Molecular Cloning of a Novel Polypeptide, DP5, Induced during Programmed Neuronal Death*

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To study the molecular mechanisms underlying neuronal programmed cell death (PCD), we performed differential display screening for genes, the expression of which was induced during PCD in the sympathetic neuron culture model deprived of NGF. We cloned a gene encoding a novel polypeptide (DP5) which consisted of 92 amino acids. DP5 polypeptide had no homology with any other known protein and contained no motif that would indicate its putative biochemical functions. DP5 mRNA levels peaked at 15 h after nerve growth factor withdrawal, concurrent with the time at which neurons were committed to die. The induction of DP5 gene expression was blocked when cell death was rescued by treatment with cycloheximide, KCl, or the cyclic AMP analogue CPTcAMP. Overexpression of the full-length DP5 in cultured sympathetic neurons was in itself sufficient to induce apoptosis. These results suggest that DP5 plays a role in programmed neuronal death.

Programmed cell death (PCD) is an indispensable phenomenon for proper development of the nervous system. Roughly half of all neurons produced by neurogenesis die during development of the mammalian nervous system (1). Neuronal survival is determined largely by neurotrophic factors such as NGF produced by target cells, and neurons that do not obtain an adequate supply of survival factors undergo apoptosis (2). Dissociated sympathetic neurons from rat superior cervical ganglia (SCG) are mainly used as an in vitro model of neuronal PCD and have been characterized extensively (3, 4). In this culture system, the majority of neurons die after removal of NGF from the culture medium. The characteristics of this neuronal death such as shrinkage of the neuronal soma with intact organelles, nuclear condensation, fragmentation of the DNA into oligonucleosomes, and blebbing of the plasma membrane are hallmarks of apoptosis (5, 6). Neuronal death in this system can be prevented by inhibitors of RNA or protein synthesis, suggesting that this phenomenon is controlled by a genetic program (7).

In the search for constituents of the genetic program, several genes have been identified using this in vitro model. Immediate early genes such as c-fos and c-jun and cell cycle-related genes such as c-myb and cyclin D1 (8, 9) are these candidates. Especially cell cycle-related genes stimulate the postmitotic neurons to attempt re-entry into the cell cycle, and these conflicting growth-regulatory signals are thought to cause neurons to undergo apoptosis. On the other hand, several anti-apoptotic proteins such as Bcl-2 (10, 11), adenovirus E1B19k (12), or cowpox virus CrmA gene products (13) have been reported. Overexpression of the bcl-2 gene products in sympathetic neurons has been shown to protect neurons from apoptosis (10). Anti-apoptotic properties of Bcl-2 have been reported to prevent the loss of mitochondrial function (14). CrmA gene products also prevent neuronal death induced by NGF deprivation. This activity is attributed to interleukin β-converting enzyme protease inhibition (13, 15).

Re-entry into the cell cycle and activation of interleukin β-converting enzyme-like proteases are closely associated with neuronal PCD, but the molecular events or cascades underlying neuronal death regulated by the genetic program still remain unclear. To elucidate the mechanisms, it is crucial to list the molecules involved in this process and to examine the relationships between them. For this purpose, using the differential display technique (16), we screened for genes, the expression of which is induced during neuronal PCD, and identified one candidate, named DP5. Here we report its structure, expression patterns, and putative biological functions.

EXPERIMENTAL PROCEDURES

Cell Culture—Sympathetic neurons from the superior cervical ganglia of 1-day-old Sprague Dawley rats were isolated and cultured as described by Ham et al. (17). Digested cells were preplated to minimize the number of non-neuronal cells on uncoated 10-cm dishes for 3 h. The resultant supernatant enriched in neurons was then plated on 10-cm dishes coated with collagen (Koken, Tokyo, Japan) in DMEM containing 10% fetal calf serum (FCS), 2 mM glutamine, 20 μM fluorodeoxyuridine, 20 μM uridine, and 50 ng/ml 2.5 S NGF (Promega). The neurons were maintained for 5–7 days in the presence of NGF before being subjected to screening, Northern blotting, and RT-PCR analyses. NGF withdrawal was carried out by changing the medium to DMEM/10% FCS lacking NGF and containing anti-NGF antibodies (CIDtech Research Inc., Ontario, Canada) diluted 1:1000.

