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GADD34 Induces p53 Phosphorylation and p21/WAF1 Transcription

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Abstract Recently, others and we have shown that one of the functions of GADD34 is a recovery from a shutoff of protein synthesis induced by endoplasmic reticulum stress. GADD34 has been shown to induce growth arrest and apoptosis. Main protein of apoptosis is p53, especially phosphorylation of p53. And one of the main proteins of growth arrest is p21/WAF1. Here we analyzed the effects of GADD34 on p53 phosphorylation and p21/WAF1 transcription. Transfected Myc-tagged p53 was dose-dependently phosphorylated at Ser15 by increasing the amount of GADD34. Transfection of GADD34 also induced the endogenous phosphorylation of p53 and enhanced p21 protein expression. Transfection of GADD34 induced p21/WAF1 promoter activity. This activity was dependent on p53, because GADD34 transfection to p53-deficient cells produced only a slight increase of p21/WAF1 promoter activity. The p21/WAF1 promoter activity was greatly enhanced by the transfection of p53. Both GADD34 and p53 transfection induced much higher p21/WAF1 promoter activity. The promoter activity of p21/WAF1 was very low in GADD34 deficient MEF. The transfection of GADD34 increased the p21/WAF1 promoter activity in GADD34 deficient MEF. J. Cell. Biochem. 90: 1242–1249, 2003.

Key words: GADD34; p53; p21/WAF1

GADD34 is a member of the protein family whose expression is up regulated by growth arrest and DNA damage [Zhan et al., 1994]. GADD34, like GADD45 and GADD153, was originally discovered as an UV-inducible transcript in Chinese hamster ovary cells [Fornace et al., 1989]. A later study demonstrated a correlation between the onset of apoptosis and GADD34 expression in selected cell lines following ionizing irradiation or treatment with the alkylating agent methyl methanesulfate [Hollander et al., 1997]. GADD34 is also induced by amino acid deprivation and several endoplasmic reticulum (ER) stresses [Mengesdorf et al., 2001; Novoa et al., 2001]. GADD34 harbors a highly conserved domain in its carboxyl-terminus, which is homologous to $\gamma_1$34.5 of HSV1. $\gamma_1$34.5 is a virulence factor that blocks the premature shutoff of protein synthesis in HSV1-infected neuroblastoma cells [Chin et al., 1997]. The carboxyl-terminal domain of the $\gamma_1$34.5 protein binds to protein phosphatase 1 (PP1). This complex specifically dephosphorylates eukaryotic translation initiation factor 2 α (eIF2α), which is evolved to preclude a shutoff of protein synthesis [He et al., 1998]. These findings suggest two possible function of cellular GADD34. One is related to apoptosis or cell cycle arrest. Another is related to shutoff of protein synthesis. Recently, we [Kojima et al., 2003] and other group [Novoa et al., 2003] have shown the latter function of GADD34 in vivo by using GADD34 knockout mice. We have shown that in GADD34$^{-/-}$ mouse embryonic fibroblasts (MEFs), recovery from a shutoff of protein synthesis was delayed, when MEF were exposed to endoplasmic reticulum (ER) stress [Kojima et al., 2003]. Several studies have demonstrated that the onset of apoptosis

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is correlated with GADD34 expression in selected cell lines after ionizing irradiation or alkylating agent of methyl methanesulfate treatment [Adler et al., 1999; Grishin et al., 2001]. HRX leukemic fusion oncogenes, the human homologue of the *Drosophila trithorax* (*trx*) gene, binds GADD34 to negatively regulate the apoptotic response [Adler et al., 1999] The expression of GADD34 in the colorectal cancer cell line SW480 has been reported to enhance IR-induced apoptosis [Adler et al., 1999]. Several proteins have been shown to be associated with GADD34. An association with proliferating cell nuclear antigen suggests that GADD34 might inhibit proliferation [Brown et al., 1997]. We have shown that GADD34 interacts with Zfp148 (also known as BFCOL1), which might affect p21/WAF1 transcription [Hasegawa et al., 1999]. Bai and Merchant [2001] have demonstrated that Zfp148 enhances p53 transcriptional activity and prevents p53 degradation by binding to p53 via its zinc-finger domain. Overexpression of Zfp148 leads to an accumulation of p53 in the nucleus, activation of p21/WAF1 and cell cycle arrest. Recently, we have shown that Zfp148 plays an essential role in the differentiation of fetal germ cells by stimulating the phosphorylation of p53 at Ser15 [Takeuchi et al., 1997]. These results indicate that GADD34 induces p21/WAF1 transcription via p53. Here, we show that transfection of GADD34 induces the phosphorylation of p53 at Ser15, and induces p21/WAF1 transcription via p53 binding site.

