Molecular cloning of rat GADD45γ, gene induction and its role during neuronal cell death

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Abstract To study the molecular mechanism of neuronal cell death, we carried out the screening of genes which were induced during the neuronal cell death of neuronal PC12. We cloned the cDNA of rat GADD45γ, the third member of the GADD45 family. Induction of GADD45γ mRNA was observed in the neuronal cell death caused by depletion of neurotrophic factor and Ca^{2+} ionophore treatment. Overexpression of GADD45γ in superior cervical ganglion neurons caused cell death. These results suggest that GADD45γ plays an important role in neuronal cell death.

Key words: Restriction landmark cDNA scanning; Microinjection

1. Introduction

Neuronal cell death is an essential process in the development of the nervous system [1]. During development, the neurons which succeed in contacting the proper target cells survive with supply of an adequate neurotrophic factor from the target cells. But nearly half of the neurons fail to contact their target cells, do not receive this factor and die [2].

To analyze cell death during neuron development, two in vitro models of neuronal cell systems are often used. One is the rat pheochromocytoma cell line PC12 and the other is the sympathetic neuron culture [3–5]. The survival of neuronal PC12 differentiated by nerve growth factor (NGF) and the sympathetic neuron culture depends on the supply of NGF. Depletion of NGF from neuronal PC12 or sympathetic neuron causes cell death. In these models, the neuronal cell death can be prevented by inhibitors of RNA or protein synthesis. This suggests that the cell death of neuronal PC12 and the sympathetic neuron by NGF depletion requires de novo RNA and protein synthesis. It indicates the existence of a special molecular mechanism of gene induction needed for cell death.

Several genes induced during neuronal cell death have been isolated [6–9]. Studies of these genes have revealed that the transcription factor c-jun and the cell cycle regulator cyclin D1 are necessary for neuronal cell death [7–9]. However, most of the molecular mechanism underlying neuronal cell death is still unclear. To elucidate it, we have screened the genes induced during the cell death of neuronal PC12 using a novel cDNA display system, restriction landmark cDNA scanning (RLCS) [10–12]. In this study, we isolated the cDNA of rat GADD45γ, the third member of the GADD45 family, and also found that induction of GADD45γ may be associated with neuronal cell death.

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Abbreviations: GADD, growth arrest and DNA damage-induced protein; NGF, nerve growth factor; SCG, superior cervical ganglia

2. Materials and methods

2.1. RLCS

RLCS analysis was carried out according to the method described previously [10]. *Mro*I (Toyobo) was used as the first-dimensional restriction enzyme and *Hin*fI (Takara) as the second-dimensional restriction enzyme.

2.2. Cell culture

PC6-3, one of the sublines of PC12 cloned by Pittman et al. [4], was used as PC12. Neuronal PC12 was maintained in RPMI medium (Nikken) containing 2% horse serum (ICN) and 1% fetal calf seru (FCS, Intergen) with 100 ng/ml 2.5S NGF (Upstate Biotechnology) on plastic dishes coated with type I collagen (Collaborative Biomedical Products). NGF was depleted by replacing the culture medium with NGF-free medium containing 40 μg/ml anti-NGF (Boehringer Mannheim).

Rat sympathetic neurons from superior cervical ganglia (SCG) were prepared and cultured as described previously [13]. Briefly, dissociated neurons from SCG of 21-day rat embryo were plated on collagen-coated dishes in Dulbecco's modified Eagle's medium (Nikken) containing 10% FCS, 2 mM glutamine (Sigma), 20 μM fluorodeoxyuridine (Sigma), 20 μM uridine (Sigma), and 75 ng/ml 2.5S NGF. SCG neurons cultured for 5–7 days were used for the following analyses.

Rat cortical neurons were prepared and cultured as described previously [14]. Briefly, dissociated neurons from the cerebral cortex of 17.5-day rat embryo were plated on polyethyleneimine-coated dishes. The neurons were maintained in glia-conditioned medium [15] for 4 days. Ca^{2+} ionophore A21387 (Sigma) 0.1 μM was added to the culture medium to induce cell death.

