Interaction between DNA-damage protein GADD34 and a new member of the Hsp40 family of heat shock proteins that is induced by a DNA-damaging reagent

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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. As bait we used the product of the partial GADD34 cDNA, including the regions rich in proline, glutamic acid, serine and threonine (PEST) and $\gamma_1$34.5 regions. A cDNA clone, named GAHSP40, which is a mouse DnaJ family protein with a high similarity to human HLJ1 was cloned. The interaction between GADD34 and GAHSP40 in cultured cells was confirmed by a co-immunoprecipitation experiment and in NIH 3T3 cells by two-hybrid analysis *in vivo*. For binding of the two proteins, the $\gamma_1$34.5-similar region of GADD34 was necessary; however, the PEST region was also involved and the C-terminus of GAHSP40, but not the J-domain, was important. GAHSP40 was detected in all mouse tissues examined, but a different transcript was found in the testis. Both GADD34 mRNA and GAHSP40 mRNA were significantly elevated by treatment with methyl methanesulphonate, although the time courses were different. In addition, both GAHSP40 and GADD34 mRNA were induced by heat shock.

Key words: methyl methanesulphonate, stress response, yeast two-hybrid system.

INTRODUCTION

Both eukaryotic and prokaryotic cells have defence mechanisms against genotoxic stress. Growth arrest is one of these important responses. DNA damage can activate a variety of cell cycle checkpoints and trigger apoptosis in some cells. GADD34 cDNA was first cloned in mammalian cells as one of the *GADD* genes on the basis of rapid induction by UV radiation in Chinese hamster ovary cells. However, it has since been found to be induced by a variety of DNA-damaging agents and certain other growth-arrest treatments. Other GADD families include GADD45, GADD153 and GADD7 [1]. GADD45 has been reported to be one of the families interacting with mitogen-activated protein kinase kinase kinase ('MAPKKK') and to be involved in the signal transduction pathway in the stress response [2]. GADD153 was found to be the transcription factor, C/EBP (CAAT/enhancer binding protein) homology protein ('CHOP') [3]. The murine *GADD34* gene is also known as MyD116, whose differentiation is induced by interleukin 6 [1,4–6], although the function of GADD34 is not yet completely known. These genes were rapidly and co-ordinately induced by the alkylating agent methyl methanesulphonate (MMS) and more slowly by growth-arrest treatments such as medium depletion. Time course and dose–response experiments suggest that these genes are often co-ordinately induced by a variety of agents. The murine MyD116, GADD34, contains 657 amino acid residues and consists of a large basic N-terminal domain, a 38-amino acid sequence repeated 4.5 times [region rich in proline, glutamic acid, serine and threonine (PEST)] [7], and a C-terminus that can substitute for the corresponding domain of the herpes simplex virus 1 $\gamma_1$34.5 protein [8].

One function of GADD34 expressed after differentiation of the myeloid leukaemic cells is its involvement in preventing terminally differentiated cells from undergoing apoptosis [9]. A second function is that, either alone or in association with other proteins, GADD34 suppresses cell division during DNA repair to preclude any stress response that could result in cell death [6,10]. The other possibility is derived from the fact that the cellular *GADD34* gene can complement a herpes simplex virus 1 $\gamma_1$34.5 deletion mutant and enable the virus to grow in neuroblastoma cells [8]. $\gamma_1$34.5 protein complexes with protein phosphatase 1α to dephosphorylate the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) and preclude the shut-off of protein synthesis by double-stranded RNA-dependent protein kinase (PKR) [11]. This means that GADD34 has a role in the viral life cycle, because it fosters the survival of infected cells and the dissemination of infectious progeny. It is therefore possible that cells that show 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 function of GADD34 and its several domains has not yet been elucidated. Here we describe the cloning of the gene whose product interacts with GADD34 and discuss the function of GADD34.