MATERIALS AND METHODS

Cell Culture

BALB3T3 10(1) cells were p53-deficient mouse fibroblast cell line, which were kindly donated from Dr. Arnold J. Levine [Harvey and Levine, 1991]. NIH3T3, 293 and 10(1) cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented

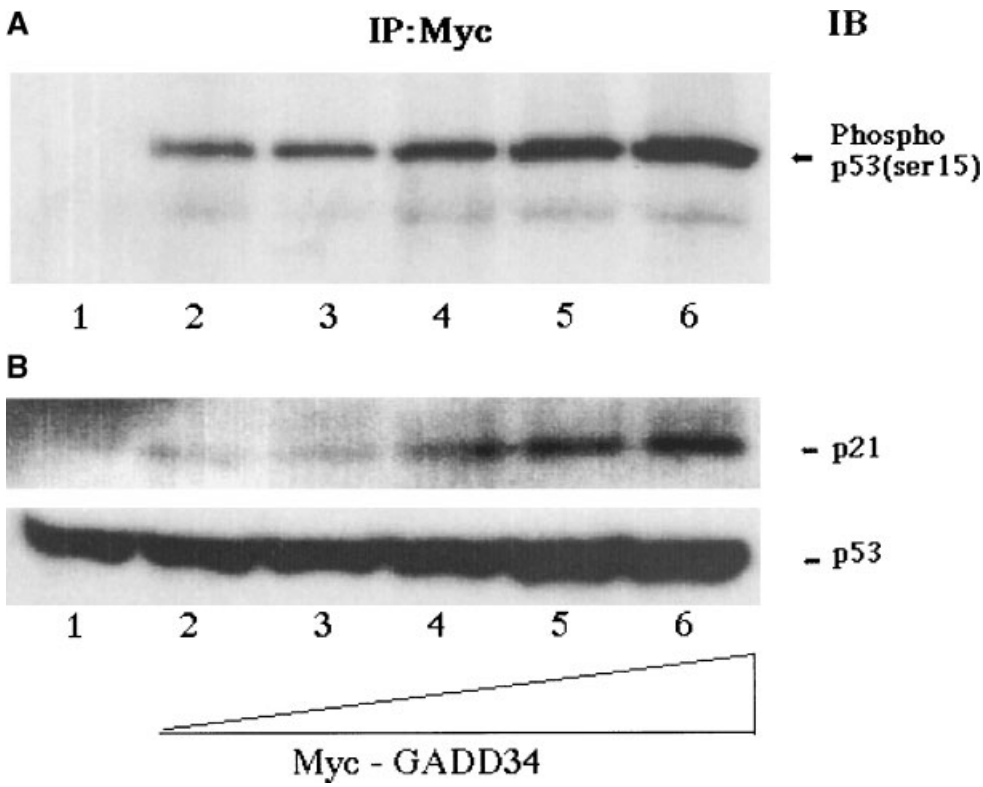

Fig. 1. Induction of p53 phosphorylation by GADD34 transfection. **A**: 293 cells were transfected with 1.0 μg of Myc-tagged vector (**lane 1**). Two hundred ninety-three cells were transfected with 0.5 μg of Myc-tagged p53 expression vector (**lanes 2–6**), together with increasing amount of Myc-tagged GADD34 expression vector (lanes 2–6; 0, 0.05, 0.1, 0.25, 0.5 μg) in six tissue culture wells. Immunoprecipitations were carried out with anti-Myc antibodies followed by immunoblot analysis with phospho-p53 (Ser 15) antibodies. **B**: Two hundred ninety-three cells were transfected with increasing amount of GADD34 expression vector (lanes 1–6; 0, 0.05, 0.1, 0.25, 0.5, 1.0 μg). Lysates were analyzed for the expression of the proteins by immunoblotting with p21/WAF1and p53 antibodies.

