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Characterization of a Murine Gene Encoding an Acidic-Basic Dipeptide Repeat That Interacts With GADD34

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Abstract GADD34 is one of a subset of proteins induced after DNA damage or cell growth arrest. To examine the function of GADD34, we used the yeast two-hybrid system to clone the protein that interacts with murine GADD34. We utilized as bait the partial product of GADD34 cDNA including the PEST region and the $\gamma_1$34.5. One cDNA clone was almost the same as MuRED, which encodes an acidic-basic dipeptide repeat; we named it G34BP. The interaction between GADD34 and G34BP was also confirmed in the NIH3T3 cells by in vivo two-hybrid analysis. For the binding of two proteins, the PEST region was important, and the C-terminal of G34BP was necessary. G34BP was detected in all the mouse tissues examined. Although GADD34 was significantly elevated with methyl methanesulfonate treatment, G34BP expression was not induced. Overexpression of G34BP in the NIH3T3 cells inhibited the cell growth analyzed by WST1 assay. J. Cell. Biochem. 77:596–603, 2000.

Key words: GADD34; two-hybrid analysis; cell growth; methyl methanesulfonate

Growth arrest is an important response to genotoxic stress in both eukaryotic and prokaryotic cells. DNA damage can activate a variety of cell cycle checkpoints in many eukaryotic cells and, in some cells, trigger apoptosis. In mammalian cells, the GADD (growth arrest and DNA damage-inducible) genes were originally isolated on the basis of rapid induction by ultraviolet radiation in Chinese hamster ovary (CHO) cells, but have subsequently been found to be induced by a variety of DNA-damaging agents and certain other growth-arrest treatments [Fornace et al., 1989]. In CHO cells, these genes were rapidly and coordinately induced by the alkylating agent methyl methanesulfonate (MMS), and more slowly induced by growth arrest treatment, such as medium depletion (starvation). Time-course and dose-response experiments suggest that these genes are often coordinately induced by a variety of agents.

The murine GADD34 gene is also known as the MyD116 gene [Fornace et al., 1989; Zhan et al., 1994]. MyD116 is one of several genes cloned as cDNA from murine myeloid leukemia cells induced to differentiate by interleukin 6 [Lord et al., 1990; McGeoch and Barnett, 1991]. Steady-state levels of MyD116 mRNA have been detected in terminally differentiated cells. Murine GADD34 contains 657 amino acids and consists of a large basic amino-terminal domain, a 38 amino acid sequence repeated 4.5 times (PEST) [Rogers et al., 1986], and a carboxyl terminus that can substitute for the corresponding domain of the HSV-1 $\gamma_1$34.5 [He et al., 1997].

One of the functions of GADD34 expressed after differentiation of the myeloid leukemic cells is to be involved in preventing the terminally differentiated cells from undergoing apoptosis [Chou and Roizman, 1994]. Another GADD34 function, alone or in association with GADD45, is to suppress cell division during

Abbreviations used: 3-AT, 3-amino-1,2,4-triazole; CHO, Chinese hamster ovary; eIF-2α, the subunit of the eukaryotic translation initiation factor 2; GADD, growth arrest and DNA damage-inducible; MMS, methyl methanesulfonate; PKR, double-stranded RNA-dependent protein kinase; PP1α, protein phosphatase 1α; SDS, sodium dodecylsulfate.

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DNA repair to preclude a stress response that could result in cell death [Zhan et al., 1994; Selvakumaran et al., 1994]. The other possibility is derived from the fact that the cellular GADD34 gene can complement an HSV-1 $\gamma_1$34.5 (fewer than 100 amino acids and homology region from GADD34) deletion mutant and enables the virus to grow in neuroblastoma cells [He et al., 1996]. The $\gamma_1$34.5 protein complexes with protein phosphatase 1α (PP1α) to dephosphorylate the α subunit of the eukaryotic translation initiation factor 2 (eIF2α) and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase (PKR) [He et al., 1997]. The GADD34 function thus plays a pivotal role in the viral life cycle, as it fosters the survival of infected cells and the dissemination of infectious progeny. It is thus possible that cells that exhibit growth arrest, differentiate, sustain DNA damage, or become infected with herpes simplex require a GADD34-like function to overcome a protein synthesis checkpoint guarded by cellular PKR.