EXPERIMENTAL

Cloning of cDNA species for polypeptides that interact with GADD34 by using a yeast two-hybrid system and DNA sequencing

The yeast strain and the procedure used were the same as those described previously [12–14]. In brief, pRS305HISpAS-GADD34, containing GADD34 cDNA and the *LEU2* gene as a selectable marker, was used for transformation to the yeast strain Y190 (*MATa*, *leu2-3,112*, *ura3-52*, *trp1-901*, *his3-Δ200*, *ade2-*

Abbreviations used: eIF-2α, α subunit of eukaryotic translation initiation factor 2; Hsp, heat shock protein; MMS, methyl methanesulphonate; PEST, regions rich in proline, glutamic acid, serine and threonine; PKR, double-stranded RNA-dependent protein kinase.

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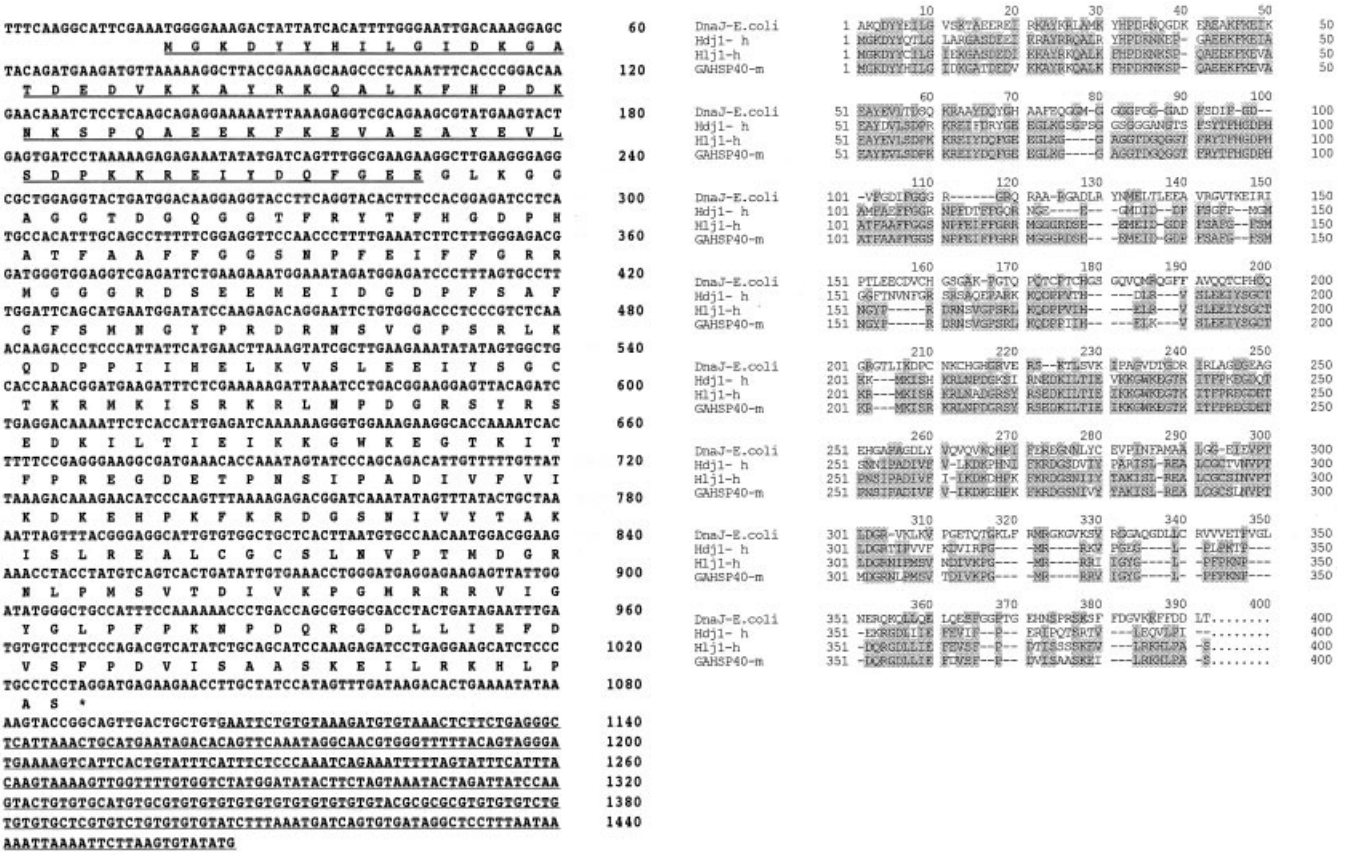

