Physical and Functional Association between GADD153 and CCAAT/Enhancer-binding Protein β during Cellular Stress*

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GADD153, a ubiquitously expressed member of the CCAAT/enhancer-binding protein (C/EBP) family is induced by a wide variety of growth-arresting and DNA-damaging agents. Functionally, GADD153 has been postulated to act as a dominant-negative regulator of C/EBPs. Therefore we sought to gain evidence for interactions between GADD153 and other C/EBPs during cellular responses to stress. In this report we have demonstrated that treatment of rat pheochromocytoma PC12 cells with sodium arsenite leads to enhanced expression of C/EBP-β and GADD153 (growth arrest and DNA damage inducible gene 153) but not other C/EBPs. Coimmunoprecipitation experiments provided evidence for the formation of endogenous GADD153-C/EBP-β complexes in arsenite-treated cells. Additional experiments were performed to determine the role of such complexes in regulating GADD153 expression. Previous studies in our laboratory demonstrated that the GADD153 promoter contains a C/EBP binding site through which other C/EBPs interact to transactivate GADD153 expression in liver hepatoma cells. Here, we demonstrate that extracts prepared from arsenite-treated PC12 cells likewise show increased amounts of factors capable of binding to the GADD153-C/EBP site and that these complexes are comprised at least in part of C/EBP-β. Forced expression of C/EBP-β was found to be capable of transactivating the GADD153 promoter in PC12 cells cotransfected with plasmids expressing a GADD153 reporter gene and C/EBP-β protein. However, overexpression of GADD153 inhibited the transactivation of the GADD153 promoter by C/EBP-β. These findings provide evidence for an autoregulatory loop in which stress-induced GADD153 feeds back to attenuate GADD153 expression during the cellular response to stress.
total RNA was isolated and analyzed for
mRNA expression by Northern blot analysis. Panels A, representative Northern blot probed consecutively with the indicated probes. Panels B and C, quantitative analysis of GADD153 and C/EBP-β mRNA levels, respectively, following normalization with 18S rRNA. Fold induction refers to the increase in mRNA levels relative to untreated controls (time 0).

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment Conditions—PC12 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 5% horse serum (Life Technologies, Inc.), and 50 μg/ml gentamicin. Cells were maintained in a humidified atmosphere containing 10% CO₂ in air. For arsenite treatment, cells were seeded at a density of 1 × 10⁶ cells/100-cm² dish 48 h prior to treatment. Sodium arsenite (Sigma) was added to the culture medium to a final concentration of 50 μM sodium arsenite. At the indicated times (h), cells were lysed, and total RNA was isolated and analyzed for GADD153 and C/EBP-β mRNA expression by Northern blot analysis. Panel A, representative Northern blot probed consecutively with the indicated probes. Panels B and C, quantitative analysis of GADD153 and C/EBP-β mRNA levels, respectively, following normalization with 18S rRNA. Fold induction refers to the increase in mRNA levels relative to untreated controls (time 0).

For coimmunoprecipitation experiments the cell lysates were preincubated with 1 h incubation with rabbit normal serum and protein A-Sepharose (Sigma) at 4°C. Antibodies specific to GADD153, C/EBP-β, or C/EBP-β (Santa Cruz Biotechnology) were added to the reaction mixture along with protein and labeled DNA Mobility Shift Assays—DNA Mobility Shift Assays—DNA Mobility Shift Assays were used to detect the presence of GADD153 protein coprecipitated with C/EBP-β or C/EBP-β during the immunoprecipitation step. Immune complexes were detected using enhanced chemiluminescence (Amersham Corp.). For detection of GADD153-C/EBP-β protein complexes shown in Fig. 3B, PC12 cells were metabolically labeled. Cells were incubated in medium with or without 400 μM sodium arsenite for 30 min, after which the medium was removed, and fresh medium minus methionine was added along with 300 μCi of [35S]methionine for 6 h at 37°C. Following immunoprecipitation using a GADD153 antibody, the samples were electrophoresed, transferred to polyvinylidene difluoride membrane, and exposed to autoradiography to detect the presence of GADD153 in the immunoprecipitates. Western analysis using a C/EBP-β antibody was then performed on the same membrane to detect the presence of C/EBP-β in the coimmunoprecipitation complexes.