However, the precise functions of the GADD34 gene and its several domains have not been established yet. In this study, we cloned the gene whose product interacts with GADD34.

MATERIALS AND METHODS

Cloning Proteins with GADD34

The yeast strain Y190 (MATa, leu2–3, 112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δgal80Δ URA3 GAL1-lacZ, LYS GAL-HIS3, cyh^r) was purchased from Clontech (Palo Alto, CA). The yeast reporter plasmid was constructed as follows. A *Sac*I-*Sal*I fragment of pAS2-1 vector (Clontech) containing the GAL4 binding domain was inserted into the *Sac*I-*Sal*I site of pRS305HIS [Hasegawa et al., 1997; Sikorski and Hieter, 1989] to generate pRS305HISpAS. An *Eco*RI-*Not*I fragment of pPC86 GADD34 was subcloned into the *Eco*RI-*Not*I site of pCITE2C vector (Novagen, Madison, WI) to make pCITE2C GADD34. The *Nco*I-*Xho*I fragment of pCITE2C GADD34 was subcloned in frame into the *Nco*I-*Sal*I site of pRS305HISpAS vector to make pRS305HISpAS-GADD34 that contained the LEU2 gene as a selectable marker. After digestion with *Cla*I, this vector was used for transformation. Yeast transformation was performed by the polyethylene glycol/lithium acetate method [Gietz et al., 1992]. Plasmid integration in the genome of yeast strains was confirmed by Southern blot analysis. Cells were then plated on a minimal synthetic dextrose plate without histidine to verify background HIS3 gene activity. One of the yeast strains that showed minimal HIS3 gene activity was selected as the strain for transformation after the initial selection. The yeast strain in which pRS305HISpAS-GADD34 was integrated was used for cDNA library transformation. cDNA libraries constructed in pPC86 vector were the same as previously described [Hasegawa et al., 1997]. cDNA plasmid (10 μg) from the libraries was transformed into the yeast strain harboring the reporter plasmid integrated into the genome and plated onto plates including 5 mM 3-amino-1,2,4-triazole (3-AT), but lacking leucine, tryptophan, and histidine. Transformation efficiency was about 1×10^5/μg cDNA plasmid. Colonies were picked after 3–5 days. Plasmid cDNAs were extracted and used for retransformation either into the same yeast strain or the yeast strain into which pRS305HISpAS plasmid had been integrated instead of pRS305HISpAS-GADD34 plasmid.

DNA Sequencing

cDNAs were subcloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. DNA sequencing was carried out using a Pharmacia (Uppsala, Sweden) ALFDNA sequencer with M13 universal and reverse sequence primers.

Cell Culture and DNA Transfections

NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Transient transfections were done by the Ca^{2+} method [Chen and Okayama, 1987].

Two-Hybrid Analysis in Mammalian Cells

GADD34 and G34BP genes were subcloned in frame into pBIND vector and pACT vector (Promega). pBIND vector construct (0.33 μg), 0.33 μg pACT vector construct, and 0.34 μg pG5luc vector (Promega, Madison, WI) were simultaneously transfected to NIH3T3 cells cultured in 24-hole plates. Forty-eight hours after transfection, cells were harvested and the amount of *Renilla* luciferase and firefly luciferase were quantitated using the Dual-Luciferase Reporter Assay System (Promega).