2.3. Cloning of GADD45γ cDNA

The cDNA fragments corresponding to the selected spots in RLCS profiles were subcloned into a pSPORT2 vector (Gibco BRL) by the PCR-mediated method [10]. Using this cDNA fragment as a probe of colony hybridization, the full-length cDNA was isolated from a PC12 cDNA library. The library was made using the SuperScript plasmid system for cDNA synthesis and plasmid cloning (Gibco BRL). The mRNA was prepared from neuronal PC12 at 18 h after NGF depletion. The sequences of the isolated cDNAs were determined on both strands. The rat GADD45γ nucleotide sequence reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB020978.

2.4. Northern blot analysis

Non-homologous regions of rat GADD45α, GADD45β and GADD45γ amplified by PCR were used as probes. The corresponding nucleotides are GADD45α, 571–772 (mouse gadd45, L28177); GADD45β, 496–708 (mouse myd118, X54149); and GADD45γ, 481–725.

2.5. Plasmid construction

The GADD45γ expression plasmid (pEF-GADD45γ) was constructed by inserting the complete ORF of rat GADD45γ cDNA (76–594) into the mammalian expression vector pEF-BOS [16], and a HA-tag sequence was fused at the N-terminal end of GADD45γ. The BAX expression plasmid (pEF-BAX) was constructed by insert-

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ing the PCR-amplified complete ORF of rat BAX (U49729) into pEF-BOS, and a HA-tag sequence was also fused at the N-terminal end of rat BAX.

2.6. Microinjection assay

Microinjection assay of SCG neuron was carried out as previously described [17,18]. Briefly, for microinjection we used a Narishige micromanipulator and needles made from glass capillaries with an inner filament (GD-1, Narishige) using a vertical electrode puller (Narishige). Plasmid DNAs were injected directly into the nucleus of SCG neurons. Each expression plasmid (0.05–0.2 μg/μl in 0.5×PBS) was injected with green fluorescent protein (GFP) expression plasmid (0.04 μg/μl pEGFP-C1, Clontech) as an injection marker. After injection, neurons were maintained with NGF, and the numbers of living cells were counted based on the fluorescence of GFP.

2.7. Anti-HA antibody and Hoechst staining

All experiments were carried out at room temperature. The neurons at 24 h after injection of pEF-GADD45γ were fixed for 20 min in 4% paraformaldehyde. They were rinsed twice with PBS and permeabilized in PBS containing 0.1% Triton X-100 for 1 min. After washing twice with PBS, the cells were incubated with a blocking solution (2% BSA in PBS) for 40 min, and then with an anti-HA antibody (Boehringer Mannheim) for 40 min. After washing twice with PBS, the cells were incubated with a fluorescein-conjugated anti-mouse IgG antibody (Boehringer Mannheim) for 40 min. Finally, the cells were washed with PBS twice, then incubated with PBS containing 1 μg/ml Hoechst 33258 (Sigma) for 5 min, and were analyzed directly under a fluorescence microscope.

3. Results

3.1. Induction of spot Mr-5 in RLCS profile during cell death of neuronal PC12

To find up-regulated genes during the cell death of neuronal PC12, we used the RLCS system, a cDNA display system on a two-dimensional gel matrix. In RLCS, each cDNA species appears as a spot at a certain position on a two-dimensional gel; the changes in the intensity of each spot reflect the changes in the expression of the corresponding gene [10]. We compared the RLCS profiles of neuronal PC12 at 18 h after NGF depletion with those of neuronal PC12 with NGF. In cell death of neuronal PC12 by NGF depletion, the time point of 18 h after NGF depletion was considered to exceed the commitment time for cell death (the time point at which 50% of the cells could be rescued by re-addition of NGF) [4]. The mRNA of genes important for cell death would have accumulated at the time point of 18 h after NGF depletion. Spot Mr-5 was derived from one of the cDNAs which showed enhanced expression by NGF depletion (Fig. 1).

