Cloning of rat *GADD45* gene and induction analysis following ionizing radiation in vivo

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Abstract A gene encoded GADD45 was isolated from rat and revealed four exons along with a p53 binding consensus sequence and a putative AP-1 site in the third intron. This suggests that the rat *GADD45* gene is also involved in the p53 signal pathway related to the G_1 cell cycle checkpoint. The rat *GADD45* mRNA was induced within 30 min in liver and increased as a function of γ-irradiation. We found that mRNA expression differed substantially in a variety of tissues (brain, liver, kidney, and spleen). The finding of in vivo induction of *GADD45* gene may provide insight into the role of *GADD45* gene in DNA repair.

Key words: Gene cloning; DNA damage inducible gene; Ionizing radiation; Induction (in vivo)

1. Introduction

To insure accurate and successful DNA replication, the pathways for DNA repair must be activated quickly in cells which have undergone significant DNA damage. In the absence of accurate and rapid DNA repair, DNA synthesis might lead to large deletions and/or translocations during the M phase of chromosome segregation [1]. Several enzymes are activated during the G1 and G2 phases in response to DNA damage. For example, the *sulA* gene, one of the RecA SOS related gene, inhibits cell growth upon DNA damage in *E. coli* [2], and the *RAD9* gene causes cell cycle arrest at G2 following X-ray irradiation in *Saccharomyces cerevisiae* [3]. Thus, these cell cycle phases function as cell cycle check points [4]. In mammals, only a few number of genes have been identified that are involved in G1 or G2 checkpoints. One such candidate gene, *GADD45* (growth arrest and DNA damage-inducible gene), was originally isolated from CHO cells after UV irradiation [5] and the expression of GADD45 was shown to be regulated by growth arrest [6] and is induced by ionizing radiation in cultured cells [7]. GADD45 protein consists of 165 amino acids, with sequence similarity to the murine *MyD118* cDNA [8].

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Abbreviations: GADD45, growth arrest and DNA damage inducible (protein); CHO, chinese hamster ovary; AT, ataxia telangiectasia; MMS, methyl methanesulfonate; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; PCNA, proliferating cell nuclear antigen.

The nucleotide sequence data reported here will be available in the GenBank/EMBL with accession number L39010.

The expression of GADD45 is regulated by p53, such that over-expression of p53 in cultured cells causes G1 arrest and prevents cells from entering the S phase [9–12]. The null mutant of p53 is unable to arrest cells at G1 after γ-irradiation, but upon transfecting the wild type p53, the G1 arrest is resumed [13]. Cells from ataxia telangiectasia (AT) patients are defective in G1 arrest and suffers massive DNA damage. In AT cells, p53 induction is not observed after γ-irradiation [14]. From these data, the expression of p53 is thought to act as a key check point in G1 arrest. p53 is a transcription factor, and forms a tetramer that binds to the specific DNA sequence (5′-RRC(A/T)(A/T)GYYYRRRC(A/T)(A/T)GYY-3′) [15,16]. The *GADD45* gene has a p53 binding consensus sequence in the third intron of the human and hamster *GADD45* genes [14,17]. Therefore, GADD45 expression seems to be regulated by p53 and GADD45 may act as a possible mediator of p53-induced G1 arrest. The roles of these p53-downstream genes of great interest because of their involvement in the G_1 cell cycle checkpoint and/or DNA repair pathway [18]. GADD45 has recently been found to be bound to theproliferating cell nuclear antigen (PCNA), one of the required components for both the Cdk–cyclin complex and DNA repair synthesis [19].

GADD45 is induced in cultured cells following UV and X-ray irradiation or by exposure to alkylating agents such as methylmethane sulfonate (MMS) [7]. We recently reported *GADD45* expression in tissues from irradiated rats [20]. Because we would like to know its mRNA expression in vivo in detail and localization of any response elements, we cloned rat genomic sequence. The deduced amino acid sequence of GADD45 is well conserved among human, mice, and hamster [7] but two divergent regions are detected when compared among species. We report here cloning of the rat *GADD45* gene and examine its mRNA induction in vivo following ionizing radiation.