DNA Mobility Shift Assays—Nuclear extracts were prepared from arsenite-treated cells, and DNA binding reactions were carried out as described previously (21). Briefly, 5 μg of nuclear extract was incubated for 30 min on ice with 10 fmol of double-stranded 32P-labeled oligonucleotide. Reactions were electrophoresed through 4% polyacrylamide gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and the ethidium bromide-stained RNA following transfer. Hybridization signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Following analysis of the GADD153 and C/EBP signals, blots were rehybridized to an end-labeled 24-base oligonucleotide complementary to 18S RNA (3), and the C/EBP signals were normalized to 18S rRNA obtained on the same blot to control for variation in loading and transfer among samples.

Protein Isolation and Analysis—Lysates from untreated or arsenite-treated cells were prepared using a buffer containing 1% Nonidet P-40 as described previously (20). The protein concentrations of the crude extracts were determined by the Bio-Rad Protein Assay Reagent.

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Interaction between GADD153 and C/EBP-β proteins in control and arsenite-treated PC12 cells. Lysates were subjected to immunoprecipitation with antibodies to either C/EBP-β, C/EBP-α, or GADD153 proteins as indicated in the rows marked IP. The immunoprecipitated complexes were subjected to Western blot analysis using antibodies indicated in the rows marked WB. Panel A, PC12 cells were treated with 50 μM arsenite for 0 or 8 h, after which cells were harvested, lysed, and immunoprecipitated. Panel B, PC12 cells were metabolically labeled following a 30-min exposure to either 0 or 400 μM arsenite as described under “Experimental Procedures.” Following immunoprecipitation, electrophoresis, and transfer to a membrane, the blot was probed with a C/EBP-β antibody. The arrows indicate the positions of C/EBP-β, GADD153 (also marked with an asterisk), and C/EBP-α. The mobility of molecular mass markers is indicated (in kDa).

RESULTS

Induction of GADD153 and C/EBP-β mRNAs by Arsenite—We have shown recently that treatment of HeLa cells with arsenite, a severe metabolic stress with genotoxic effects, results in the rapid induction of GADD153 mRNA (22). As shown in Fig. 1, A and B, GADD153 mRNA is also highly induced in PC12 cells following treatment with 50 μM sodium arsenite. The biphasic pattern of induction seen here is consistent with that observed in HeLa cells. To determine the effects of arsenite treatment on the expression of other C/EBP family members, the same RNA samples were examined for expression of the other C/EBP family members. As expected, based on its known tissue-restricted expression, no C/EBP-α mRNA expression was detected in PC12 cells (data not shown). Similarly, no C/EBP-β induction was apparent in these cells. However, C/EBP-β mRNA was detected readily even in untreated cells, and its expression was elevated further in response to arsenite treatment (Fig. 1, A and C). It is worth noting that while C/EBP-β mRNA levels begin to decline after 6 h of treatment, this is not reflected in C/EBP-β protein levels, which remain high for the duration of the arsenite treatment (Fig. 2). It is possible that differences in mRNA stability or protein turnover rates account for such differences.

GADD153 and C/EBP-β Protein Expression in Arsenite-Treated Cells—Western blot analysis was used to determine whether induction of GADD153 and C/EBP-β mRNAs was associated with increased amounts of the respective proteins. As seen in Fig. 2, arsenite treatment of PC12 cells resulted in a time-dependent increase in both GADD153 and C/EBP-β proteins. C/EBP-β was present as a doublet, consistent with previous observations by others in PC12 cells (23). In contrast to that seen for GADD153 and C/EBP-β, C/EBP-δ protein expression was low and did not change in response to arsenite treatment. These findings are consistent with the lack of C/EBP-δ mRNA expression noted above.