PC12 cells were maintained in DMEM containing 5% FCS and 10% heat-inactivated horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Neuronal PC12 cell cultures were prepared by treatment with 50 ng/ml NGF in DMEM containing 5 μg/ml transferrin, 5 μg/ml insulin, and 0.02 μM progesterone for 7–10 days. Deprivation of NGF
was performed as for SCG neurons.

**Screening by Differential Display**—Total RNA was isolated by the acid guanidinium thiocyanate-phenol/chloroform method (18) from SCG neurons cultured for 15 h in medium containing NGF and those cultured in medium from which NGF had been removed. Differential display was carried out essentially as described previously (19). Oligonucleotides were synthesized from total RNA with reverse transcriptase after DNase I treatment, and PCR was performed using a random primer with the synthesized cDNA as a template. We used about one hundred different random primers (12-mers, Common primer, Bex Inc.) for screening. After separation by 5% polyacrylamide gel electrophoresis, cDNA bands of interest were cut from the gel, gel-purified using the same primers from the eluted cDNA solution, and then cloned into the pGEM-T vector (Promega).

cDNA Library Screening—A 630-bp DP5 cDNA fragment (DP-PCR) obtained by differential display was used to display 1 × 10^6 plaques from three new baby brains containing 300 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in a 60-μl reaction mixture in the presence of 2.5 μM oligo(dT) primer and 20 μM dNTP mixture for 60 min at 37 °C. For PCR amplification, specific oligonucleotide primer pairs (0.5 μM each) were incubated with 1 μl of cDNA, 1 μl of Taq polymerase, 1 × Taq buffer (10 M Tris-Cl (pH 8.3), 50 M KCl, 1.5 M MgCl2), 10 μM dNTP mixture, and 10 μl of cDNA. Oligonucleotides used for amplification of DP5 cDNA were DP5-5′, 5′-AGACACAGC-CGGACCGAGCAA-3′, 5′-347–368; DP5-3′, 5′-ATAGCACTGAGGTG-GCTATC-3′, 5′-516–535. Typical cycle parameters were 1 min at 94 °C, 1 min at 60 °C, and 30 s at 72 °C for 22–24 cycles followed by 72 °C for 5 min. 10-μl aliquots of each reaction mixture were electrophoresed through 5% polyacrylamide gels, and the dried gels were subjected to autoradiography and quantitative analysis (Scanning Image, Molecular Dynamics). Control experiments were performed to determine the range of PCR cycles over which amplification efficiency remained constant. The identity of each PCR product was confirmed by subcloning the amplified cDNAs into pGEM-T vector (Promega) and sequencing.

**Protein Production, Preparation of Anti-DP5 Polypeptide Antibody, and Western Blotting**—Sf21 insect cells were infected with recombinant baculovirus expressing glutathione S-transferase (GST)-DP5 fusion protein. Recombinant fusion proteins were extracted with Triton X-100 and purified by affinity chromatography on glutathione-agarose (Pharmacia Biotech Inc.). Typical yield of fusion protein was 1 mg per 2 × 10^6 infected Sf21 cells. Antibodies were raised against the recombinant GST-DP5 fusion protein. Immunization of rabbits and screening of antisera were performed as described previously (20).

For detection of DP5 polypeptide, 1 × 10^6 SCG neurons were plated per 10-cm dish and incubated for 7 days in the presence of NGF. One dish was used per immunoprecipitation. Neurons were harvested and then lysed in 1 ml of buffer containing 10 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1% EDTA, 10 μg/ml aprotinin (Sigma). Lysates were centrifuged at 13,000 rpm for 5 min to remove large cellular debris. For each immunoprecipitation, 3 μl of the anti-GST-DP5 fusion antibody was used. Samples were incubated for 1 h at 4 °C on a rocker with the antibody. Anti-rabbit IgG agarose (Sigma, 20 μl) was added to each sample followed by another 1-h incubation at 4 °C on a rocker. The beads were then washed five times in the lysis buffer to reduce nonspecific binding. On the last wash, all buffer was removed, and reducing sample buffer was added to each sample. Samples were boiled and loaded onto a 4–20% gradient SDS-polyacrylamide gel. After electrophoresis, gels were transferred to Immobilon P (Millipore). Blots were preblocked in phosphate-buffered saline (PBS) containing 5% nonfat milk, and washes were performed using PBS containing 0.1% Tween 20 (PBS-T). Primary antibodies were used at 0.2–0.5% (v/v), and detection was performed with the alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim) in PBS-T with 5% nonfat milk by the alkaline phosphatase method.