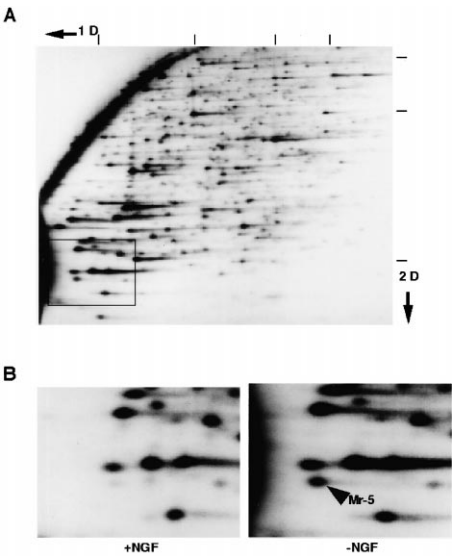


Fig. 1. RLCS analysis of neuronal PC12 during cell death. A: Whole profile of neuronal PC12 at 18 h after NGF depletion. Arrows show the directions of first- and second-dimensional electrophoresis (1D and 2D, respectively). The scales expected from previous studies are 2, 1.5, 1, and 0.5 kbp (from right to left) in 1D, and 1.5, 1, and 100 bp (from top to bottom) in 2D. The region around spot Mr-5 (surrounded by a square) is magnified in B. B: Profiles around spot Mr-5 of neuronal PC12 with NGF (+NGF) and neuronal PC12 at 18 h after NGF depletion (−NGF). Arrowhead shows spot Mr-5.

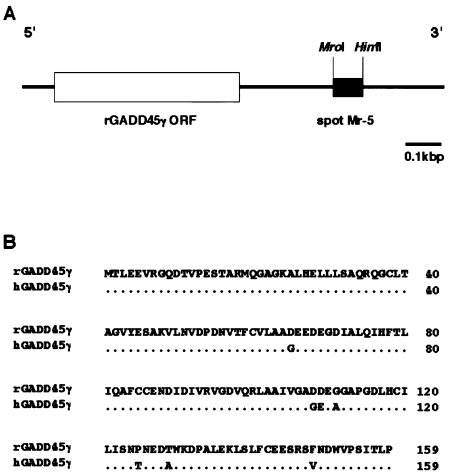
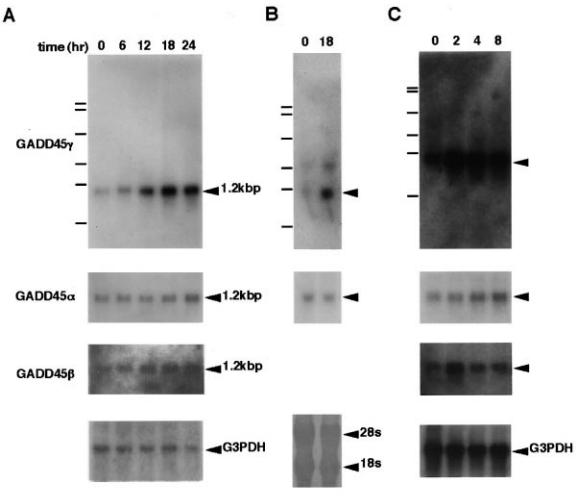


Fig. 2. Structure of rat GADD45γ. A: Schematic representation of rat GADD45γ (rGADD45γ) cDNA. The open box shows the 480 bp coding region of rGADD45γ. The filled box shows the DNA fragment corresponding to spot Mr-5. B: Primary structure of rGADD45γ. The deduced amino acid sequences of rGADD45γ is shown. Alignment of the amino acid sequence to human GADD45γ (hGADD45γ, AF078078) is also shown. In the hGADD45γ sequence, amino acids identical to the rat ones are shown by dots.