2. Experimental

2.1. Animals and γ-irradiation

Two-month-old male rats (Wistar; Charles River) were used for cloning and in vivo induction experiments. γ-Irradiation was generated from Cs^{137} source gamma-ray irradiator (Gammacell 40, Atomic Energy of Canada) at 1.2 Gy per minute. Rats were irradiated with variable doses (15 to 60 Gy) of γ-rays. After 0.5 to 4.5 h for recovery, rats were sacrificed and tissues were dissected and stored at −80°C until use. Control rats were kept in the same γ-ray generator but not irradiated.

2.2. Cells and chemical treatments

Three rat cell lines, N1-S1 (Rat hepatoma; CRL-1604), Clone 9 (Normal rat liver; CRL-1439), and BRL 3A (Buffalo rat liver; CRL-1442) were obtained from American type culture collection. These cell lines were grown according to the manufacturer's description and treated with the alkylating agents, ethyl methanesulfonate (EMS) (Aldrich) and methyl methanesulfonate (MMS) (Aldrich) at concentra-

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tions from 5 μg/ml to 100 μg/ml. After 4 h incubation with chemical agents, cells were harvested and mRNA was extracted for in vitro induction experiments.

2.3. Polymerase chain reaction (PCR), cloning and sequencing

PolyA-containing RNA was extracted from the liver of irradiated and control rats. The first strand cDNA was synthesized from polyA-containing RNA using cDNA Synthesis System Plus (Amersham). The cDNA was used for the PCR reactions. Oligonucleotides were synthesized by PCR Mate (Applied Biosystems). Several conserved regions between the rat and hamster *GADD45* cDNA sequences and portions of *GADD45* genomic fragments were selected for designing PCR primers (see Table 1). PCR was performed by DNA Thermal Cycler (Perkin Elmer-Cetus) as previously described [21]. Reaction conditions for primers P1, P1′, P9, P10, P12 were 35 PCR cycles of 1 min at 94°C for denaturation, 1 min 15 s at 55°C for annealing, 3 min 15 s at 72°C for polymerization, while that for primers P2, P3, P4, P5, P6 were 1 min at 94°C for denaturation, 1 min 15 s at 46°C for annealing, 3 min 15 s at 72°C for polymerization. PCR products were gel-isolated and subcloned into pGEM-T Vector (Promega). Procedures of plasmid extraction and sequencing were as described previously [20,21]. The sequencing data were analyzed with NCBI Gen Info compact library series Re. 5.0 Entrez.

2.4. RNase protection assay and Northern blot analysis

RNase protection was performed as previously described [22,23] with a minor modification using ^{35}S-labeled *GADD45* and phosphoglycerate kinase (PGK) as a control [24]. Complementary RNA probes were generated by in vitro transcription using SP6 polymerase and [α-^{35}S]UTP (800 Ci/mmol, DuPont/NEN) and purified through gel-electrophoresis. PolyA-containing RNA (1 μg each) from control and irradiated rat liver was hybridized with the ^{35}S-labeled *GADD45* cRNA probe at 50°C in the presence of *E. coli* tRNA (10 μg) as a carrier in a 30 μl reaction mixture containing 80% formamide and 40 mM PIPES buffer (pH 6.4). After 16 h of incubation, digestion was carried out with RNase T1 (300 U/μl, BRL) and RNase A (10 mg/ml) for 30 min at 37°C. The RNase-treated samples were separated on 4% polyacrylamide gel containing 7.5 M urea, and protected RNAs were visualized by autoradiography on X-ray film or by phosphor imager (ImageQuant, Molecular Dynamics).

Northern blot analysis was performed essentially as previously described [23] using a rat *GADD45* cRNA probe. Poly(A)-containing RNA (5 μg) was separated by electrophoresis in 1% agarose-formaldehyde gel, and transferred on to a nylon membrane (GeneScreen Plus, DuPont/NEN). ^{35}S-labeled *GADD45* cRNA probe (residues 221–413, Fig. 1) was synthesized as described above and purified through a push-column (Stratagene).

3. Results

3.1. Isolation of the gene encoding rat GADD45

A series of overlapping genomic fragments were isolated by PCR using the *GADD45* primers, i.e. P1 through P12 (Table 1). Nucleotide sequence analysis of these PCR products revealed that the *GADD45* gene consists of four exons (Fig. 1). The coding region of *GADD45* mRNA starts at residue 22, and ends at residue 1603. In the third intron a p53-binding consensus sequence (GAGCGTGTCTAAGCTCAT) and a putative AP-1 site (TGAGTCA) were present. These two elements were among few conserved regions in the intron of the previously reported *GADD45* genes from other species [17].