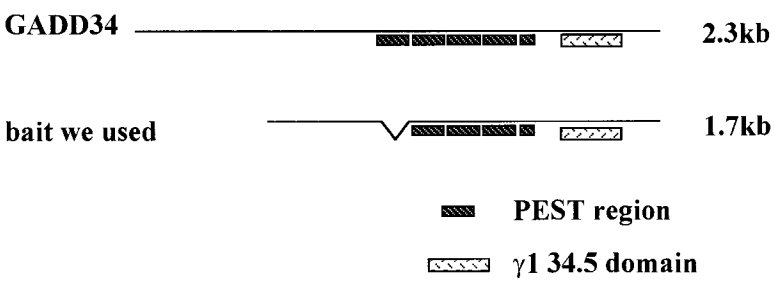

Fig. 1. Schematic representation of GADD34 used for the yeast two-hybrid screening.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from mouse tissues and cell lines as previously mentioned [Hasegawa et al., 1997]. About 20 μg of each total RNA preparation was electrophoresed on 1% agarose gels containing 1.1 M formaldehyde. The RNA was transferred to Genescreen plus membrane (NEN Life Science, Boston, MA). mRNA was detected with a ^{32}P-labeled GADD34 (random prime labeling kit, Takara (Ohtsu, Japan)) after hybridization for 18 h at 42°C in 5× SSPE, 5× Denhart's solution, 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 50 μg/ml heat-denatured salmon testis DNA, and radioactive probe. Membranes were washed twice for 15 min each at 65°C in a solution containing 2× SSC and 0.1% SDS, then once in 1× SSC with 0.1% SDS for 30 min at 65°C, and finally twice for 15 min each in 0.1% SSC with 0.1% SDS at room temperature. The membranes were then autoradiographed at −80°C using Fuji RX film. GAPDH was used as an internal control.

WST1 Assay

The G34BP was constructed in the mammalian expression vector and transfected to NIH3T3 cells by Qiagen (Hilden, Germany) transfect reagent. Forty-eight hours after transfection, WST1 assay was performed according to the manufacture's protocol (Cell Counting kit, Dojindo, Japan).

RESULTS

Cloning of cDNAs for Polypeptides that Interact With GADD34

We used as bait about 1.7 kb of the GADD34 gene containing the PEST region comprised of a 38-amino acid sequence repeated 3.5 times and the $\gamma_1$34.5 homology region to clone cDNAs for proteins interacting with GADD34 using the yeast two-hybrid system (Fig. 1) [Hasegawa et al., 1999]. This cDNA was chosen to obtain the cDNA clones that interact with not only the $\gamma_1$34.5 homology region but also the PEST region. We screened the mouse embryonic cDNA library primed with oligo-dT. In the yeast strain used for selection, plasmid pRS305pASGADD34 was integrated into the genome. In this plasmid, a GADD34 fragment was fused to the GAL4 binding domain. After screening three million independent colonies from the library, 30 histidine-positive colonies were selected. Among them, four cDNA plasmids gave positive colonies upon retransformation of the parental strain. These four cDNAs could specifically activate the HIS3 gene and β-galactosidase gene of the yeast strain containing the pRS305pASGADD34 plasmid without activating the HIS3 or β-galactosidase gene of the strain with the pRS305pAS control plasmid. Sequence analysis revealed that one clone was almost the same as MuRED [Assier et al., 1999]. Figure 2 presents the entire sequence of the cDNA we obtained. The open-reading frame starting from the first methionine in the cDNA is 1,671 nucleotides long and encodes a putative polypeptide of 557 amino acids same as MuRED. However, the cDNA we obtained had approximately 260 bp more nucleotides in the 3′-untranslated region. In addition, there were several small regions whose sequences were different. We named it G34BP and use this term throughout this article.

In Vivo Binding Experiments

To confirm that the G34BP could bind to the GADD34 in vivo other than in the yeast two-hybrid condition, we carried out an in vivo mammalian two-hybrid analysis. We transfected G34BP cDNA and its several deletion mutants inserted in the pACT vector to NIH3T3 cells together with GADD34 cDNA in the pBIND vector and pG5luc vector. Then we assayed the luciferase activities that corre-

