNA damage, we focused on identifying the effects of various protein kinases and phosphatase inhibitors on the induction of GADD153 mRNA following treatment of cells with DNA-damaging agents. Because many DNA-damaging agents result in cellular oxidative stress, we used a scavenger of free radicals to test for the role of oxidative stress in mediating the GADD153 activation following DNA damage. Based on our findings presented herein, we propose a model in which the induction of GADD153 expression by DNA damage represents an alternate pathway for gene activation following genotoxic stress that is independent of either PKC or tyrosine kinases and is separate from the pathway regulating expression following oxidative stress, at least with respect to the initiating signals.

Materials and Methods

Cell Culture and Treatments. HeLa cells were grown and maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (HyClone) and 50 μg/ml gentamicin (GIBCO). Cell treatments have all been described previously (13) and include 100 μg/ml MMS (Aldrich) treatment for 4 h, 30-kl/ml UV radiation followed by a 2-h recovery period, 200 μM cadmium chloride (Sigma) treatment for 1 h followed by a 3-h recovery, and 1 μg/ml TPA (Sigma) treatment for 24 h. Treatments with kinase inhibitors were initiated 30-60 min prior to genotoxic treatments, at the indicated time points.

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3 The abbreviations used are: PKC, protein kinase C; H7, 1-(5-isoquinolylsulfonyl)-2-methylpipерazinе; MAP, mitogen-activated protein; MMS, methyl methanesulfonate; NAC, N-acetylcysteine; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; cDNA, complementary DNA.
The kinase inhibitors utilized were H7 (Sigma), 2-aminopurine (Sigma), calphostin C (Kamiya Biomédical), staurosporine (Sigma), genistein (GIBCO), lavendustin A (GIBCO), herbimycin A (GIBCO), and tyrphostin (GIBCO). Treatments with the phosphatase inhibitors okadaic acid (Calbiochem) and sodium orthovanadate (Sigma) were performed similarly. NAC (Sigma) was prepared fresh and added 1 h prior to the genotoxic treatments.

RNA Isolation and Analysis. Cells were lysed at the indicated times after treatment and homogenized in guanidine isothiocyanate, followed by centrifugation through a cesium chloride gradient. For Northern analysis, equal amounts (10 μg) of total RNA, based on spectrophotometric analysis, were denatured in 2.2 M formaldehyde and fractionated on 1% agarose gels containing 2.2 M formaldehyde. RNA was subsequently transferred to GeneScreen Plus (Du Pont) membranes in 10X standard saline citrate (1X = 0.15 M sodium chloride, 0.15 M sodium citrate), followed by baking of the membranes for 2 h at 80°C. Filters were then hybridized and washed according to the method of Church and Gilbert (16), using cDNA probes labeled by the random primer method (17). The full length human GADD153 cDNA was cloned in the laboratory (15). The human β-actin cDNA used for normalization purposes was obtained from Dr. Larry Kedes (18).

Results

Effects of Serine-Threonine Kinase Inhibitors on the GADD153 DNA Damage Response. Because it is a strong inducer of GADD153 mRNA expression, we used the alkylating agent MMS to monitor the effects of various protein kinase inhibitors on the DNA damage-induced expression of GADD153 mRNA levels. Pretreatment of HeLa cells with staurosporine, a general inhibitor of both serine-threonine and tyrosine kinases (19), had no effect on either basal or MMS-induced GADD153 mRNA levels. Pretreatment of HeLa cells with the phosphatase inhibitor okadaic acid (Calbiochem) and sodium orthovanadate (Sigma) were performed similarly. NAC (Sigma) was prepared fresh and added 1 h prior to the genotoxic treatments.

Fig. 1. Effect of protein kinase inhibitors on MMS-induced GADD153 mRNA expression. HeLa cell cultures were treated with protein kinase inhibitors for 30 min prior to the addition of MMS, except for TPA treatment, in which 1 μg/ml TPA was added to the cells 24 h before MMS addition. Total RNA was harvested 4 h after MMS addition and was analyzed for GADD153 mRNA expression by Northern analysis (10 μg RNA/lane). The same Northern blot was stripped and reprobed for β-actin mRNA expression, to normalize for loading consistency. Treatments included: 50 μM H7, 10 mM 2-aminopurine (2 AP), 10 nM staurosporine (STAURO), and 1 μM calphostin C (CALPH).

The kinase inhibitors utilized were H7 (Sigma), 2-aminopurine (Sigma), calphostin C (Kamiya Biomédical), staurosporine (Sigma), genistein (GIBCO), lavendustin A (GIBCO), herbimycin A (GIBCO), and tyrphostin (GIBCO). Treatments with the phosphatase inhibitors okadaic acid (Calbiochem) and sodium orthovanadate (Sigma) were performed similarly. NAC (Sigma) was prepared fresh and added 1 h prior to the genotoxic treatments.