3.2. In vivo induction of rat GADD45 *mRNA following γ-irradiation in liver*

Northern blot analysis revealed a *GADD45* mRNA band of 1.4 kb detectable at a low level in the liver of control rats, but substantially induced in irradiated animals (Fig. 2). The induction of *GADD45* mRNA became apparent even at 30 min after γ-irradiation, while the control PGK mRNA levels were unchanged. In order to examine the dose response, we performed RNase protection assays. The 420 nucleotide-long protected RNA fragment was detected in the control sample at a low level seen in Fig. 3A. The band intensity was increased in the RNA sample from 15 Gy-irradiated rats, and further induced at 60 Gy. By contrast, control PGK mRNA remained unchanged with γ-irradiation. The induction levels of *GADD45* mRNA as normalized to PGK mRNA are presented in Fig. 3B, and reveal that *GADD45* mRNA is induced nearly 10-fold at 60 Gy of γ-irradiation.

3.3. In vivo induction of GADD45 mRNA in various tissues

We examined the expression of *GADD45* mRNAs in various rat tissues (brain, liver, kidney and spleen) following γ-irradiation. Expression of *GADD45* mRNA was detected in several tissues. The greatest induction of *GADD45* mRNA by 45 Gy of γ-irradiation was observed in liver (5.5-fold) when compared with non-irradiated samples after 30 min. In brain, kidney and spleen, the induction of GADD45 mRNA was only slightly (up to 1.8-fold) increased over baseline levels after 30 min (Table 2). After 4.5 h, the induction of *GADD45* mRNA in liver was increased and substantial induction was also observed in brain by Northern blot analysis and in situ hybridization (not shown).

3.4. In vitro induction after treatment of rat cell cultures with alkylating reagents

We examined the in vitro expression of *GADD45* using rat cell lines following treatment with EMS and MMS in order to classify the response as either DNA damage inducible gene class I or II according to Fornace et al. [5]. *GADD45* mRNA

Table 1
Properties of PCR oligonucleotide primers

Primer	Sequence	Residues of GADD45	cDNA/gene	Direction
P1	CTGGCTGCGGATGAAGACGAC	386 to −409	rc	+
P1′	CTGGCTGCGATGAAGACGAC	386 to −409	rc	+
P2	CGGGAAAGTCGCTACATGGATCA	1549 to 1571	rc	−
P2′	CGGGAAAGTCGCTACATGGATCA	1549 to 1571	rc	−
P3	GGCAGGAGCAGCCCGCACGCC	−21 to −1	hc	+
P4	GAAGGAAGCTGTGTTGAAA	1784 to 1803	hc	−
P5	GCAATATGACTTTGGAGGAATT	17 to 38	rc	+
P6	TGCTGCGAGAACGACATCAACATCCTGCG	459 to 484	rc	−
P9	GCCTGAGAATGTTTTTCAGAAG	1230 to 1251	rg	−
P10	GTTTAAACGAGCCCTCTCCA	991 to 1010	rg	−
P12	AGATTAGATAAAGCCACATTA	811 to 826	rg	−

rc: rat cDNA; rg: rat gene; hc: hamster cDNA.