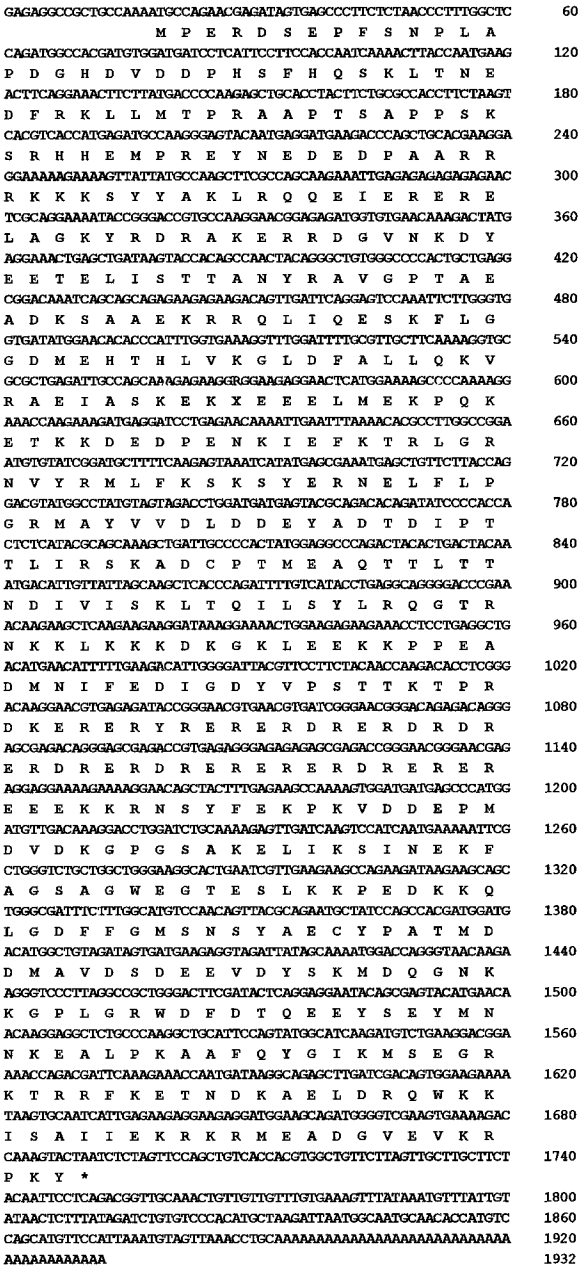

Fig. 2. Nucleotide sequences and predicted amino acid sequence of the G34BP cDNA. Numbers correspond to the DNA sequence.

spond to the binding strength by luminometer. As shown in Fig. 3A, a full-length G34BP construct showed more than 20 times the luciferase activity as the control vector. We could also confirm this result in the other deletion mutant (*Eco*RI-*Sca*I), which is lacking a 3′-untranslated region. However, we could not detect any luciferase activity over the control in another deletion mutant (*Eco*RI-*Hinc*II), which is lacking 179 amino acids in the C-terminal end; nor could we detect higher luciferase activity in the *Eco*RI-*Eco*RV mutant, which is lacking 161 more amino acids than the *Eco*RI-*Hinc*II mutant. These results suggest that G34BP could bind to the GADD34 in mammalian cells, and that the C-terminal 179 amino acid region is necessary for such binding. We then asked which regions of GADD34 are important for the interaction of the two proteins. This time we transfected GADD34 cDNA and its several deletion mutants inserted in the pBIND vector to NIH3T3 cells together with the full-length G34BP cDNA in the pACT vector. Fig. 3B shows that the luciferase activities decreased in the construct that is lacking the 5′ and 3′ end (lanes 4 and 2, respectively). We could not detect any luciferase activity over the control in the vector that contains only the $\gamma_1$34.5 region (lanes 5 and 6). However, we could detect lower luciferase activity in the construct lacking the $\gamma_1$34.5 region (lane 3). These results suggest the PEST region is necessary for binding. However, the $\gamma_1$34.5, 5′ and 3′ end regions also support binding of the two proteins.