GADD153 and C/EBP-β Proteins Interact In Vivo—Experiments employing either recombinant proteins in vitro or vectors overexpressing the proteins in transiently transfected cells
have provided evidence that the GADD153 and C/EBP proteins can form dimers (14). Whether such interactions occur between endogenous proteins in vivo has not been demonstrated. Since both GADD153 and C/EBP-β show increased expression in response to arsenite treatment, we sought evidence for their functional interaction in vivo. To examine this possibility, two-stage experiments were performed in which C/EBP-β, C/EBP-δ, and GADD153 proteins were first immunoprecipitated from control (0 h) and arsenite-treated (8 h) cells under mild non-denaturing conditions, after which the immunoprecipitates were analyzed for the presence of GADD153 protein by Western analysis with the same antibody used in the IP experiment. As shown, immunoprecipitation of each of the three proteins, C/EBP-β, C/EBP-δ, and GADD153, with their respective antibodies followed by Western analysis with the same antibody resulted in the expected size bands (lanes 1–3 and 6), verifying the specificity of the various antibodies and successful immunoprecipitation of each protein. Although Western analysis of C/EBP-β immunoprecipitates with an anti-GADD153 antibody showed little or no GADD153 protein in control cells (lane 4), a significant amount of GADD153 protein coprecipitated with C/EBP-β in 8-h arsenite-treated cells (lane 7). Similar experiments with C/EBP-δ immunoprecipitates showed no evidence of GADD153 protein in either control or arsenite-treated cells (lanes 5 and 8).

To confirm the specificity of the GADD153-C/EBP-β interactions, the inverse experiment was performed. GADD153 antibody immunoprecipitates were analyzed for the presence of C/EBP-β by Western analysis in both untreated and arsenite-treated cellular extracts (Fig. 3B). Immunoprecipitation using a GADD153 antibody revealed a novel band of 29 kDa which corresponded to the molecular mass of GADD153. This band was seen only in extracts prepared from arsenite-treated cells (lanes 9 and 10). Western analysis of the same GADD153 immunoprecipitates using antibodies against C/EBP-β resulted in detection of C/EBP-β only in lanes in which GADD153 was also detected (lanes 11 and 12). These results are consistent with the results shown in Fig. 3A and lend further support for GADD153-C/EBP-β interactions in response to arsenite treatment.

C/EBP-β Binds to the GADD153 Promoter—We previously identified a functional C/EBP binding site in the GADD153 promoter and demonstrated that this site is responsible for the transactivation of a GADD153-CAT reporter construct by C/EBPs in human hepatoma HepG2 cells (24). Since C/EBP-β and GADD153 are induced by arsenite in PC12 cells, it was of interest to determine whether, in this cell type, C/EBP-β would bind to this same promoter region and thereby contribute to the activation of GADD153. Nuclear extracts isolated at various times after arsenite treatment were analyzed for binding activity to a radiolabeled oligonucleotide encompassing the GADD153-C/EBP site (Fig. 4A). A time-dependent increase in protein binding to the GADD153-C/EBP oligonucleotide was observed with two regions of binding activity. A faster migrating band (labeled A) was maximum in abundance 2 h after arsenite treatment but decreased in activity thereafter. The second, slower migrating band (labeled B) increased at later time points (maximum at 6 h). A third band (labeled C) was present in both treated and untreated extracts. As shown in Fig. 4B, binding activity associated with all bands was competed out by the addition of excess cold (unlabeled) GADD153-C/EBP oligonucleotide (self), but not by the addition of oligonucleotides containing binding sites for unrelated transcription factors (NFκB or SP-1).

Supershift analysis was used to determine the presence of C/EBP-β and GADD153 proteins in the DNA-binding complexes of nuclear extracts prepared from cells treated with arsenite for either 2 or 6 h (Fig. 4C). Extracts preincubated with labeled probe were incubated further with nothing (−), preimmune (normal) serum (NS), or antibodies specific to...
C/EBP-β (β) or GADD153 prior to gel loading (Fig. 4C). Lanes containing anti-C/EBP-β antibody showed the appearance of a novel band or supershift, indicating that C/EBP-β contributes to the DNA-binding complexes seen with extracts from arsenite-treated cells. The absence of GADD153 in the DNA-binding complexes, as evidenced by the lack of a supershift, is consistent with the notion that GADD153 contains a nonfunctional DNA binding domain. Similar analysis using antibodies specific for C/EBP-α and C/EBP-β also showed no evidence of a shift in the DNA binding pattern, indicating that these proteins do not contribute to the DNA-binding complexes (data not shown).