**Plasmid Construction**—The DP5 cDNA DP-TW was subcloned in both sense and antisense orientation into the XbaI site of pEF-BOS (21, pEFDP5 and pEFAntidP, respectively). For the control vectors, the lacZ gene (3.5-kb cDNA containing the open reading frame of β-galactosidase) was introduced previously into pCH111, CLONTCHE1 were cloned into pEF-BOS (pEFlacZ). The rat-bcl2 cDNA was PCR using RNA from rat brain and was also cloned into the XbaI site of pEF-BOS. Microinjection Assay—Microinjection was carried out using a Narishige microinjection. Microinjection needles were pulled from glass capillaries using a vertical electrode puller and loaded using Eppendorf microinjectors. Microinjections were made directly into the nucleus. Each expression vector, pEFDP5, pEFAntidP, and pEFlacZ, was injected in 0.5 × PBS at a concentration of 0.05–0.2 μg/ml. Approximately 80–90% of SCG neurons survived after microinjection.

For analysis of the effects of expression vectors on neuronal cell death, neurons were plated at a density of 100 per cover (3 × 3 mm) and used 5–7 days after plating. Fluorescein isothiocyanate-labeled goat anti-mouse IgG (The Jackson Laboratory) was used to mark the injected cells in these experiments and was added to the injection mixture at a final concentration of 0.5 μg/ml. The neurons were maintained in the presence of NGF, and the numbers of living cells were counted based on morphological criteria and trypan blue staining at various time points after microinjection. For X-gal staining of neurons expressing β-galactosidase, neurons were fixed in 4% formaldehyde (in 0.1 M sodium citrate) for 2 min on ice. After washing twice, in situ labeling of apoptosis-induced DNA strand breaks was performed using an In situ Cell Death Detection Kit, fluorescein (Boehringer Mannheim). The cells were analyzed directly under a fluorescence microscope.

**RESULTS**

**Identification of DP5, a Gene Induced during Neuronal PCD**—Previously, the commitment point for cell death in the culture model, as defined by the ability of half of the neurons to be rescued from death by readdition of NGF or addition of cycloheximide (CHX), has been shown to occur after approximately 15–17 h (23, 24), and this was confirmed by our results (data not shown). Sufficient quantities of “death gene” or “death-related gene” products and their mRNA would have accumulated by this time. Therefore, we collected mRNAs from SCG cultures 15 h after NGF withdrawal. The control mRNAs were isolated from SCG neurons which were cultured in medium containing NGF. These mRNAs were reverse-transcribed and screened for genes induced during neuronal PCD by the differential display technique (see “Experimental Procedures”). Although most of the bands observed in this screening showed the same patterns in both control neurons and those in which PCD was induced, we isolated one clone (DP5), the expression of which was strongly induced during cell death, and the length of the cDNA fragment amplified by PCR was 630 bp (designated as clone DP-PCR, Fig. 1). The nucleotide sequence of the cDNA fragment showed no homology with those of known genes registered in the EMBL, GenBankTM, and DDBJ data bases. By Northern blot analysis, a cDNA probe synthesized from 630 bp of the DP5 cDNA DP-PCR was shown to hybridize with a single mRNA species of about 5.5 kb, and expression of DP5 mRNA was specific to the neurons in which PCD was induced (Fig. 2A).

The sequence of events that occur following removal of NGF from differentiated PC12 cells is very similar to that in sympathetic neurons following removal of NGF (3), and the commitment point in the neuronal PC12 cell model system is considered to be 12–15 h after NGF deprivation (25). To show that induction of DP5 is not specific to SCG neurons, we examined expression of the DP5 gene during PCD of neuronal PC12 cells.
As shown in Fig. 2B, PC12 cells cultured in medium containing NGF expressed DP5 mRNA only faintly, whereas those deprived of NGF showed elevation of DP5 mRNA expression. The level of expression of DP5 mRNA was higher 15 h after removal of NGF, and this time was consistent with the commitment point for PCD in this model.

We next examined tissue distribution of DP5 mRNA. Among the various adult rat tissues examined, only the brain showed a hybridizing band for the DP5 mRNA (Fig. 3B). No signals were detected in other tissues examined even if the membrane was exposed to film for 7 days. Therefore, it appears that expression of the DP5 gene could be highly specific to the nervous system.