Northern Blot Analysis

To determine the expression pattern of G34BP, a Northern hybridization experiment was performed. One major RNA transcript was identified in all tissues examined (Fig. 4). The expression was rather strong in the testis (lane 9); moderate in the thymus (lane 2), lung (lane 6), spleen (lane 6), kidney (lane 7), and colon (lane 8); and weak in the brain (lane 1), heart (lane 4), and liver (lane 5). However, we could also detect a larger minor band in the organs where major transcripts were detected. We think this additional faint upper band is probably processing intermediate. We then analyzed the mRNA levels in the MMS-treated NIH3T3 cells by Northern blot analysis. We checked the influence of MMS treatment on G34BP induction, because GADD34 mRNA was reported to be greatly induced by MMS treatment. As shown in Fig. 5, GADD34 greatly increased after 2 h (lane 4 in upper panel), and the induction continued until 8 h. However, G34BP expression was not induced in the time period examined (Fig. 5, lower panel).

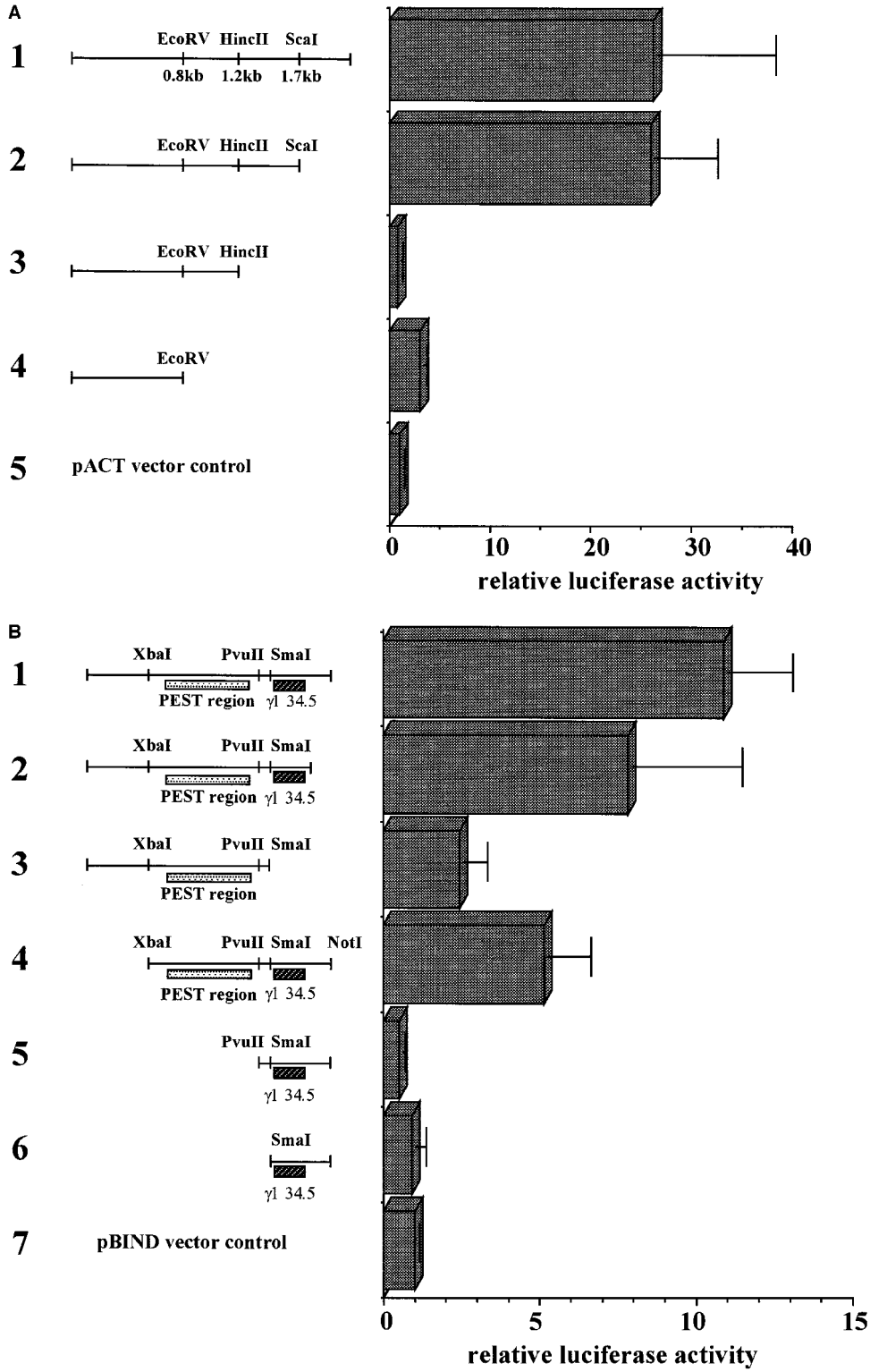

Fig. 3. Mammalian two-hybrid analysis of GADD34 and G34BP. **A:** pBINDGADD34 and the deletion constructs of pACTG34BP were cotransfected with pGluc5 vector to NIH3T3 cells by the Ca^{2+} method. Firefly luciferase activities were normalized by *Renilla* luciferase activities and compared with activities with pACT vector within the same transfection experiment. Each construct was assayed in duplicate transfection in at least three separate experiments. **B:** pACTG34BP and the deletion constructs of pBINDGADD34 were cotransfected with pGluc5 vector to NIH3T3 cells by the Ca^{2+} method.

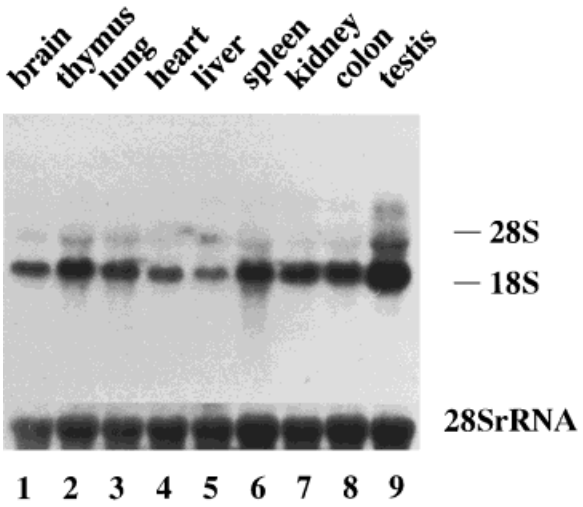

Fig. 4. Northern blot analysis. Total RNA was extracted from C57BL/6 mouse tissues, electrophoresed, blotted, and hybridized with ^{32}P-labeled G34BP cDNA probe. Human 28SrRNA probe was used as an internal RNA-loading control.