3.2. Cloning of rGADD45γ cDNA

Using the cDNA fragment extracted from spot Mr-5 as a probe, we isolated the 1088 bp cDNA from the PC12 cDNA library. As shown in Fig. 2A, in this cDNA there are *Mro*I and *Hin*fI sites corresponding to spot Mr-5 at positions near the 3′-terminus (719–852 bp) which was expected from the RLCS profile. The Mr-5 containing cDNA includes open reading frames (ORF) at the 5′-terminus (89–568 bp) and a poly(A) signal at the 3′-terminus (1048 bp) followed by a poly(A) sequence. The ORF encodes a protein which consists of 159 amino acid residues. From the database analysis, the amino acid sequence coded in this ORF had homology with GADD45 [19,20] and MyD118 [21]. The identity of the amino acid sequence was 53% with mouse GADD45 (accession number L28177) and 54% with MyD118 (accession number X54149). The Mr-5 containing cDNA was expected to encode a new member of the GADD45 family. Recently the third member of the GADD45 family, human GADD45γ (hGADD45γ), was cloned [22]. From alignment of amino acid residues, the Mr-5 containing cDNA is a counterpart of the GADD45γ rat homolog (Fig. 2B). The identity of the amino acid sequence between human and rat was 96%. According to Takekawa et al. [22], we gave the name GADD45 to GADD45α, and MyD118 to GADD45β.



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	PC12 NGF depletion					SCG neuron NGF depletion		cortical neuron Ca^{2+} ionophore treatment			
time (hr)	0	6	12	18	24	0	18	0	2	4	8
GADD45γ	1.0	1.3	3.9	4.2	7.2	1.0	5.4	1.0	3.5	2.7	1.9
GADD45α	1.0	0.78	0.95	0.80	2.1	1.0	0.88	1.0	1.4	2.0	2.0
GADD45β	1.0	1.5	3.1	2.5	3.2	N. D.	N. D.	1.0	4.3	2.5	2.0

Fig. 3. Northern blot analysis of GADD45γ mRNA expression during neuronal cell death. A: 1.5 μg poly(A)$^+$ RNA prepared from neuronal PC12 at the indicated time (hours) after NGF depletion was blotted in each lane. The sizes of the RNA ladder (Gibco BRL) are indicated in the left (9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kbp from top to bottom). The expression of GADD45γ mRNA is shown in the top panel. The expression of GADD45α and GADD45β mRNA is also shown in the middle panels. The hybridization signals of G3PDH in each lane are shown in the bottom panel. B: 12.5 μg total RNA prepared from SCG neuron culture at indicated times (hours) after NGF depletion was blotted in each lane. The expression of GADD45γ mRNA and GADD45α mRNA is shown in A. The methylene blue-stained ribosomal RNAs (28S and 18S) in each lane are shown in the bottom panel. C: 1.5 μg poly(A)$^+$ RNA prepared from cortical neuron culture at the indicated time (hours) after Ca^{2+} ionophore treatment was blotted in each lane. Panels are displayed as in A. D: The fold induction of GADD45γ, GADD45α and GADD45β was calculated. The intensities of bands were measured using the software NIH image. The values were normalized using G3PDH or 28S ribosomal RNA and the value at 0 h was set at 1.

3.3. Induction of rGADD45γ mRNA during neuronal cell death

We examined the mRNA induction of GADD45γ and other GADD45 family members during the cell death of neuronal cells by Northern blotting. First, we confirmed the time course of the mRNA induction profile of GADD45γ during the cell death of neuronal PC12 by NGF depletion. As shown in Fig. 3A, a 1.2 kbp transcript of GADD45γ was observed. The size of the transcript estimated from Northern blot analysis corresponds well with the size of cDNA (1088 bp). Neuronal PC12 cultured with NGF expressed GADD45γ mRNA only faintly, whereas GADD45γ mRNA was induced by NGF depletion. The expression level of GADD45γ mRNA was increased 4.2 times at 18 h after NGF depletion. At 18 h after NGF depletion, the cells were considered to be past the commitment time of cell death and showed some apoptotic aspect (cell membrane blebbing and nuclear condensation). GADD45β mRNA (1.2 kbp) was induced similarly to GADD45γ mRNA by NGF depletion, but the expression level of GADD45α mRNA (1.2 kbp) did not change (Fig. 3A).