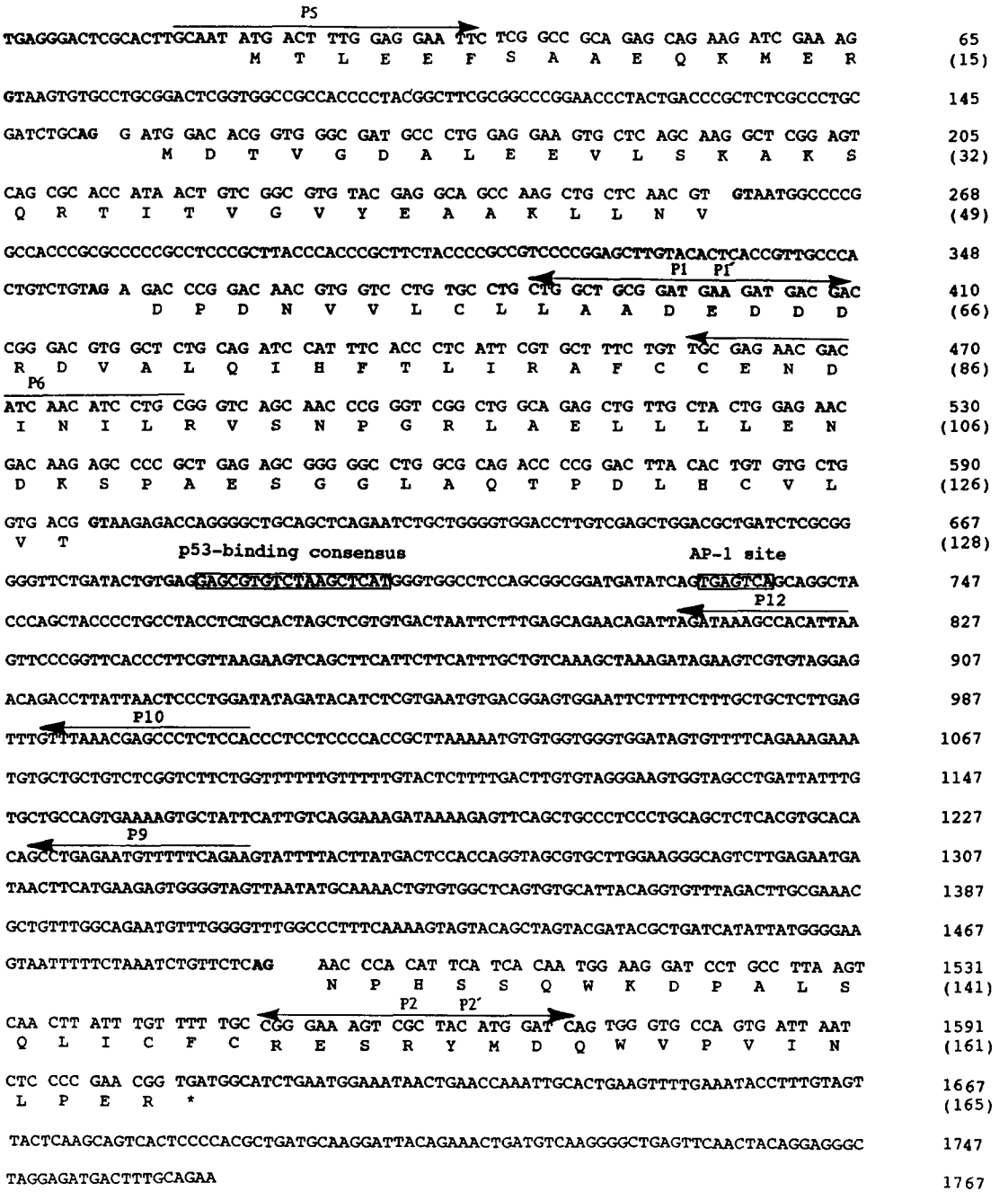

Fig. 1. Genomic structure of the rat *GADD45* gene. Four exons are shown along with deduced amino acid sequences indicated below. Splicing donor and acceptor sequences are shown by bold letters and a termination codon is indicated by asterisk. p53-binding consensus site and AP-1 site are boxed. The positions of oligoprimers are indicated by arrows.

induction after treatment with MMS was substantially greater than with EMS. *GADD45* mRNA levels increased 24.2 fold after MMS treatment (100 μg/ml), but slightly changed (1.1 times) when treated by EMS (100 μg/ml) (see Fig. 4).

3.5. Predicted structural features of GADD45

When the nucleotide sequence of *GADD45* was translated into amino acid sequences, most of substitutions at the nucleotide level were offset, and most of amino acid substitutions were in two distinct regions. Deduced amino acid sequences of the rat GADD45 was aligned with the previously reported human and hamster GADD45 sequences [7] (Fig. 5). The alignment revealed conserved regions and two clusters of divergent amino acid sequences: divergent region I (residues 9–31) and divergent region II (residues 106–119), regardless of species. Examination of possible secondary structures revealed divergent region I as α-helix permissible, and divergent region II as either random coil structure or omega-loop structure by the Garnier-Robson [25] and Chou-Fasman [26] algorisms, respectively. Constant regions are almost devoid of amino acid replacements (residue