with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. MEFs were prepared from 14.5-day embryos of GADD34 knockout and wild-type control mice [Kojima et al., 2003]. The cultures were maintained in Dulbecco's modified essential medium with 10% FCS. Cells were plated $1-2 \times 10^6$/10 cm plate for subculture and experimentation.

Plasmid Constructs

The mouse p21/WAF1 promoter luciferase reporter plasmids and the mutant of p53-binding site (mutants 1; p21 (−1)) were constructed as described previously [Xiao et al., 1997, 1999]. Full-length cDNA of GADD34 was constructed by mRNA of NIH3T3 cells. We used RT-PCR using sense primer as GGAATTCCAGACACATGCCCCCGAGC and antisense primer as ACGCGTCGACGCCCCGCCTCCCAAG. They were cut with *Xba*I/*Hin*III and inserted into partial GADD34 expression vector [Hasegawa et al., 1999] based on pcDNA3.1/Myc-His A vector (Invitrogen, Bethesda, MA). Myc-His tagged p53 expression vector was constructed from the p53 cDNA donated from Dr. Vogelstein, We used GCTCTAGATGGAGGAGCCGCAG (sense) and CCCAAGCTTGTCTGAGTCAGGCCCTTCTGT (antisense) for PCR primers and p53 cDNA as target. The PCR product was cut with *Xba*I/*Hin*D and inserted into Myc-His tagged (Invitrogen). pTA-luc vectors were purchased from Clontech (Palo Alto, CA). The pTA-luc contains the firefly luciferase (*Luc*) gene fused to a TATA-like (TAL) promoter region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter. The pp53TA-Luc (Tal-p53) contains the firefly luciferase gene and has the p53-binding sequence fused to pTA-luc vector.

Transfection Assays

Transient transfection to 293, NIH3T3 and 10(1) cells was carried out using SuperFect reagent (Qiagen, Hilden, Germany) as previously described [Maehara et al., 2001]. In general, the day before transfection, 2×10^5 cells were plated in 24-well tissue culture plates. A total of 0.5–0.6 μg of DNA consisting of 0.45–0.55 μg of the indicated luciferase plasmid and 0.05 μg of the pRL-thymidine kinase control