Figure 1 Characterization of GAHSP40

Left panel: nucleotide sequence and predicted amino acid sequence of GAHSP40 cDNA. The conserved J-domain is doubly underlined (at the top of the panel). The cDNA used for Northern blot analysis is underlined (at the bottom of the panel). Numbers correspond to the DNA sequence. Right panel: amino acid similarity. Each set of sequence data was obtained from GenBank and the multiple homology search was done by DNASIS.

101, *gal4*Δ*gal80*Δ *URA3 GAL1-lacZ*, *LYS GAL-HIS3*, *cyh′*). Yeast cells were then plated on a minimal synthetic dextrose plate without histidine to verify background *HIS3* gene activity. One of the yeast transformants that had minimal *HIS3* gene activity was selected as the strain for the transformation after the initial selection. Plasmids (10 μg) containing cDNA libraries constructed in the pPC86 vector were then transformed into the yeast strain harbouring the reporter plasmid integrated into the genome and plated on plates including 5 mM 3-amino-1, 2,4-triazole but lacking leucine, tryptophan and histidine. Colonies were isolated after 3–5 days. Plasmid containing cDNA species were extracted and used for retransformation either into the same yeast strain or the yeast strain into which pRS305HISpAS instead of pRS305HISpAS-GADD34 had been integrated. Positive cDNA species were subcloned into the pCR2.1 vector (Invitrogen) and sequenced. DNA sequencing was performed by using a Pharmacia ALFDNA sequencer with M13 universal and reverse sequence primers.

Cell culture and DNA transfections

Cos-1 cells were obtained from the RIKEN cell bank (Wako, Japan). NIH 3T3 fibroblasts and Cos-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal-calf serum. Transient transfections were performed using the Ca^{2+} method [15].

Co-immunoprecipitation and Western blotting

Cos-1 cells in 10 cm dishes were transfected with 2.5 μg of each of the Xpress-tagged pcDNA3.1/His (Invitrogen) and FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys)-tagged pFLAG-CMV-2 (Kodak) 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: 1.5 μg (anti-Xpress; Invitrogen) or 1 μg (anti-FLAG M2; Sigma) 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 [16] with the first antibodies, which were diluted 1:500 (anti-FLAG M2) and 1:2500 (anti-Xpress).

Two-hybrid analysis in mammalian cells

The *GADD34* and *GAHSP40* genes were subcloned in-frame into the pBIND vector and pACT vector (Promega). pBIND

vector construct (0.33 μg), pACT vector construct (0.33 μg) and pG5luc vector (0.34 μg) (Promega) were simultaneously transfected to NIH 3T3 cells cultured in 24-hole plates. At 48 h after transfection, cells were harvested and the amount of *Renilla* luciferase and firefly luciferase were quantified with the Dual-Luciferase™ Reporter Assay System (Promega).

RNA isolation and Northern blot analysis

Total RNA was extracted from mouse tissues and cell lines as described previously [10]. Approximately 20 μg of each total RNA preparation was subjected to electrophoresis on 1 % (w/v) agarose gels containing 1.1 M formaldehyde. The RNA was transferred to Genescreen Plus membrane (NEN Life Science); mRNA was detected with a ^{32}P-labelled GADD34 cDNA (random prime labelling kit; Takara). The membranes were then autoradiographed at −80 °C with Fuji RX film. GAPDH was used as an internal control.