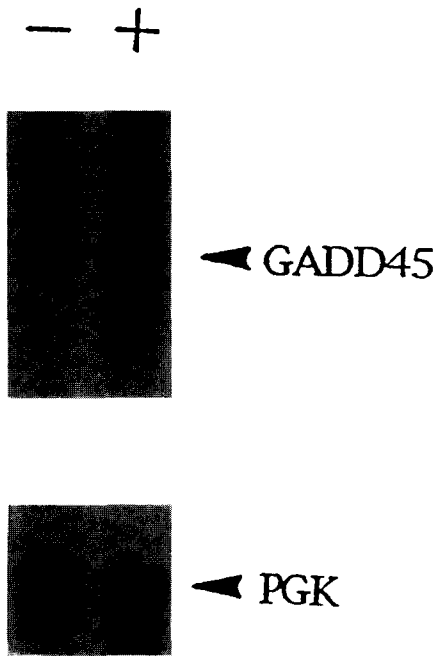

Fig. 2. *GADD45* mRNA is induced in vivo in the liver following γ-irradiation. Northern blot analysis of polyA-containing RNA from the non-irradiated (−), or γ-ray irradiated (+) rat liver 30 min following γ-irradiation. The positions of *GADD45* (1.4 kb) and PGK (1.8 kb) mRNA are indicated by arrow heads.

1–8, 32–105, 120–165). Six cystein residues (dot in Fig. 5) were present in all three species, which may conform the GADD45 protein tertiary structure.

4. Discussion

We report here the cloning of rat *GADD45* gene and the induction of *GADD45* mRNA in vivo following γ-ray irradiation. While *GADD45* mRNA is induced in several tissues in vivo (e.g. brain, kidney and spleen), it appears to be highest in liver tissues. *GADD45* mRNA induction is detected in as short as 30 min after γ-irradiation in vivo. This finding implies the *GADD45* mRNA is induced rapidly in response to DNA damage in vivo.

The exon/intron organization of the rat *GADD45* gene is essentially the same as the previously reported *GADD45* genes in human, hamster and mouse [17]. All *GADD45* genes have one p53-binding consensus sequence in the third intron. The location of this putative p53-regulatory site is in a similar location in all *GADD45* genes, suggesting that it may be involved in the downstream function of p53 and in the G1 cell cycle arrest together with other p53 mediators including the WAF-1/Cip-1/Sdi-1/p21 [27] and RB genes [28]. Although GADD45