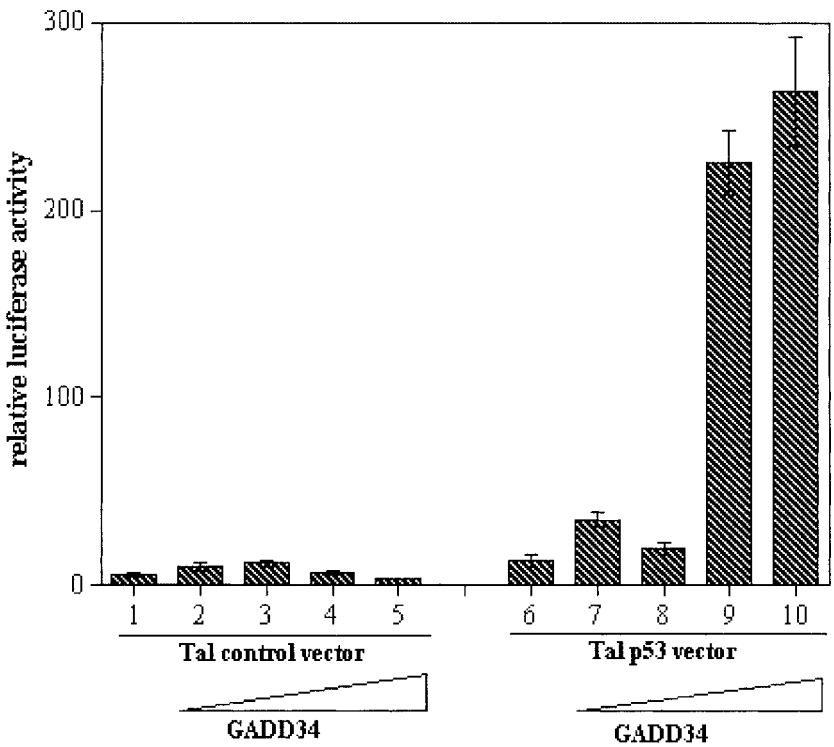

Fig. 2. Induction of Tal-p53 promoter activity by GADD34 transfection. Two hundred ninety-three cells were co-transfected with 0.05 μg of control vector (**lanes 1–5**) or 0.05 μg of Tal-p53 (**lanes 6–10**) and GADD34 expression vector (lanes 1–5 and lanes 6–10; 0, 0.05, 0.1, 0.25, 0.5 μg) in 24 tissue culture wells. Results were expressed as the mean ± standard errors (SE) of three independent experiments, each performed in triplicate.

vector (pRL-TK) (Promega, Southampton, England) per plate was used for transfection studies. After being harvested, the cells were assayed by the Dual-Luciferase Reporter Assay System (Promega), using a luminometer (Lumat; Berthold Technologies, Germany). Protein concentrations of the cell lysates were determined by the method of Bradford with the Bio-Rad protein assay dye reagent. Promoter activities were expressed as relative light units (RLU), normalized against the concentration of the protein. All transfection experiments were repeated three times.

Immunoprecipitation and Western Blotting

Human 293 cells in 6-well plates were transfected with 0.5μg each of the Myc-tagged pcDNA3.1/His-Myc (Invitrogen) expression plasmids. At 36 h after transfection, cells were dissolved in 1 ml of lysis buffer [25 mM Tris/HCl (pH 8.0) /150 mM NaCl/10% (v/v) glycerol/5 mM $MgCl_2$/2 mM EDTA/0.3% (v/v) Nonidet P40/ 5 mM NaF/0.5 mM PMSF/2 μg/ml aprotinin], and debris was discarded after centrifugation. Whole cell lysate was measured for protein quantity; 300 μg was used in the following steps: 1 μ (anti-MycSanta Cruz) of antibody was added to the lysates, which were then rotated at 4°C for 1 h. Then 20 μl of protein G/A—Sepharose beads was added and rotated for a further 2 h at 4°C. The beads were washed with lysis buffer three times and with PBS once. Proteins were eluted with SDS/PAGE sample buffer and boiled for 5 min. Western blotting was performed as described (Maehara et al., 2001) with the first antibodies, which were diluted 1:400 (anti-GADD34; Santa Cruz, CA) and 1:500 (anti-p21; Santa Cruz). Anti-p53 and anti-phospho Ser15-p53 (all rabbit polyclonal antibodies) were purchased from Cell Signaling Technology (New England Biolabs, Inc.).

RESULTS

GADD34 Induces p53 Phosphorylation

Previous studies have shown that the phosphorylation of p53 at Ser15 induces p53-dependent growth suppression [Fiscella et al., 1993].