RESULTS

Cloning of cDNA species for polypeptides that interact with GADD34

We used as bait a gene of approx. 1.7 kb that contained the PEST region, which consists of a 38-residue sequence repeated 3.5 times, and the $\gamma_1$34.5 analogous region to clone cDNA species for proteins interacting with GADD34 by using the yeast two-hybrid system [14]. This cDNA was chosen to obtain the cDNA clones that interacted with not only the $\gamma_1$34.5 analogous region but also the PEST region. We screened the mouse embryonic cDNA library primed with a random hexamer oligonucleotide. In the yeast strain that was used for selection, plasmid pRS305pASGADD34 was integrated into the genome. In this plasmid, the GADD34 fragment was fused to the GAL4-binding domain. After screening 3×10^6 independent colonies from the library, an initial six histidine-positive colonies that could grow in the absence of histidine, owing to the activation of the *HIS* gene, were picked. Of these, one cDNA could specifically activate the *HIS3* gene and the gene encoding β-galactosidase in the yeast strain containing the pRS305pASGADD34 plasmid without activating the *HIS3* gene or the gene encoding β-galactosidase in the strain with the pRS305pAS control plasmid. This cDNA clone had sequence similarity to human HLJ1, which has been cloned as a new member of the Hsp40 family of heat shock proteins [17]. This cDNA clone was designated GAHSP40 (for GADD34-associated Hsp40). Figure 1 (left panel) presents the sequence of GAHSP40 cDNA that we obtained. The open reading frame starting from the first codon in the cDNA was 993 nt long and encoded a putative polypeptide of 331 residues. The GAHSP40 belongs to the DnaJ family and has high similarity to HLJ1 at the amino acid level (Figure 1, right panel).

Binding experiments *in vitro*

To confirm that the GAHSP40 could bind to the GADD34 *in vitro* as well as in the yeast two-hybrid condition, we performed co-immunoprecipitation experiments by transfection of GADD34 and GAHSP40 expression vectors with sequences encoding the epitope for anti-Xpress or anti-FLAG antibody. A protein immunoprecipitated with anti-Xpress antibody from the COS1 cells transfected with Xpress-GAHSP40 and FLAG-GADD34 expression vectors was detected by anti-FLAG antibody (Figure 2A, upper panel, lane 1). A protein immunoprecipitated with anti-Xpress antibody from the COS1 cells

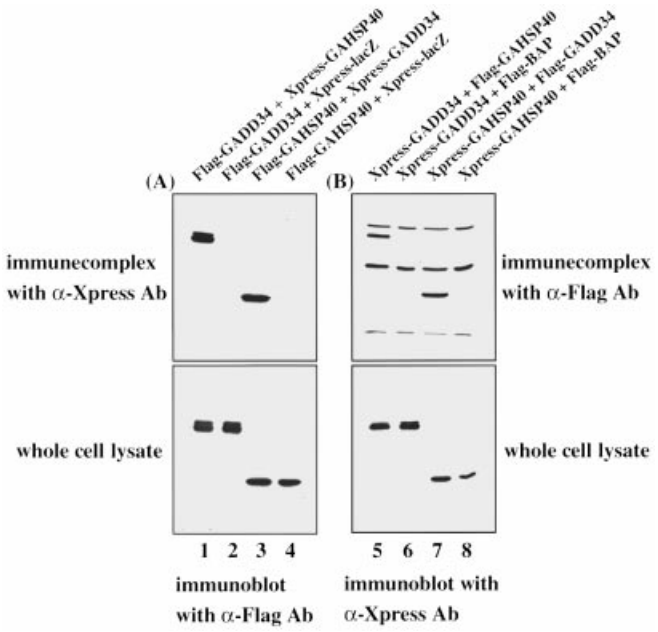

Figure 2 Co-immunoprecipitation of GADD34 and GAHSP40

Cos-1 cells were co-transfected with different combinations of expression constructs carrying the Xpress-tag or FLAG-tag as indicated at the top of the panel. FLAG-BAP and Xpress-lacZ were control vectors. Co-immunoprecipitation was conducted as described in the Materials and methods section. Immunocomplexes were eluted and analysed by Western blotting (upper panels). Whole cell lysates were analysed as control (lower panels). Abbreviations: α-Xpress Ab, antibody against Xpress; α-Flag Ab, antibody against FLAG.

transfected with Xpress-GADD34 and FLAG-GAHSP40 expression vectors was also detected by anti-FLAG antibody (Figure 2A, upper panel, lane 3). We obtained the same results with the protein precipitated with anti-FLAG antibody (Figure 2B, upper panel, lanes 5 and 7). Thus we confirmed that GADD34 and GAHSP40 interacted *in vitro* as well as in the yeast two-hybrid condition.