Fig. 2. Effects of genistein on MMS- and UV-induced GADD153 mRNA expression. HeLa cells were treated with the indicated doses of genistein for 60 min before further treatment with MMS (100 μg/ml, for 4 h) or UV (30 J/m²). Total RNA was harvested 4 h after MMS addition to the medium or 2 h after UV exposure. Northern blots were prepared and probed as described in the legend to Fig. 1.

Fig. 3. Effects of various tyrosine kinase inhibitors on the MMS and UV induction of GADD153. HeLa cells were treated with the indicated doses of inhibitors for 60 min before addition of 100 μg/ml MMS or exposure to 30 J/m² UV. Total RNA was extracted and analyzed as described in the legend to Fig. 1. The inhibitors included: lavendustin A (LAV), herbimycin A (HERB), and tyrphostin (TYP).

Effects of Tyrosine Kinase Inhibitors on the GADD153 DNA Damage Response. To investigate whether enhanced tyrosine kinase activity resulting from treatment with DNA-damaging agents has a role in the induction of GADD153 mRNA expression, a number of tyrosine kinase inhibitors were examined for their ability to influence MMS- and UV-induced GADD153 expression. Genistein, a well established inhibitor of tyrosine kinases (24), has been shown to significantly inhibit c-jun mRNA induction by UV radiation but not by TPA (8). Pretreatment of HeLa cells with genistein at concentrations ranging from 1 to 100 μM did not inhibit the induction of GADD153 mRNA expression in response to either MMS or UV treatment (Fig. 2). Treatment with other tyrosine kinase inhibitors, including tyrphostin, lavendustin A, and herbimycin A, had no effect on either basal GADD153 mRNA levels or MMS-induced GADD153 mRNA expression (Fig. 3). Increased expression of GADD153 mRNA following UV exposure was partially inhibited by pretreatment with lavendustin A (10 μM), herbimycin A, and tyrphostin (35%, 50%, and 25% inhibition, respectively) (Fig. 3). However, because the inhibitory effects were slight and variable between the different inhibitors and because genistein had no inhibitory effects on UV-induced GADD153 mRNA levels, we cannot conclude that tyrosine kinases are responsible for this UV induction.

Effects of Phosphatase Inhibitors on the GADD153 DNA Damage Response. Another approach to analyzing the role of phosphorylation in GADD153 expression following DNA damage is to examine the effects of phosphatase inhibitors on the response. Okadaic acid is an inhibitor of serine-threonine protein phosphatases 1 and 2A (25). Fig. 4 demonstrates the ability of okadaic acid to induce GADD153
expression in HeLa cells in a dose-dependent manner. Interestingly, cotreatment with MMS did not significantly alter the level of the response seen with optimum doses of okadaic acid alone. In contrast, treatment of cells with the tyrosine phosphatase inhibitor orthovanadate (26) had no effect on GADD153 mRNA levels, nor did pretreatment with the inhibitor alter the level of GADD153 expression observed in response to MMS (Fig. 4).

**Effects of N-Acetylcyesteine on the GADD153 DNA Damage Response.** Many DNA-damaging agents result in cellular oxidative stress either through the production of free radicals (e.g., UV radiation, via lipid peroxidation) or by reaction with free sulfhydryl groups (e.g., MMS and other alkylating agents) (1, 2). We used NAC to test the role of reactive oxygen intermediates generated by DNA-damaging and oxidative stress-inducing agents in mediating GADD153 expression. MMS was used as the DNA-damaging agent, and cadmium chloride was used as an oxidative stress. Cadmium chloride is presumed to generate oxidative stress by interacting with and decreasing the levels of cellular glutathione (27). NAC is rapidly converted within cells to the reduced form of glutathione, acting as a free radical scavenger (28). While NAC had no effect on GADD153 induction following MMS treatment, it completely prevented induction of the gene by cadmium chloride (Fig. 5).

**Discussion**

We have focused on understanding the molecular mechanisms associated with the regulation of GADD153 expression in response to genotoxic agents. We have shown by RNA analysis that, although broad-spectrum kinase inhibitors with specificity for serine-threonine kinases (H7 and 2-aminopyridine) inhibited GADD153 expression in response to MMS, neither specific inhibitors of PKC nor inhibitors of tyrosine kinases had any effect on the induction of GADD153 following MMS or UV treatments. Likewise, the tyrosine phosphatase inhibitor orthovanadate was without effect. However, the phosphatase inhibitor okadaic acid, which prevents dephosphorylation at serine-threonine residues, substantially increased GADD153 mRNA levels. These results indicate that neither PKC nor tyrosine kinases play essential roles in regulating GADD153 expression following DNA damage but, rather, they suggest that some other serine-threonine kinase is likely to be involved. The target for this unidentified serine-threonine kinase is likely to be one or more transcription factors which influence GADD153 transcription. The observation that okadaic acid induced GADD153 expression but did not further enhance gene expression upon cotreatment with MMS suggests that a similar, or the same, kinase plays a role in both of these induction pathways. Interestingly, okadaic acid also induces expression of the c-jun, c-fos, and EGR1 genes, both by increasing their transcription through the AP-1 site of c-jun and the serum response elements of c-fos and EGR1 and by increasing the stability of their mRNAs (29-31).