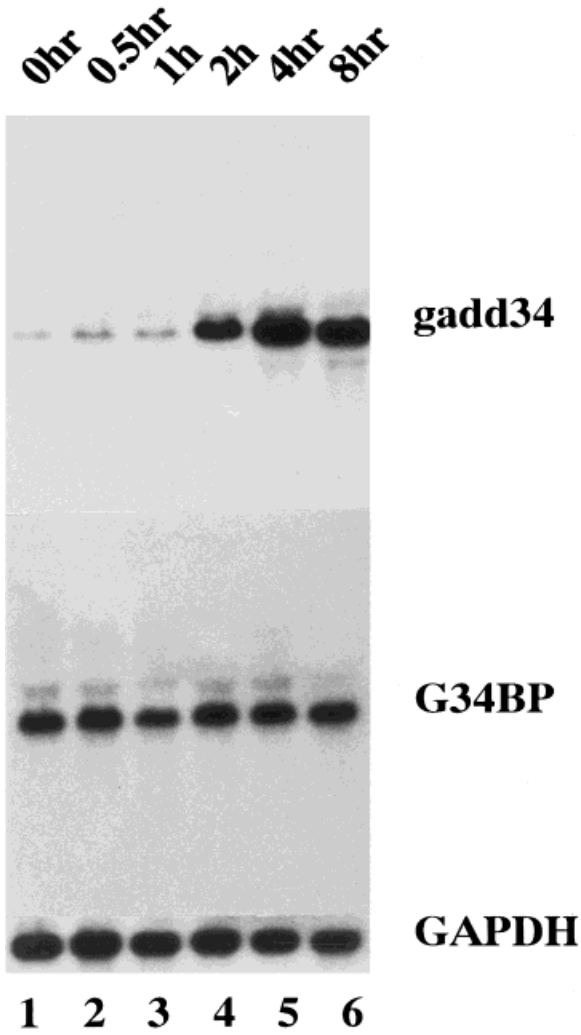

Fig. 5. Northern blot analysis. NIH3T3 cells were treated with 100 μg/ml methyl methanesulfonate for the indicated time, and total RNA was extracted and subjected to Northern blot analysis. The upper panel represents GADD34 mRNA expression, and the lower panel represents G34BP. GAPDH probe was used as an internal RNA-loading control.

WST1 Assay

Finally we checked the function of G34BP. GADD34 was reported to suppress cell growth once it is overexpressed in mammalian cells. To examine the effect of G34BP, we transfected the G34BP expression vector in the NIH3T3 cells and analyzed the cell growth by WST1 assay. We transfected the G34BP expression vector with the expression vector alone. As we show in Fig. 6, although the transfection itself inhibited cell growth slightly (compare lanes 1 and 2), cell growth measured by WST1 assay decreased by the transfection of the G34BP expression vector dose dependently. The growth was inhibited 50% as no-transfection control. These results suggest that G34BP might be involved in the cell-growth or cell-death pathway.

DISCUSSION

GADD34 was first cloned in mammalian cells as one of the GADD genes on the basis of rapid induction by ultraviolet radiation in CHO cells. Other GADD families include GADD45, GADD153, GADD7, and so on. Recently GADD45 has been reported to be the family interacting with mitogen-activated protein kinase kinase kinase and is involved in the signal transduction pathway in stress response [Takekawa and Saito 1998]. GADD153 was found to be the transcription factor CHOP [Ron and Habener 1992]. The murine GADD34 gene is also known as the MyD116 gene, but its function has yet to be completely revealed. To help us understand the function of GADD34, GADD34-interacting proteins might provide clues. We tried to clone the genes whose products could interact with the GADD34 gene product. One cDNA (described in this paper as G34BP) has almost the same nucleotide sequence as MuRED [Assier et al., 1999]. However G34BP has a nucleotide approximately 260 bp long stretching from the 3′ end of MuRED. There are some differences in the open-reading frame between MuRED and G34BP. MuRED and G34BP may derive from