Binding experiments *in vivo*

We then performed mammalian two-hybrid analysis *in vivo*. We transfected GAHSP40 cDNA and its several deletion mutants inserted in the pACT vector to NIH 3T3 cells together with GADD34 cDNA in the pBIND vector and pG5luc vector. Then we used a luminometer to assay the firefly luciferase activities, which corresponded to the binding strength. As shown in Figure 3 (upper panel), the full-length GAHSP40 construct had more than 20-fold the luciferase activity of the control vector. We confirmed this result by using another *Eco*RI–*Hin*cII deletion mutant that had no 3′ untranslated region. This mutant also had more than 20-fold the luciferase activity of the control vector. We detected the lower luciferase activity in the other deletion mutant, the *Eco*RI–*Bgl*II mutant, which lacked more than half of the open reading frame. However, we could not detect any luciferase activity higher than control in the shortest construct, which contained the J-domain of Hsp40. These results suggest that GAHSP40 and GADD34 can also interact in mammalian cells as well as *in vitro* and in the yeast two-hybrid condition, and that regions other than the J-domain of GAHSP40 which might interact with Hsp70 are necessary for the binding. We then asked which region of GADD34 is important for the interaction of the two proteins. In this experiment we transfected GADD34 cDNA

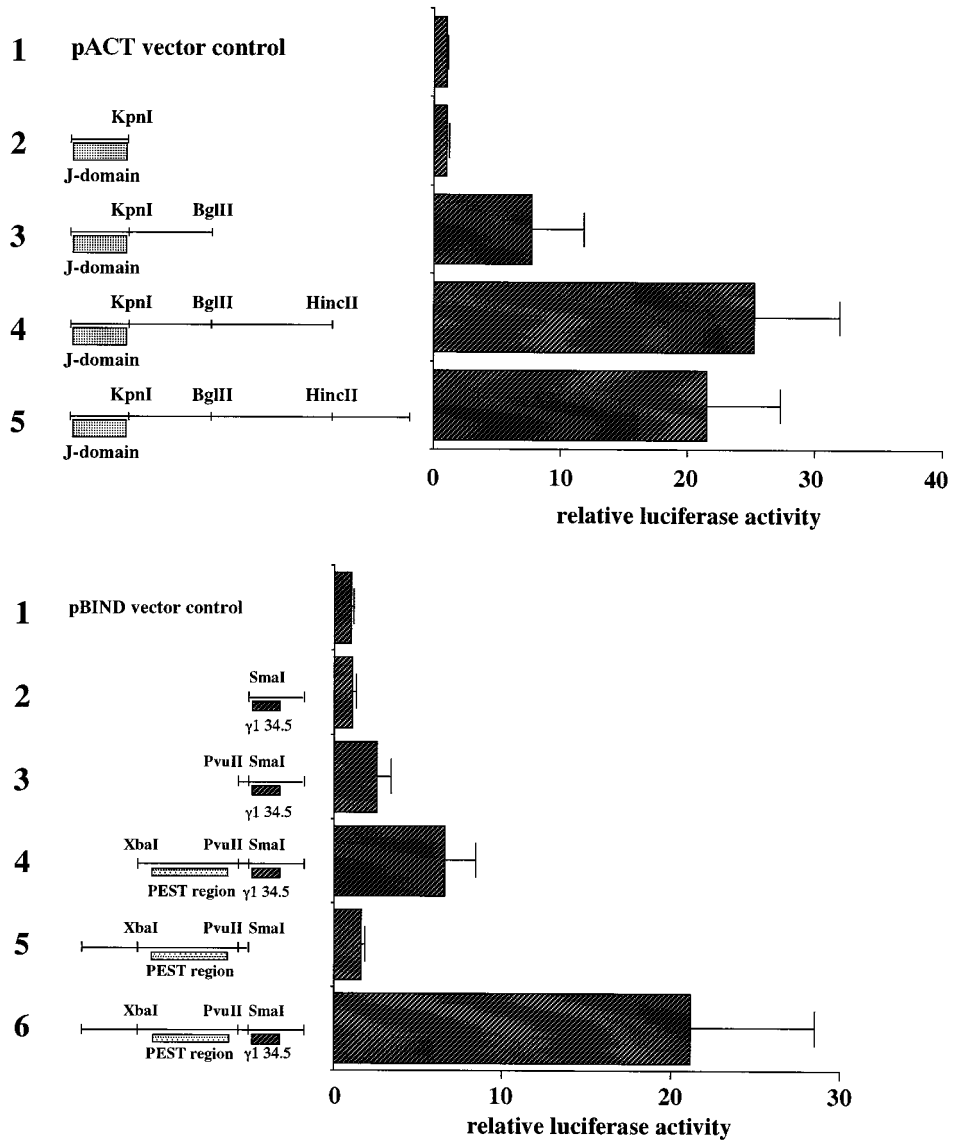

Figure 3 Mammalian two-hybrid analysis of GADD34 and GAHSP40

Upper panel: pBINDGADD34 and the deletion constructs of pACTGAHSP40 were co-transfected with pG5luc vector to NIH 3T3 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 with a duplicate transfection in at least three separate experiments. Lower panel: pACTGAHSP40 and the deletion constructs of pBINDGADD34 were co-transfected with pG5LUC vector to NIH 3T3 cells by the Ca^{2+} method.