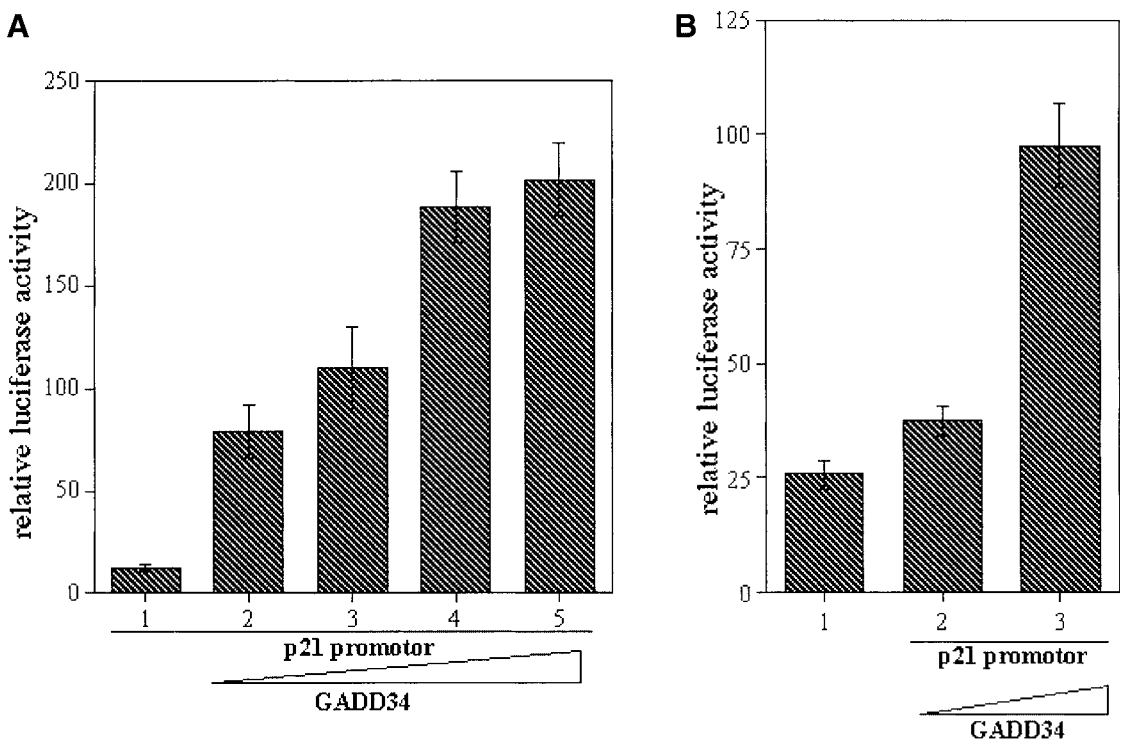

Fig. 3. Induction of p21/WAF1 promoter activity by GADD34 transfection. **A:** Two hundred ninety-three cells were co-transfected with 0.05 μg of p21/WAF1 promoter (**lanes 1–5**) and GADD34 expression vector (lanes 1–5; 0, 0.05, 0.1, 0.25, 0.5 μg) in 24-well tissue culture wells. Results were expressed as the mean ± SE of three independent experiments, each performed in triplicate. **B:** NIH3T3 cells were co-transfected with 0.05 μg of p21/WAF1 promoter (**lanes 1–3**) and GADD34 expression vector (lanes 1–3; 0, 0.05, 0.1 μg) in 24-well tissue culture wells. Results were expressed as the mean ± SE of three independent experiments, each performed in triplicate.

We first analyzed the phosphorylation of p53 induced by the transfection of GADD34 expression vector. We transfected Myc-tagged p53 together with increasing amounts of GADD34 into 293 cells. The phosphorylation of Ser15–p53 was increased by increasing the amount of GADD34 (Fig. 1A). Transfection of GADD34 also induced the endogenous phosphorylation of p53 (data not shown) and p53 protein expression (Fig. 1B).

Enhancement of Reporter Plasmid Containing p53 Responsive *Cis*-Element

Several target genes have p53-responsive *cis*-elements [El-Deiry et al., 1993; Thut et al., 1997; Zhao et al., 2001], such as GADD45, MDM2 and p21/WAF1. In order to examine whether GADD34 induces p53-responsive reporter plasmid, we transfected 293 cells with Tal–p53 reporter plasmid together with increasing amounts of GADD34 expression vector. The transfection of GADD34 up-regulates Tal–p53 promoter activity. (Fig. 2). Then we examined the expression of p21/WAF1, which is one of the target genes of p53 protein.