DP5 Encodes a Unique Polypeptide—We then isolated and determined the full nucleotide sequence of DP5 cDNA. The cDNA consists of 5253 nucleotides, and many stop codons were found in this nucleotide sequence. There were three possible open reading frames (ORF) in all three frames. In all cases, these ORFs encoded small polypeptides which consisted of about 100 amino acids. To determine the ORF of DP5, we isolated a cDNA of mouse DP5 homolog from newborn mouse brain and characterized it. Comparison of a 279-bp segment of an ORF at its 5' end, which has the 5'-most ATG among the cDNA clones isolated, from rat and mouse revealed only one nucleotide change, and this change did not alter the predicted amino acid sequence of the ORF (Fig. 3B). In contrast, the other regions of mouse DP5 cDNA including another two possible ORFs showed 84% homology with those of rat cDNA. These observations suggest that there is selective pressure to conserve this 279-bp segment as a protein coding region, and DP5 encodes a polypeptide of 92 amino acids. Data base analysis showed that DP5 had no homology with any other known protein and contained no motif that would indicate its putative biochemical functions. Hydrophobicity analysis indicated that DP5 does not contain a signal sequence or any transmembrane regions.

To demonstrate that the DP5 polypeptide is indeed expressed during neuronal death, a rabbit polyclonal anti-GST-DP5 fusion protein antibody was raised against proteins produced in a baculovirus expression system, and immunoprecipitation followed by Western blotting was carried out. An immunoreactive band of approximately 10 kDa was detected in Sf21 insect cell homogenates expressing a 1.5-kbp cDNA fragment containing the ORF (clone DP-7W, Fig. 4). This immunoreactive band was also observed in SCG neurons 24 h after NGF deprivation, but not in those of control neurons.

Expression of DP5 mRNA during PCD of SCG Neurons—To examine the temporal changes in levels of DP5 mRNA during PCD, we reverse-transcribed mRNAs isolated from SCG cultures before and at varying times after NGF withdrawal and analyzed proportions of DP5 cDNAs using RT-PCR (Fig. 5A). We also analyzed the expression patterns of control cellular marker genes including the neuronal gene neurofilament-M (NF) and the glial and Schwann cell marker S-100β. Expression of S-100β did not significantly change over the 25-h period of NGF withdrawal. PCR products of NF diminished over the course of PCD. Thus, mRNA levels for neuronal and non-neuronal genes decreased or did not change during PCD. The DP5 expression pattern during PCD was in contrast with the patterns of expression of the control markers described above. A relatively low level of DP5 expression was detected before
NGF withdrawal. This signal increased at 5 h and was maximal at 15 h after removal of NGF with a subsequent reduction after this time point. The temporal changes in DP5 mRNA expression during PCD matched the expression pattern predicted for putative cell death genes or death-related genes, i.e. the peak of DP5 expression occurred concurrently with the commitment point for PCD as determined by CHX rescue after NGF withdrawal.

We next examined the changes in DP5 mRNA expression on treatment of SCG neurons with various agents which prevent neuronal PCD, i.e. CHX, KCl (26, 27), and the cyclic AMP analogue CPTcAMP (24, 28). To test the abilities of various agents to promote survival, SCG neurons deprived of NGF were cultured for 24 h with or without various agents, and the number of survived neurons were counted. Without additives, approximately 90% of the cells died, while good survival was promoted in the presence of CHX, KCl, or CPTcAMP (Fig. 5B). After neuronal cultures were deprived of NGF for 15 h in the presence or absence of various agents, total RNA was extracted and analyzed for changes in DP5 gene expression by RT-PCR. Levels of products amplified by DP5-specific primers were markedly decreased in all cases treated with cell death blockers (Fig. 5C). Quantification of the DP5 signals showed that the expression levels of DP5 in SCG neurons treated with various agents were approximately 5–30% of those in control neurons (Fig. 5D). The degrees of cell death prevention corresponded well with the reduction of DP5 gene expression by treatment with these agents.