and its several deletion mutants inserted in the pBIND vector to NIH 3T3 cells together with the full-length GAHSP40 cDNA in the pACT vector. Figure 3 (lower panel) shows that no luciferase activity was detected higher than control in the vector, which contained only the $\gamma_1$34.5 region. However, we detected only very low luciferase activity in the construct lacking the $\gamma_1$34.5 region. These results suggest that this region is necessary, but not sufficient, for binding and that the other regions including the PEST region are also involved in the interaction of the two proteins.

Northern-blot analysis

To determine the expression pattern of GAHSP40, a Northern hybridization experiment was performed. First we examined the mRNA expression in mouse tissue. In this case we used a ribosomal RNA probe as an internal control to confirm that the same amount of total RNA was applied, because the same GAPDH signal was not correlated with the same total RNA applied. Only one 2.4 kb RNA transcript was identified in all tissues examined except in testis when we used the 3′ untranslated region probe (Figure 4). The mRNA size was the same when we used the coding region DNA probe (results not shown). The reason for the discrepancy between the size of the mRNA from the Northern blot and the cDNA that we obtained is that there might exist 5′ and/or 3′ untranslated regions in the mRNA of GAHSP40. The expression was rather strong in heart (Figure 4, lane 4) and moderate in thymus (lane 2) and lung (lane 3). The expression was weak in brain (Figure 4, lane 1), liver (lane 5), spleen (lane 6) and colon (lane 8). However, we detected a 2.4 kb band and a stronger 1.4 kb band in testis (Figure 4, lane 9). Because GAHSP40 seemed to be one of the heat shock proteins,

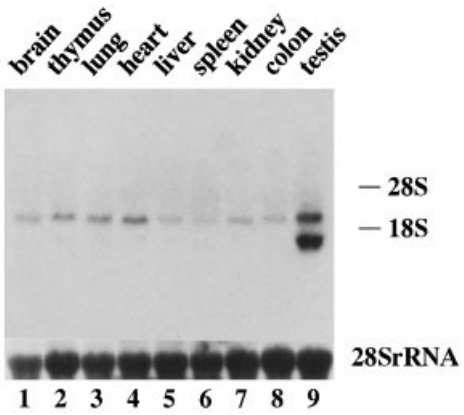

Figure 4 Northern blot analysis

Total RNA was extracted from C57BL6 mouse tissues, subjected to electrophoresis, blotted and hybridized with approx. 300 bp of ^{32}P-labelled GAHSP40 cDNA probe. Human 28 S rRNA (28SrRNA) probe was used as an internal RNA-loading control.