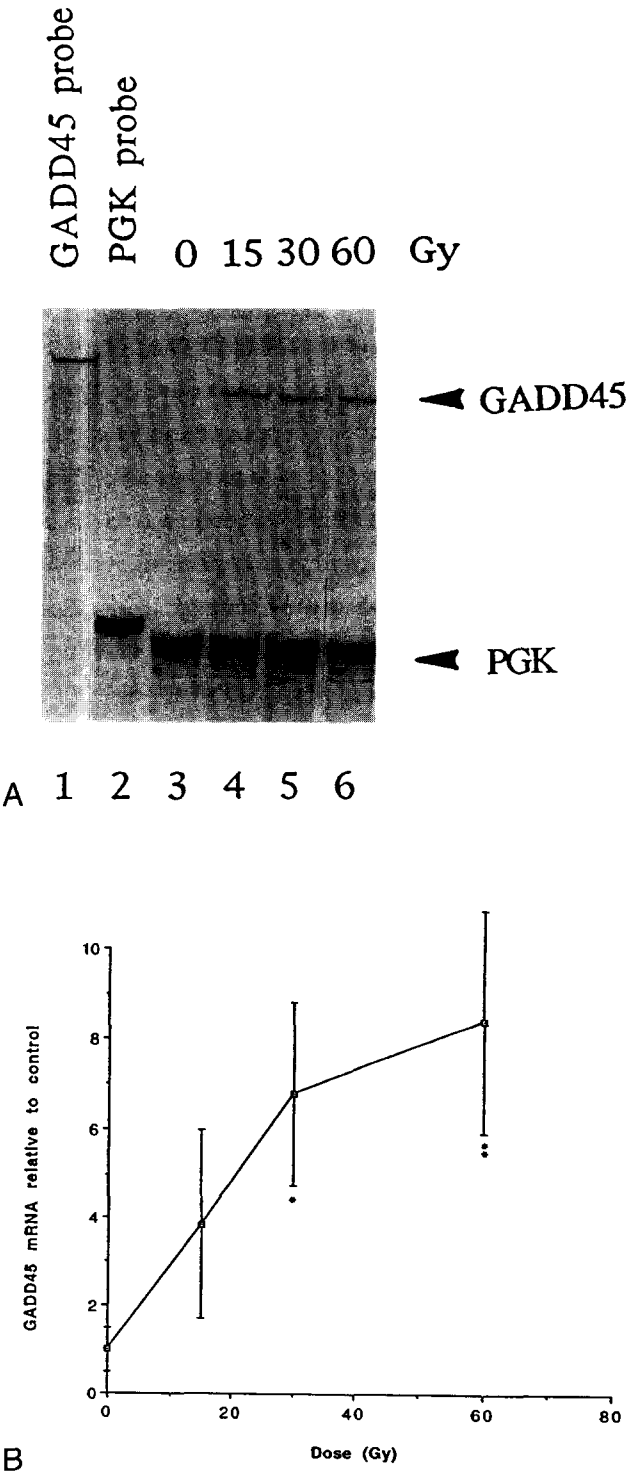

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Fig. 3. In vivo induction of *GADD45* mRNA is correlated with the dose of γ-irradiation. The induction of *GADD45* mRNA was measured by RNase protection assay 30 min following γ-irradiation. (A) Autoradiogram of RNase protection assay. Lane 1,2, probes only. Lane 3–6, Rnase protection using the *GADD45* and PGK probes from 0, 15, 30, 60 Gy of γ-irradiated rats. The positions of GADD45 and PGK are indicated by arrow heads. (B) Dose response of *GADD45* mRNA induction with γ-irradiation. Induction levels of *GADD45* mRNA were normalized to PGK mRNA levels and are plotted against the γ-ray dose. Note that induction of *GADD45* mRNA is significantly different from control levels at γ-ray doses 30 and 60 Gy. Induction of *GADD45* mRNA was not significantly different from control at 15 Gy γ-ray dose. Statistics were performed using a paired Student's *t*-test. Significance was set at $P < 0.1$ (represented as * in the figure) and $P < 0.05$ (represented as ** in the figure). Each data point represents results from three independent experiments. S.E.M.s about the mean are shown. The level of *GADD45* mRNA in controls was arbitrarily set at 1.

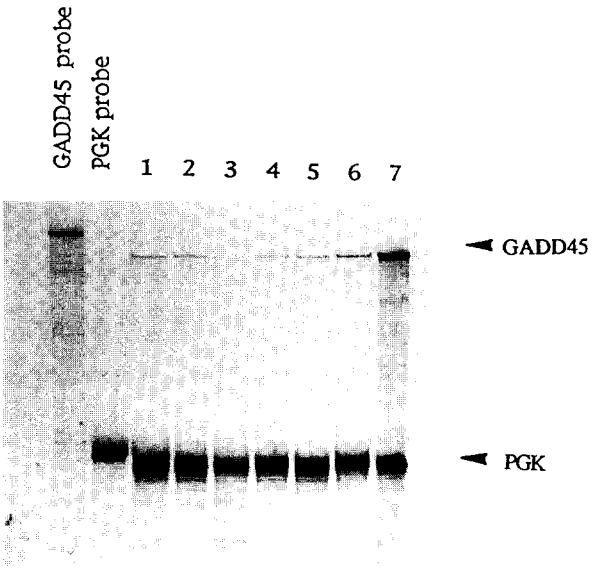

Fig. 4. In vitro induction of *GADD45* mRNA after alkylating agents. The induction of *GADD45* mRNA by EMS-treatment (Lane 2–4) and by MMS-treatment (Lane 5–7) in cultured rat cells using an RNase protection assay. Lane 1, control; Lane 2–4 and 5–7 represent a concentration of 5 μg/ml, 20 μg/ml, 100 μg/ml for each mutagen, respectively. Arrowheads indicate *GADD45* signals and PGK signals 4 h following treatment with alkylating agents.

interacts with proliferating cell nuclear antigen (PCNA) and participates in the cell cycle checkpoint and DNA repair [19], further studies of GADD45 protein are necessary to understand the roles of GADD45 in cellular responses following ionizing irradiation.

An amino acid sequence comparison of the three *GADD45* genes (human, rat, hamster) reveals that GADD45 sequences are well conserved among mammals. Interestingly, amino acid replacements among the species occur in two regions. The two regions may form variable loops that are exposured on the surface from the conserved core domain, which may be constrained by three potential cystine bonds involving the six cysteins at residues 57, 82, 83, 124, 145, 147 (see Fig. 5). Although the amino acid sequence alignment and the secondary structural prediction suggests a putative structural model of the GADD45 protein, determination of the GADD45 structure depends on future biochemical and biophysical studies.