**Ability of DP5 to Induce Neuronal PCD**—Having demonstrated that expression of DP5 increases after NGF withdrawal and that DP5 is closely associated with neuronal PCD, it was of interest to determine whether overexpression of DP5 alone is sufficient to cause the death of SCG neurons. We microinjected neurons with a full-length DP5 polypeptide expression vector (pEFDP5) and with antisense DP5 vector (pEFantiDP) or a β-galactosidase expression vector (pEFlacZ) as a negative control. The neurons were maintained in the presence of NGF, and the numbers of surviving cells were counted at various time points after injection. Most cells injected with pEFlacZ and pEFantiDP at a concentration of 0.1 mg/ml survived at 24 h after injection. In contrast, about 70% of neurons injected with pEFDP5 at an equivalent concentration died by this time point (Fig. 6). The cell death caused by introduction of DP5 gene began at 3 h after injection and surviving cell number was rapidly decreased at 6 h. The dying cells overexpressing DP5 showed morphological characteristics of apoptosis such as shrinkage of the cell body, pyknotic nuclei, and blebbing of the plasma membrane (Fig. 7, A–D). TUNEL (TdT-mediated dUTP nick end-labeling) staining revealed positive reactivity in the nuclei of shrinking cells injected with pEFDP5 (Fig. 7F), but those of cells injected with pEFlacZ and pEFantiDP were negative for TUNEL staining (data not shown). These results suggested that cell death induced by DP5 involved DNA fragmentation.

To verify that this type of cell death is caused by apoptosis but not by nonspecific stress or toxicity of overproduction of DP5 polypeptide, we coinjected expression plasmids of bcl-2 gene together with the DP5 gene into the SCG neurons. Most of the injected neurons did not die 24 h after microinjection of bcl-2 and DP5 expression vectors (Fig. 8). This suggested that Bcl-2 can prevent cell death induced by DP5 and that this type of cell death is not simply caused by toxicity of overproduction of DP5 polypeptide. This notion was also supported by the facts that neither β-actin nor GST expression vectors killed SCG neurons (data not shown).

**DISCUSSION**

PCD in a variety of systems requires de novo gene and protein expression, evidenced by the ability of transcriptional or translational inhibitors to block PCD (3, 29–33). This indicates that cell death genes and proteins which control the cell
death program are induced during PCD. We isolated one clone, DP5, strongly induced during PCD of cultured SCG neurons. The putative ORF of DP5 gene was very small and was located at the 5' end of the 5.3-kbp DP5 cDNA. Although this was not a usual position of ORF, we consider it valid for the following two reasons. 1) The mouse DP5 homolog shows a very high degree of conservation of the nucleotide sequence within this ORF. 2) In Western blotting analysis, the polyclonal DP5 antibody reacted with a 10-kDa band in SCG neurons undergoing PCD, which is consistent with the size of the protein predicted from ORF. This gene and gene product showed no homology with any other known genes or proteins and contained no motif that may indicate a biochemical function. DP5, therefore, may represent the prototype of a novel type of cell death gene. Another unique feature of DP5 gene is that it is expressed only in the brain in adult rats, and no expression was detected in other tissues. If the DP5 gene is also closely associated with the death program in vivo as shown in the in vitro study, cell death cascades in the nervous system, which accompany induction of DP5, may differ from those of other tissues. In support for this

**Fig. 5. RT-PCR analysis of DP5 expression.** A, time course of DP5 mRNA expression during cell death of SCG neurons. Primary cultures (10-cm dishes) were maintained with NGF for 5–7 days and then deprived of NGF for the indicated intervals. Total RNA was isolated, and 3-μg aliquots of RNAs were reverse-transcribed. Then 1/60 volume of cDNA solution was used to examine expression of the following genes by PCR (number of PCR cycles in parentheses): DP5 (24 cycles), neurofilament-M (NF, 22 cycles), and S-100β (24 cycles). The amplified DNAs were separated on 5% polyacrylamide gels and visualized by autoradiography. The identity of each PCR product was confirmed by DNA sequencing. B, neuronal death prevented by various agents. After SCG cultures were deprived of NGF for 24 h in the presence or absence of 1 μg/ml CHX, 35 mM KCl, or 400 μM CPTcAMP, the number of dying cells was counted. The data (mean ± SD) shown were the percentages of dead cells. Data were collected from at least five independent experiments. C, using RT-PCR assay, analysis of changes of DP5 expression 15 h after NGF deprivation in the cases treated with various cell death blockers. NF was used as an internal control. D, quantification of changes of DP5 expression suppressed by cell death blockers. Changes in the levels of DP5 were quantified by Scanning Imager (Molecular Dynamics) analysis of polyacrylamide gels such as that shown in C. Changes were determined relative to the DP5 expression levels in the control with deprivation of NGF and represent the means from three analyses.
The expression of DP5 was not detected during cell death of thymocytes induced by glucocorticoid (data not shown). Whether DP5 overexpression can induce cell death in other types of cells is an interesting point and is now under investigation in our laboratories.