we then analysed the mRNA expression in NIH 3T3 cells treated by heat shock. In this case we used GAPDH as an internal control because the mRNA of GAPDH was proportional to total RNA in the analysis. The NIH 3T3 cells were cultured at 42 °C for 1 h and then returned to 37 °C. The mRNA level of GAHSP40 started to increase even in the first 30 min culture at 42 °C (Figure 5, left panel, lane 2), and reached a maximum after 1 h (lane 3). The level then continued for 3 h after returning to 37 °C (Figure 5, left panel, lanes 4–6). The mRNA returned to the previous level after 5 h (a total of 6 h; Figure 5, left panel, lane 7). GADD34 mRNA induction was detected after 1–2 hours (Figure 5, left panel, lanes 3–5). We then checked the influence of treatment with MMS on GAHSP40 induction, because GADD34 mRNA was reported to be greatly induced by MMS treatment. As shown in Figure 5 (right panel), GADD34 mRNA was greatly increased after 2 h (lane 4) and the induction continued until 8 h (lanes 5 and 6). GAHSP40 mRNA was also induced after 2 h (Figure 5, right panel, lane 4) and decreased gradually (lanes 5 and 6).

DISCUSSION

One of the clues to the functions of GADD34 has been derived from the analysis of the $\gamma_1$34.5 domain, which is similar to herpes simplex virus and interchangeable with it. Thus GADD34 has a role in the regulation of protein synthesis in the response to a stress such as viral infection. However, the function of the total gene product, including the PEST region and $\gamma_1$34.5 domain, is unknown. We hypothesized that GADD34 might have a role together with other proteins. Here we have tried to clone the genes whose products could interact with GADD34. We used as bait the regions including the PEST region and the $\gamma_1$34.5 domain. One of the products of the cloned gene was the Hsp40 family, which was very similar to the human *HLJ1* gene [17]. HLJ1 was cloned by yeast two-hybrid screening with the G-protein β subunit of *Saccharomyces pombe* as bait and showed that HLJ1 is preferentially expressed in heart, skeletal muscle and pancreas. Moreover, the results of that Northern blot showed two major and two minor transcripts. Our results