Next, to show that the induction of GADD45γ mRNA was not specific to the cell death of neuronal PC12, we examined the mRNA induction of GADD45 family members during the cell death of sympathetic neuron. The SCG sympathetic neuron culture was also the well-known neuronal model [3]. The sequential events that occur following NGF depletion from SCG neurons are quite similar to those of neuronal PC12 [23]. In agreement with the results of the cell death of neuronal PC12, an about 5-fold increase of GADD45γ mRNA expression was observed during the cell death of SCG neurons, but GADD45α mRNA did not change its expression level (Fig. 3B). GADD45β mRNA could not be detected in SCG neurons. We also examined the mRNA induction of GADD45 family members during the cell death of cortical neurons by Ca^{2+} ionophore treatment. This is the model of neuronal cell death by excitotoxicity [14]. GADD45γ mRNA and GADD45β mRNA were highly induced at 2 h after Ca^{2+} ionophore treatment, but GADD45α mRNA did not change its expression level (Fig. 3C). In this model, the earlier induction of GADD45γ mRNA and GADD45β mRNA than NGF depletion might reflect a shorter time course of cell death.

3.4. Capability of rGADD45γ to cause neuronal cell death

We demonstrated that GADD45γ and GADD45β mRNA was induced during neuronal cell death, suggesting a close association between its induction and neuronal cell death. To examine whether overexpression of GADD45γ induces neuronal cell death, we microinjected GADD45γ expression plasmid (pEF-GADD45γ) into SCG neurons. Since only

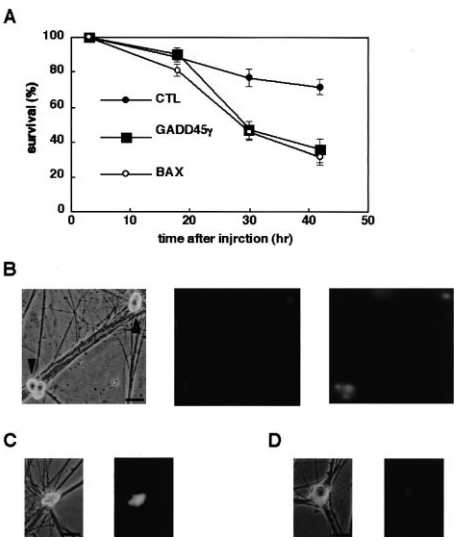

Fig. 4. Effect of GADD45γ expression in SCG neurons. A: Expression vector pEF-GADD45γ (GADD45γ), pEF-BAX (BAX) or pEF-BOS (CTL) was microinjected into SCG neurons. All expression plasmids were injected at 0.1 μg/μl. Changes in the survival of neurons at specified time points after injection were determined relative to the number of neurons at 3 h after injection. The results are presented as the mean percentages of surviving neurons and standard deviations observed in three independent experiments (50–100 neurons were injected for each vector in each experiment). B: Phase-contrast image of neurons at 24 h after injection with pEF-GADD45γ (left panel). Injected neurons are indicated by arrowheads. The same area is shown after anti-HA staining (middle panel) and Hoechst 33258 staining (right panel). Bar = 20 μm. C, D: Phase-contrast image (left panel) and the fluorescence image of GFP (right panel) of neurons at 30 h after injection with pEF-GADD45γ. Neurons were co-injected with pEGFP-C1 as an injection marker. Bar = 10 μm.