Transactivation of the GADD153 Promoter by C/EBP-β and Its Inhibition by Overexpression of GADD153—The ability of C/EBP-β to transactivate the GADD153 promoter in PC12 cells is shown in Fig. 5A. Cotransfection of a GADD153-promoter-luciferase reporter construct (GADD153-LUC) with increasing amounts of a plasmid that constitutively expresses high levels of C/EBP-β resulted in a dose-dependent increase in luciferase activity. Next, we examined whether overexpression of GADD153 protein could block GADD153 promoter transactivation by C/EBP-β. For this experiment, 1 µg of GADD153-LUC reporter construct was transfected into PC12 cells along with 3 µg of C/EBP-β expression vector and varying amounts of expression vectors containing the GADD153 CDNA sequence in either the sense (solid bars) or antisense (hatched bars) orientation. As shown in Fig. 5B, transfection of cells with the plasmid overexpressing GADD153 in the sense orientation prevented transactivation of the GADD153 promoter by C/EBP-β, whereas the antisense expressing vector was without effect. Taken together, these findings suggest that GADD153 is itself a C/EBP-regulated gene and that expression of GADD153 protein serves to feedback and suppress transactivation of GADD153.

DISCUSSION

Although in vitro studies with purified proteins and in vivo experiments using vectors overexpressing GADD153 and C/EBP-β indicated that these proteins could in fact form heterodimers, prior to this report no studies had shown evidence for an interaction between endogenous C/EBP-β and GADD153. In this study we provide evidence for such an interaction. Furthermore, we demonstrate that GADD153-C/EBP-β heterocomplexes increase in arsenite-treated cells. Since both C/EBP-β and GADD153 are ubiquitously expressed and are up-regulated in response to numerous treatments, C/EBP-β and GADD153 heterodimers are likely to function not only in regulating gene expression in response to arsenite, but also during other conditions of stress.

We had shown previously that GADD153 expression increases in rat liver during the acute phase response following lipopolysaccharide injection (24). We suggested that in liver, GADD153 was itself a C/EBP-regulated gene and perhaps was involved in the attenuation of the acute phase response through its interference with C/EBP activity. Our findings presented here are consistent with this view and further extend the role of GADD153-C/EBP interactions to other conditions of stress, as well as to other cell types.

Based on our current knowledge, we propose the model depicted in Fig. 6 in which GADD153 protein plays a role both in self-regulating GADD153 expression as well as in the attenuation of other C/EBP-β-regulated genes. According to the proposed model, in the absence of stress both C/EBP-β and GADD153 protein levels are relatively low, although levels of C/EBP-β are higher than GADD153. Following stress, C/EBP-β DNA binding activity increases as a result of posttranslational modification of preexisting C/EBP-β as well as from increased levels of new C/EBP-β protein. C/EBP-β then acts to activate GADD153 transcriptionally (as well as other C/EBP-β-regulated genes). As GADD153 protein levels increase, GADD153 and C/EBP-β form heterodimers, resulting in a relative decline in C/EBP-β dimers capable of binding to the promoter. Therefore, it is the ratio of GADD153 and C/EBP-β proteins which determines whether transcriptional activity will be enhanced or suppressed. Although it is presented in a simplistic form in Fig. 6, this model does not preclude the interaction of C/EBP-β with other regulatory proteins. Furthermore, it is important to note that although neither our current nor our previous studies on the acute phase response have provided evidence to suggest any interaction between GADD153 and C/EBP-β, these do not preclude the possibility for such interactions in other cell types or in response to other stresses where C/EBP-β is highly expressed. Finally, it is also possible that, like other C/EBPs, GADD153 interacts with other classes of leucine zipper proteins to modify their DNA binding activity, and such interactions could have either an inhibitory or stimulatory (through relief of repression) effect on gene transcription. Whatever the outcome, the GADD153 protein, through its interaction with C/EBPs, is likely to participate in the regulation of gene expression during the cellular response to a variety of stressful conditions.

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