While in vitro induction of GADD45 after X-ray irradiation or by DNA-alkylating reagents has been reported previously [7], there was little known about expression of GADD45 in vivo [8,20]. Our analysis of the induction of *GADD45* mRNA in rat tissues after ionizing radiation provides strong evidence that GADD45 has a significant role in response to DNA-damage in vivo. Although *GADD45* mRNA was induced in all tissues examined, the induction of the *GADD45* mRNA was much higher in the liver than other tissues. The higher induction of the *GADD45* mRNA in liver tissue may suggest that *GADD45* gene might have tissue selectivities in response to DNA damage after ionizing radiation in vivo. However, the expression of GADD45 in brain tissue is more prominent after 4.5 h (not shown). The induction kinetics of *GADD45* mRNA in liver occurred after only 30 min following γ-ray exposure, and correlated well with the dose of γ-ray up to 60 Gy. The GADD45 induction kinetics in liver was similar to the induction in cultured cells in vitro [7], although the energy strength of ionizing radiation that is required for the in vivo and in vitro induction was different: X-ray vs. γ-ray in in vitro and in vivo experiments, respectively. Our finding on the induction of *GADD45* mRNA in vivo following ionizing radiation may offer important clues for further understanding of the roles of GADD45 in DNA repair processes in response to DNA-damage.

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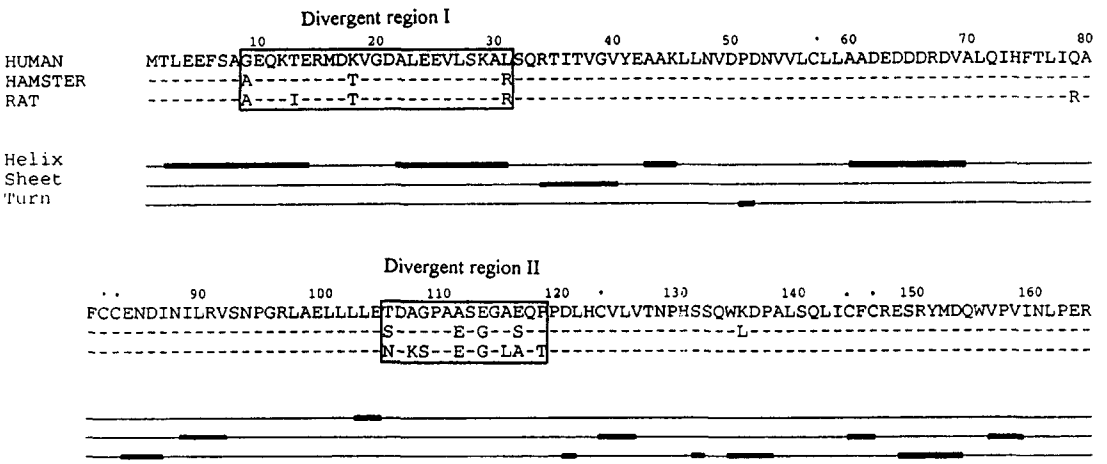

Fig. 5. Amino acid sequence alignments of GADD45 sequences reveal conserved and variable regions. The amino acid differences are shown in single letter fashion and identical amino acid sequences are indicated by dashes. The two variable regions are boxed, and the six cystein residues are shown by dots. The criteria for scoring each amino acid residues are as follows: α-helix structure was predicted by > 200 and < 100 scores of α-helix, β-sheet possibilities, respectively. β-sheet was by < 100 and > 200 scores of α-helix, β-sheet possibilities, respectively. Helical turn conformation was by > 150 and < 150 scores of helical turn and random coil possibilities, respectively. All scores of prediction are according to Garnier-Robson methods.

Table 2
Induction of rat *GADD45* mRNA in various tissues

	Brain		Liver		Kidney		Spleen	
	−	+	−	+	−	+	−	+
GADD45 mRNA	5.21 (±0.84)	5.23 (±0.46)	4.36 (±0.68)	24.24 (±1.20)	19.19 (±0.74)	34.57 (±4.17)	3.16 (±0.58)	3.28 (±0.60)

GADD45 mRNA relative signal intensities normalized to PGK mRNA of +: γ-ray (45 Gy) irradiated, −: control; S.E.M.s are indicated in parentheses.

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