The induction of DP5 mRNA during neuronal PCD was demonstrated using RT-PCR and Northern blotting. During PCD, DP5 mRNA began to accumulate 5 h after NGF withdrawal and reached maximal levels at 15 h, with a subsequent gradual decrease by 25 h. The peak of DP5 expression correlated well with the commitment point for cell death (15–17 h) as determined by CHX rescue after NGF deprivation (23, 24). DP5 was not induced immediately early, but many hours after the initial stimulus of NGF removal, suggesting that DP5 was induced in response to intracellular events. Indeed, the induction of DP5 expression was blocked by CHX. This finding suggests that DP5 could be a part of a cellular genetic program that required ongoing protein synthesis.

Cell death was prevented by treatment with high K\(^+\), which depolarized neurons and raised intracellular Ca\(^{2+}\) levels (26, 27), or by treatment with cAMP analogue CPTcAMP (23, 28). In these cases, the expression of DP5 was markedly suppressed. The degrees of suppression of DP5 expression corresponded with those of cell death blockade by these agents. The mechanisms by which these agents prevent the death program remain unclear. However, the results suggest that DP5 expression is closely associated with proceedings of neuronal death, and DP5 is likely to be a constituent of the death program in SCG neurons.

Neurons microinjected with pEFDP5 to overexpress DP5 gene products underwent apoptosis even in the presence of NGF. Cells into which DP5 was introduced showed typical morphological characteristics of apoptosis such as shrinkage of the cell body, blebbing of the plasma membrane, and DNA fragmentation as evidenced by TUNEL staining. Deckwerth and Johnson (24) reported morphological changes of sympathetic neurons after removal of NGF. In this paper, 19 h after NGF withdrawal, atrophy was first detectable in half of the neuronal somas, and the plasma membrane lost its smooth appearance and its cellular outline became increasingly irregular. These observations corresponded with morphological changes of neurons injected with DP5 expression vectors, suggesting that the cells into which the DP5 constructs were introduced could be activated death pathway and die in an apoptotic fashion. In contrast, cells injected with antisense DP5, lacZ, \(\beta\)-actin, or GST expression vectors showed no morphological changes and did not die. In addition, most of the neurons co-injected with \(\beta\)cI-2 and DP5 did not die. Taking these results into account, it is unlikely that the cell death was caused by nonspecific toxicity of DP5 overproduction. Injecting DP5 kills neurons rapidly even in the presence of NGF. This suggests that DP5 may be having an effect late in the death processes unlike most other “killer genes” such as c-jun and myc. As described above, the induction of DP5 expression was inhibited by CHX or some other cell death blocker, i.e. it is required for ongoing protein synthesis and may be needed for execution of cell death. However, we cannot unveil the molecular cascades of neuronal apoptosis induced by DP5 in the present study. There are several precedents which can induce apoptosis of neuronal cells, such as Bak (34) and c-Jun (17). Whether DP5 interacts or associates with these proteins must be analyzed further to understand the mechanisms of DP5-induced cell death.

In conclusion, the present study strongly suggests that DP5 is likely to be one of the constituents of the genetic program of neuronal PCD. Whether DP5 is required for this phenomenon hypothesis, the expression of DP5 was not detected during cell death of thymocytes induced by glucocorticoid (data not shown). Whether DP5 overexpression can induce cell death in other types of cells is an interesting point and is now under investigation in our laboratories.

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In conclusion, the present study strongly suggests that DP5 is likely to be one of the constituents of the genetic program of neuronal PCD. Whether DP5 is required for this phenomenon
or not is open for further analysis. Experiments to test this question are currently in progress, using gene knockout technologies. Furthermore, identification of the protein(s) which interact(s) with the DP5 polypeptide and transcription factors which regulate DP5 gene expression are necessary to elucidate the putative DP5-related cell death cascade.

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Addendum—When this manuscript was accepted for publication, we noticed a paper that described cloning of a gene, harakiri (Hrk), which was significantly homologous with DP5 (35). Judging from the differences of sizes of the transcripts and expression patterns, DP5 seems not to be a rat homologue of Hrk, but to constitute a gene family with Hrk.

Both proteins can induce apoptosis when overexpressed in the cultured cells. We do not know whether DP5 can specifically interact with BCL-2 and BCL-Xl as Hrk does. This aspect is now under investigation.

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