GADD45γ mRNA induction was observed (Fig. 3B) during the cell death of SCG neuron, we examined the capability of GADD45γ to cause neuronal cell death in SCG neurons. We also injected BAX expression plasmid (pEF-BAX), which was reported to cause cell death [17], as a positive control. To count living cells, GFP expression plasmid was co-injected as an injection marker. The injected SCG neurons were maintained in the presence of NGF, and the number of living cells was counted at various time points after injection. As shown in Fig. 4A, most cells injected with vector plasmid only survived at 42 h after injection. In contrast, about 70% of the neurons injected with pEF-GADD45γ had died by this time, similar to the case of pEF-BAX. The cell death caused by the injection of pEF-GADD45γ was observed from 18 h after injection, and most of the surviving cells died following 6 h. The result of BAX expression was the same as previously reported [17]. In Fig. 4A we show the result of the microinjection assay at 0.1 μg/μl of each expression plasmid. We also examined microinjection at 0.05 μg/μl and at 0.2 μg/μl of each expression plasmid. The rate of dead cells was lower at 0.05 μg/μl of pEF-GADD45γ injection than at 0.1 μg/μl and we obtained almost the same result at 0.2 μg/μl of pEF-GADD45γ injection as at 0.1 μg/μl. The rate of dead cells depends on the injected pEF-GADD45γ concentration (data not shown). The expression of GADD45γ was confirmed by anti-HA staining (Fig. 4B, middle panel). The dying cells overexpressing GADD45γ showed some apoptotic features, nuclear condensation (Fig. 4B, right panel), cell fragmentation and membrane blebbing (Fig. 4C,D).

4. Discussion

In this study, we identified a cDNA encoding rat GADD45γ, which corresponds to the RLCS spot Mr-5, induced during cell death of neuronal PC12. In the profile of RLCS, the position of the first dimension of the spot reflects the length from the 3′ poly(A) sequence to the landmark restriction enzyme site (*Mro*I site) and the position of the second dimension reflects the length of the cDNA fragment (the length of Mr-5) (Fig. 1A, Fig. 2A and [10]). Since reasonable lengths were found in the cDNA encoding rat GADD45γ as the position of Mr-5 in the RLCS profile, we were able to confirm that the spot Mr-5 is a part of rat GADD45γ cDNA.

GADD45γ is the third member of the GADD45 family. The expression of GADD45α (GADD45) is induced by DNA damage (UV, *N*-acetoxy-2-acetyl-aminofluorene, methyl methanesulfonate (MMS)) in CHO and some cells [20,24]. The expression of GADD45β (MyD118) is induced in terminally differentiated myeloid precursor cells [21] and by MMS treatment in CHO and some cells [24]. The expression of GADD45γ is also induced by MMS treatment as GADD45α and GADD45β in ML-1, the myeloid leukemia cells [22]. We also obtained the same result using NIH3T3 cells (data not shown). But only GADD45γ and GADD45β were induced during the neuronal cell death caused by NGF depletion from neuronal PC12 or SCG neurons and Ca^{2+} ionophore treatment of the cortical neurons. These findings suggest that the expressions of GADD45γ and GADD45β are induced by different pathways from GADD45α. The induction of GADD45γ and GADD45β was observed in models of neuronal cell death not only by trophic factor depletion but also by excitotoxicity. We consider that GADD45γ and GADD45β might play a role in the closely related pathway of neuronal cell death.

Recently a new function and ability of the GADD45 family was reported [22]. All GADD45 family members were shown to activate the p38/JNK (c-Jun N-terminal kinase) MAPK (mitogen-activated protein kinase) pathway via binding and activation of MTK1/MEKK4, cytoplasm MAPKKK (MAPK kinase kinase). We demonstrated that the induction of GADD45γ and GADD45β during neuronal cell death and the overexpression of GADD45γ causes the cell death of SCG neurons. Induction of GADD45γ and GADD45β might activate the MTK/MEKK4-p38/JNK pathway and the activation of p38/JNK causes neuronal cell death. Indeed, activation of the p38/JNK pathway was observed during neuronal cell death [25–27] and the forced activation of this pathway induces neuronal cell death [28].

Some molecules are closely associated with neuronal cell death. For example, BAX-related protein DP-5 was induced during neuronal cell death and its overexpression caused neuronal cell death [18]. Also essential for neuronal cell death were p38/JNK pathway-related molecules, c-JUN [7,8], MKK3 [28] and Cdc42 [29]. The relationship among these molecules, GADD45γ and GADD45β in the cascade of the neuronal cell death is not clear, but GADD45γ and GADD45β are thought to play an important role in the cascade of neuronal cell death.

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