GADD34 Enhances the p21/WAF1 Promoter Activity

The expression of p21/WAF1 protein was increased by the GADD34 transfection (Fig. 1B). In order to determine whether or not p21/WAF1 promoter activity depends on GADD34, we cotransfected GADD34 expression vector with p21/WAF1 promoter. As shown in Figure 3, p21/WAF1 promoter activity was greatly enhanced by the increase of GADD34 transfection both in 293 cells (Fig. 3A) and NIH3T3 cells (Fig. 3B). Then in order to show that GADD34 induces p21/WAF1 promoter activity depends on p53, we used p53-deficient 10 (1) cells for promoter assay. Without p53 co-transfection, p21/WAF1 promoter activity was very low (Fig. 4, lane 5), and GADD34 co-transfection showed only a slight increase in promoter activity (Fig. 4,

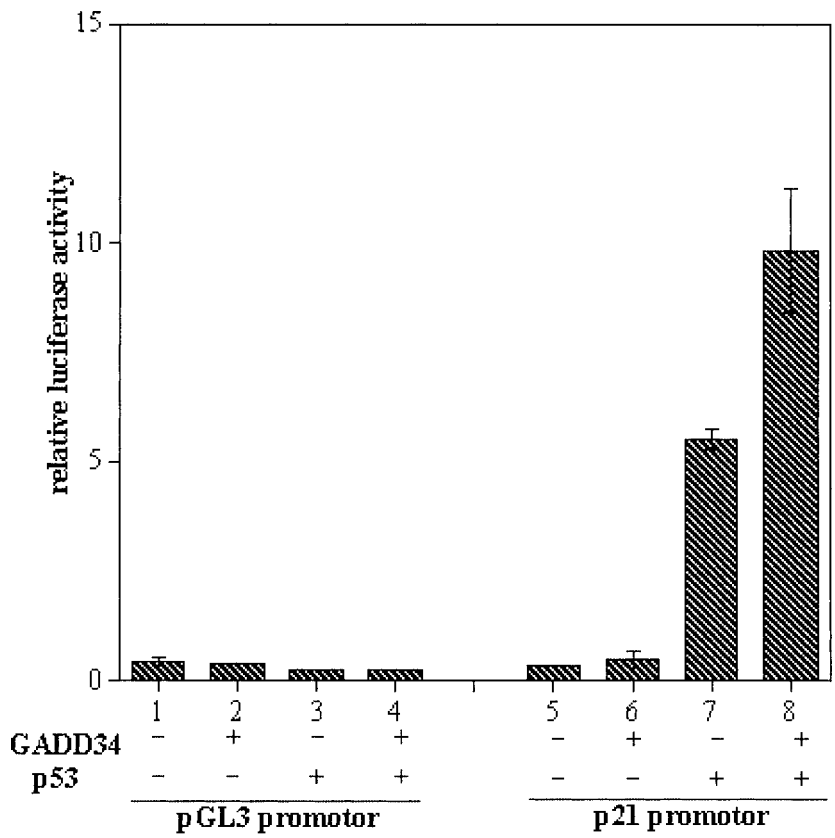

Fig. 4. GADD34-induced p21/WAF1 promoter activity in p53 deficient cells. Because p21 promoter vector was constructed in pGL3 luciferase vector, we used pGL3 vector as control. Promoter activities were measured in 10(1) cells transfected with 0.05 μg of control pGL3 promoter vector (**lanes 1–4**) or 0.05 μg of p21/WAF1 promoter vector (**lanes 5–8**) in 24 tissue culture wells. They were co-transfected with 0.25 μg of GADD34 expression vector (lanes 2, 6) , 0.25 μg of p53 expression vector (lanes 3, 7), and 0.25 μg of each GADD34 and p53 expression vector (lanes 4, 8) Results were expressed as the mean ± SE of three independent experiments, each performed in triplicate.