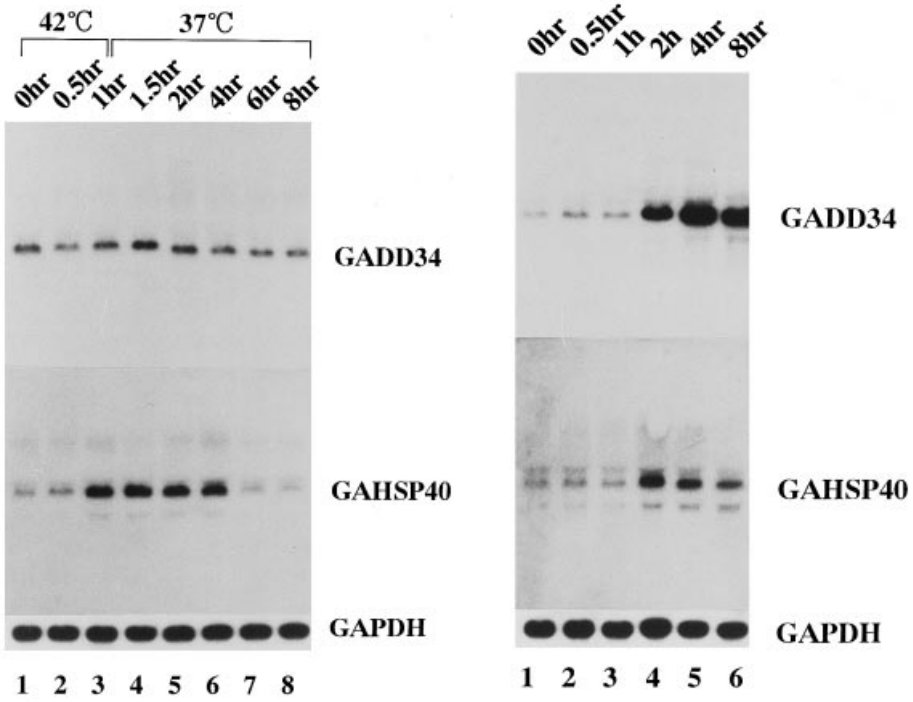

Figure 5 Northern blot analysis

Left panel: NIH 3T3 cells were incubated at 42 °C for 1 h and then returned to 37 °C. Total RNA was extracted at the indicated times and hybridized. The expressed mRNA species are indicated; the GAPDH probe was used as an internal RNA-loading control. Right panel: NIH 3T3 cells were treated with 100 μg/ml MMS for the indicated durations; total RNA was extracted and subjected to Northern blot analysis. The expressed mRNA species are indicated; the GAPDH probe was used as an internal RNA-loading control.

revealed one major 2.4 kb band in all except the two major bands in the testis. We also detected very faint bands in this analysis with longer exposure; faint bands other than 2.4 kb were detected in the analysis of the NIH 3T3 cell lines. The different pattern of mRNA expression between HLJ1 and GAHSP40 might be due to species differences.

We examined the effect of heat shock on the expression of mRNA. The mRNA expression of GAHSP40 increased as early as 30 min after heat shock, then decreased very rapidly after removal of the heat shock. We also showed for the first time that GADD34 mRNA was induced by heat shock. In addition, we demonstrated that GAHSP40 was increased by drug treatment. To our knowledge this is the first report indicating that Hsp40 family proteins are overexpressed by treatment with MMS. Samali and Cotter [18] reported that heat shock proteins such as Hsp27 or Hsp70 increase resistance to apoptosis induced by actinomycin-D, camptothecin and etoposide. They propose that heat shock proteins might protect against apoptosis by inhibiting the synthesis or modification of one or more of the death proteins. Overexpression of GADD34 has been reported to suppress cell growth [6]. From the results showing that GADD34 and GAHSP40 could interact with each other and that both were induced with MMS treatment, it is suggested that GAHSP40 might prevent apoptosis.

Hsp40 family proteins contain the regions which are homologous to the DnaJ protein of *Escherichia coli*. The J-domain of DnaJ is known to be the region that interacts with DnaK [19] or the DnaK analogous region of Hsp70 family proteins, and to have a role in the activation of ATPase activity of DnaK [20]. Here we have shown that a domain other than the J-domain of GAHSP40 can interact with GADD34. This means that GADD34 could form a complex with Hsp70. As mentioned previously, the biological role of GADD34 is not clearly understood. The study of the $\gamma_1$34.5 domain suggests that GADD34 is involved in the regulation of protein synthesis. In other words, the $\gamma_1$34.5 domain complexes with protein phosphatase 1α (PP1α), dephosphorylates eIF-2α and prevents the shutting-off of protein synthesis. This process is controlled also by PKR [9], which phosphorylates eIF-2α and leads to the shutting-off of protein synthesis. The activity of PKR is regulated by p58^{IPK} [21,22] and I-P58^{IPK}, which has been shown to be Hsp40 (Hdj 1) [23]. Thus eIF-2α is controlled in two different ways. The GADD34–GAHSP40 complex might be involved in both processes. GAHSP40 could be the subunit of the complex of protein phosphatase 1α with GADD34 or it might disrupt the complex. For the control of p58^{IPK}, GAHSP40 could bind p58^{IPK} like Hsp40; GADD34 might be the modulator of this complex. Direct evidence to substantiate such hypotheses must be obtained in further studies that will also serve to clarify the stress response reaction including the GADD34 pathways.

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