In concordance with its differential regulation by kinases, GADD153 differs from the majority of DNA damage-inducible genes in several other respects. First, it is not responsive to phorbol ester stimulation in HeLa cells (as well as most other cell types), despite the presence of a conserved AP-1 element in both the human and hamster gene promoter regions (15). Second, GADD153 expression is more frequently associated with growth-inhibitory stimuli (11), whereas most DNA damage-inducible genes are responsive to proliferative stimuli (1, 2). Third, the GADD153 response to DNA damage is somewhat selective, in that the gene is not induced by X-radiation or X-ray-mimetic agents, although most other forms of DNA damage cause increased GADD153 expression (13). This selective responsiveness is not altogether unique, because the long terminal repeat of the human immunodeficiency virus is inducible only by UV and UV-mimetic agents and not by alkylating agents or X-ray mimetic agents (32). Finally, although DNA-damaging treatments increase binding to the GADD153 AP-1 regulatory element and we have provided evidence to suggest that this element contributes to the response, it is certainly not the sole critical element mediating activation of GADD153 in response to DNA damage (1, 33). This is in sharp contrast to findings with the collagenase gene, where the AP-1 element is required and crucial for both UV and TPA responsiveness (3).

In Fig. 6, we have depicted the possible signaling pathways involved in the mammalian DNA damage response as they have thus far been described. We emphasize that the induction of GADD153 by DNA damage occurs via a pathway distinct from those described for c-jun and collagenase (8, 9). We have also included the GADD45 gene induction in response to most DNA-damaging agents along the same pathway as GADD153, since its responsiveness to different types of DNA damage is similar, in that it lacks PKC and tyrosine kinase involvement (20). The exception is the induction of GADD45 by X-irradiation, which has been described as occurring through a unique pathway mediated via the tumor suppressor gene product p53 (34). p53 is activated following exposure of cells to ionizing radiation and

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has been shown to specifically bind to a sequence in the first intron of the GADD45 gene, resulting in its induction (34).

As represented in Fig. 6, there is also considerable overlap between the responsiveness of many genes to DNA damage and to oxidative stress. We do not feel that it is clear at this point that these inducers always follow identical pathways or that these pathways are mutually exclusive. However, this issue of overlapping signals underlies an important question: what initiates the DNA damage response? Is it the immediate oxidative stress caused by the DNA-damaging agents, as suggested by Devary et al. (8)? Is it the DNA damage itself acting synergistically with oxidative damage? Or are these distinct pathways which occur simultaneously and merge, perhaps at the point where the phorbol ester signals also merge, with the requirement for Raf-1 kinase?

This issue with respect to GADD153 is further complicated when all of the evidence is weighed. For example, GADD153 expression is induced by numerous carcinogenic and oxidative stresses which include, in addition to UV and MMS treatments, DNA-cross-linking agents, DNA intercalators, topoisomerase inhibitors, cadmium chloride, and hydrogen peroxide (13). Furthermore, with respect to DNA-damaging agents, the magnitude of GADD153 induction appears to correlate with the number of DNA lesions, seemingly being dependent on both the dose of the agent and the repair capacity of the cells. In cells from patients with xeroderma pigmentosum, which cannot repair UV lesions, a much lower dose of UV can induce maximal CADD153 expression than in repair-proficient cells, presumably due to an accumulation of unrepaired DNA lesions (13). However, the dose-response curves are essentially the same when the incurred DNA damage is the type which is repaired proficiently in both cell types, i.e., DNA alkylation (13). In more recent studies, we have examined the induction of GADD153 in HeLa cells, which are resistant to cisplatin damage (35). In the resistant cells, the magnitude of GADD153 induction is significantly lower than the induction seen in the wild-type parental cells from which they were derived. With regard to the role of oxidative stress in mediating the response to treatment with genotoxic agents, the observation that NAC fails to block MMS-induced expression of GADD153 but successfully blocks the cadmium chloride induction suggests that the early events leading to GADD153 activation in response to these agents are distinct. The fact that NAC does block UV-induced activation of c-jun transcription further emphasizes the complexity of the pathways mediating the mammalian genetic response to DNA damage, which differ depending on the gene, the damaging agent, and the cell type.

References


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