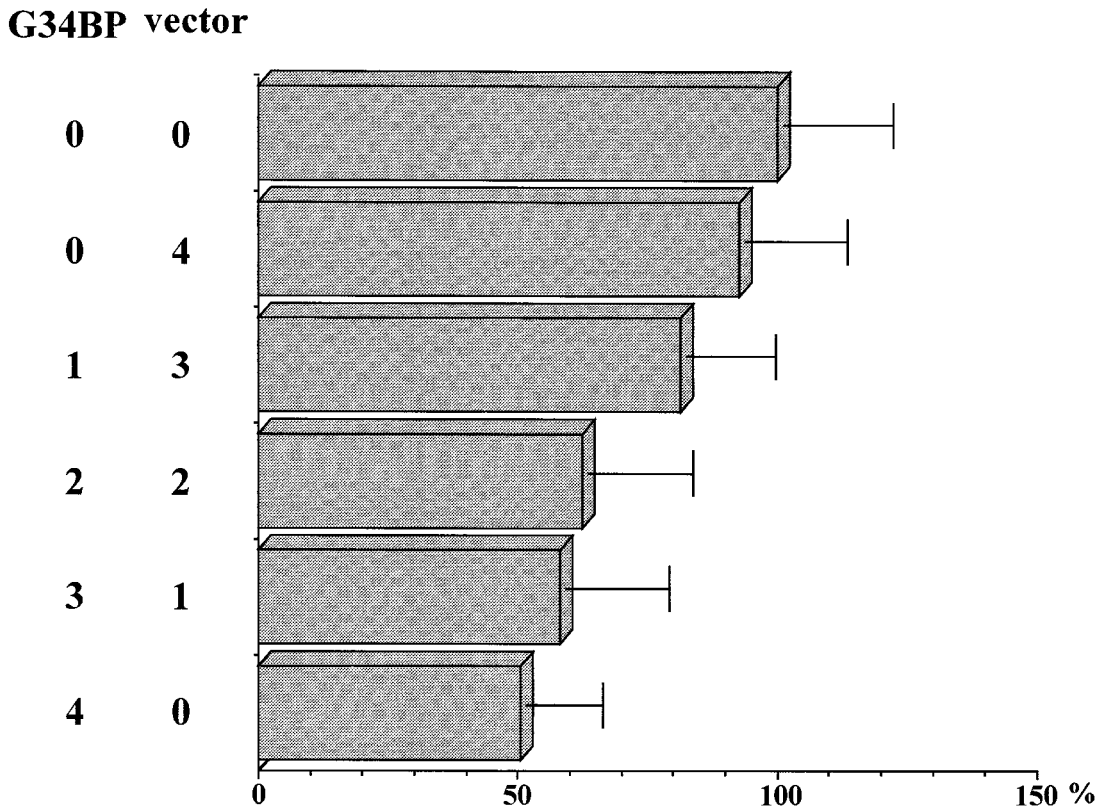

Fig. 6. WST1 assay. G34BP expression vector and control expression vector were transfected to NIH3T3 cells by Qiagen superfect reagent at indicated ratio (total 0.5 μg). Three hours after transfection, cells were washed and cultured for 40 h more. Then the WST1 assay was performed following the method provided by the manufacturer. Each construct combination was assayed in duplicate transfection in at least three separate experiments.

one genome. The possibilities of the differences between two genes are probably due to alternate splicing or strain differences, but we cannot conclude that at this moment. MuRED was cloned from the murine erythroleukemia cell lines TFP10, and G34BP was cloned from normal C57BL/6 tissue.

As mentioned earlier, the biologic role of GADD34 has not been clearly understood. The studies of the $\gamma_1$34.5 domain suggest that GADD34 is involved in the regulation of protein synthesis. Thus, the $\gamma_1$34.5 domain complexes with PP1α dephosphorylate the eIF2α and preclude the shutoff of protein synthesis by PKR. However the functions of the other regions, including the PEST region, remain unclear. In this article, we showed that although the $\gamma_1$34.5 domain is important for binding the two proteins, it is not essential to bind G34BP, because the deletion mutant lacking the $\gamma_1$34.5 domain could bind to G34BP. This means that the PEST region itself is necessary for binding of the two proteins. One can concern the issue of charge, because GADD34 is very acidic throughout much of its sequence. However the binding pattern of the same deletion mutant of GADD34 between Translin gene is different from between G34BP gene [Hasegawa and Isobe, 1999]. This suggests that the binding is rather sequence specific, not by the charge effect. Finally, we showed that the transfection of GADD34 inhibits cell growth, leading to apoptosis. This result contrasted with the earlier finding that the $\gamma_1$34.5 domain prevents the shutoff of protein synthesis and may not relate to the situation in vivo. However, one possibility is that G34BP can function in the presence of GADD34, especially PEST domain. By the stress such as MMS, GADD34 expression is upregulated. GADD34 binds to G34BP via PEST region other than $\gamma_1$34.5 domain, and G34BP can induce apoptosis.

So far we have reported that GADD34 interacts with the BFCOL1 transcription factor that may be involved in murine type I collagen promoter [Hasegawa et al., 1997] and p21 pro-

moter [Hasegawa et al., 1999]. In addition, we found that GADD34 interacts with Translin, which binds to the recombination hot spot found in malignant lymphoma [Hasegawa and Isobe, 1999]. These data suggest that GADD34 might function in transcription and/or DNA recombination. Assier et al. (1999) suggested that MuRED is localized in the nucleus and might be involved in transcription. Gel shift assay could not detect recombinant G34BP protein directly binding to the promoter sequence of collagen or p21 promoter (data not shown). However, judging from the data, it is possible that G34BP and MuRED could form a complex with GADD34 and transcription factors such as BFCOL1, and may be involved in transcription.

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