lane 6). However, p53 transfection greatly enhanced p21/WAF1 promoter activity (Fig. 4, lane 7). Both p53 and GAD34 co-transfection produced a further enhancement of p21/WAF1 promoter activity (Fig. 4, lane 8). To further examine the p53 dependency of GADD34-induced p21/WAF1 promoter activity, we used the mutant of one of p53-binding sites (p21 (−1)). As shown in Figure 5, enhancement of p21 (−1)/WAF1 promoter activity by the p53 transfection (lanes 7,8) was lower than that of wild type p21/WAF1 promoter activity (lanes 11,12). Finally in order to show that GADD34 really induces p21/WAF1 transcription, we used GADD34-deficient mouse embryonic fibroblasts (MEF). Without GADD34 transfection, p21/WAF1 promoter activities were very low in GADD34 deficient MEF (Fig. 6, lane 3). The p21/WAF promoter activity was increased by the transfection of full length GADD34 cDNA (Fig. 6, lane 4).

DISCUSSION

Here, we showed that GADD34 induced p53 phosphorylation and p21/WAF1 reporter activities. Because GADD34 has been cloned as one of Growth arrest and DNA damage inducible proteins, it has been suggested that GADD34 induces cell cycle arrest or apoptosis. Hollander et al. [2001] showed that in a short-term transfection assay, more than 30% of GADD34-transfected cells exhibited nuclear fragmentation by 48 h. The most critical protein of cell cycle arrest is p21/WAF1. The transcription of p21/WAF1 is mainly regulated by p53 protein. Phospo-p53 binds to p21/WAF1 binding sites. Here, we showed that GADD34 induced p53 phosphorylation and p21/WAF1 reporter activities.

Others and we have shown that GADD34 interacts with a diverse array of proteins within the cell [Adler et al., 1999; Hasegawa et al., 1999; Hasegawa et al., 1999, 2000; Connor et al., 2001; Grishin et al., 2001]. Some of these interactions facilitate growth suppression/apoptosis. Expression of GADD34 in the colorectal cancer cell line SW480 has been reported to enhance IR-induced apoptosis [Adler et al., 1999]. In addition, the results of GADD34-induced apoptosis are extended to two other cell lines, HEK293 and HeLa, by the DNA-damaging agent MMS. These data suggest that GADD34 is a positive

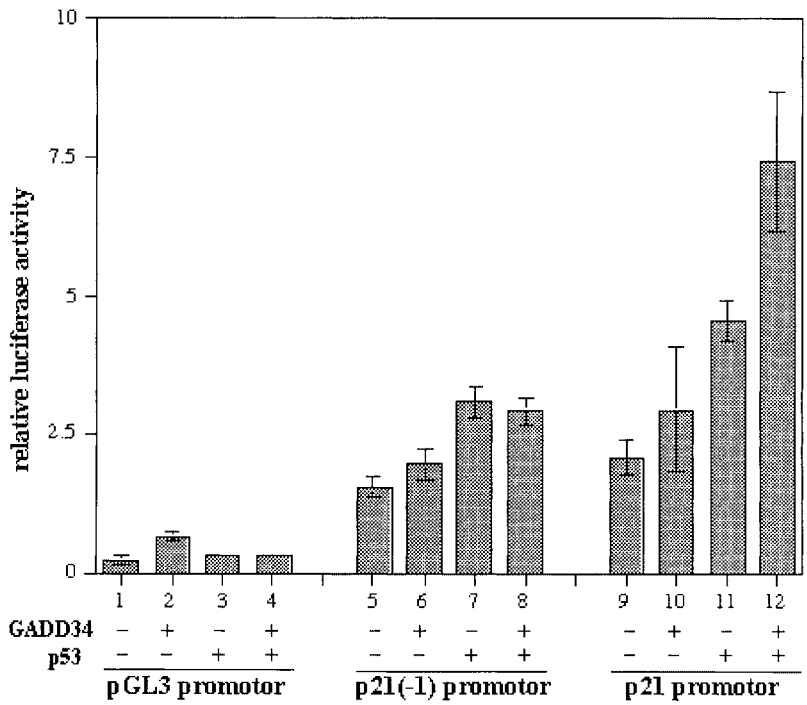

Fig. 5. Effects of p53-binding *cis*-elements in GADD34-induced p21/WAF1 promoter activity. 10(1) cells were transfected with 0.05 μg of pGL3 promoter vector (**lanes1–4**), 0.05 μg of p21(−1) promoter vector (**lanes 5–8**) or 0.05 μg of p21 promoter vector (**lanes 9–12**) in 24 tissue culture wells. They were co-transfected with 0.25 μg of GADD34 expression vector (lanes 2, 6, 10) , 0.25 μg of p53 expression vector (lanes 3, 7, 11), and 0.25 μg of each GADD34 and p53 expression vector (lanes 4, 8, 12). Results were expressed as the mean ± SE of three independent experiments, each performed in triplicate.

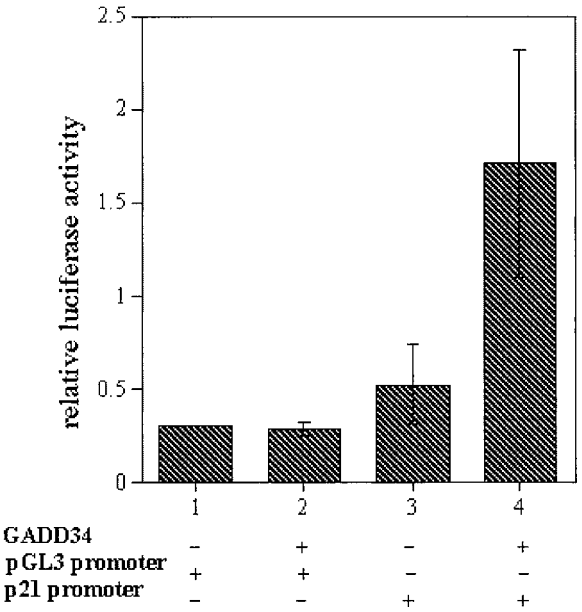

Fig. 6. Decreased p21/WAF1 promoter activity in GADD34-deficient MEF was recovered by GADD34 transfection. MEF were co-transfected with 0.05 μg of pGL3 promoter vector (**lanes 1**, **2**) or 0.05 μg of p21/WAF1 promoter (**lanes 3**, **4**), and control Myc-tagged vector (lanes 1, 3; 0.25 μg) or GADD34 expression vector (lanes 2, 4; 0.25 μg) in 24 tissue culture wells. Results were expressed as the mean ± SE of three independent experiments, each performed in triplicate.

regulator of apoptotic processes, whose activity is modulated by interaction with other proteins. It has been reported that human SNF5 protein (hSNF5/INI1) associates with GADD34, and that both proteins can coexist in a trimeric complex with chimeric leukemic HRX fusion proteins [Adler et al., 1999]. Lee et al. [2002] have shown that several subunits of the human SWI/SNF complex bind to the tumor suppressor protein p53 in vivo and in vitro. Overexpression of dominant negative forms of either hSNF5 or BRG-1 have inhibited p53-mediated cell growth suppression and apoptosis. These reports suggest that GADD34 itself induces apoptosis or cell cycle arrest. However, the molecular mechanisms or signaling cascades from GADD34 to apoptosis or cell cycle arrest have not been elucidated. Here we showed that GADD34 itself induced p53 phosphorylation and p21/WAF1 expression (Fig. 1). GADD34-induced p21/WAF1 expression was mainly dependent on p53 (Fig. 4). By using mutant of p21/WAF1 promoter, luciferase activity of p21/WAF1 was dependent on p53 binding site (data not shown). More precise biochemical mechanism of interaction between GADD34 and p53 are now ongoing.

Recently, it has been shown that human GADD34 has been induced by the differentiation and growth arrest of melanoma [Jiang et al., 2000] and glioma [Su et al., 2003]. Further adenovirus infection of melanoma differentiation-associated gene-7 (*mda-7*), which induces cell cycle arrest and apoptosis of melanoma, induces GADD34 via p38 MAPK phosphorylation [Su et al., 2003]. These works and our works presented here open the possibility to treat cancer by GADD34 induction.

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