The p53-activated Gene, PAG608, Requires a Zinc Finger Domain for Nuclear Localization and Oxidative Stress-induced Apoptosis*

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The p53-activated gene PAG608, which encodes a nuclear zinc finger protein, is a p53-inducible gene that contributes to p53-mediated apoptosis. However, the mechanisms by which PAG608 is involved in the apoptosis of neuronal cells are still obscure. In this study, we demonstrated that expression of p53 was induced by 100 μM 6-hydroxydopamine (6-OHDA), accompanied by increased PAG608 expression in PC12 cells. On the other hand, transient or permanent transfection of antisense PAG608 cDNA into PC12 cells significantly prevented apoptotic cell death induced by 100 μM 6-OHDA or 200 μM hydrogen peroxide but not by 250 μM 1-methyl-4-phenylpyridinium ion. The 6-OHDA-induced activation of caspase-3, DNA fragmentation, loss of mitochondrial membrane potential, and induction of p53 and Bax were also prevented in PC12 cells that stably expressed antisense PAG608 cDNA. These results suggest that PAG608 is associated with the apoptotic pathway induced by these oxidative stress-generating reagents, upstream of the collapse in the mitochondrial membrane potential in PC12 cells. Interestingly, transient transfection with PAG608 cDNA increased p53 expression in both PC12 cells and B65 cells, indicating that PAG608 induced by p53 is able to induce p53 expression in these cells inversely. Furthermore, transient transfection of a truncated mutant PAG608 cDNA, lacking the first zinc finger domain, inhibited 6-OHDA-induced cell death and altered the nuclear and nucleolar localization of wild-type PAG608 in PC12 cells. These results suggest that PAG608 may induce or regulate p53 expression and translocate to the nucleus and nucleolus using its first zinc finger domain during oxidative stress-induced apoptosis of catecholamine-containing cells.

PAG608 expression has been reported to increase in ischemia-sensitive brain regions, accompanied by accumulation of p53, caspase-3, and phospho-e-Jun (3–5), and overexpression of PAG608 resulted in apoptotic cell death of human p53-null tumor cells (1, 6). Therefore, PAG608 is thought to be activated by p53 and to be associated with neuronal apoptotic cell death.

The pathogenesis of some neurodegenerative disorders has been linked to apoptosis (7–9). It is well known that 6-hydroxydopamine (6-OHDA)1 is a neurotoxin that causes selective degeneration of tyrosine hydroxylase-positive neurons and has been used to generate animal models of Parkinson's disease. Recently, using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and hematoxylin staining, apoptotic morphological changes were detected in the substantia nigra of parkinsonian rats lesioned by 6-OHDA (10, 11). Moreover, 6-OHDA is known to induce p53 expression and neuronal apoptotic cell death in cultured tyrosine hydroxylase-expressing neuronal cells (12–14). Therefore, we investigated whether PAG608 is associated with 6-OHDA-induced apoptotic cell death. We present evidence that PAG608 and p53 expression was increased by 6-OHDA treatment in PC12 cells and that 6-OHDA-induced apoptotic events, such as up-regulation of p53 and Bax, collapse in mitochondrial membrane potential and the appearance of apoptosis-related morphological changes, were inhibited in PC12 cells transfected with antisense PAG608 cDNA. We also found that overexpression of PAG608 increased p53 expression in neuronal cells. Furthermore, the first zinc finger domain-deleted mutant of PAG608 dramatically inhibited 6-OHDA-induced cell death and disturbed the nuclear and nucleolar localization of PAG608, suggesting that this first zinc finger domain is necessary for induction of apoptosis and the nuclear and nucleolar localization of PAG608.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells (Japanese Cancer Research Resources Bank, Osaka, Japan) were cultured at 37 °C in 5% CO2 in RPMI 1640 culture medium (Invitrogen) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin (Invitrogen). Rat neuroblastoma, B65 cells (European Collection of Animal Cell Culture, Salisbury, UK) were cultured at 37 °C in 5% CO2 in RPMI 1640 culture medium (Invitrogen) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin (Invitrogen).

1 The abbreviations used are: 6-OHDA, 6-hydroxydopamine; Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp p-nitroanilide; ANOVA, analysis of variance; BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; GEP, green fluorescent protein; His, polyhistidine metal-binding; JC-1, 5,5’,6,6’-tetrachloro-1,1’3,3’-tetraethylbenzimidazolycarbocya-nine iodide; MPP+, 1-methyl-4-phenylpyridinium ion; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VST, 1,2-(4-Iodophenyl)-3-(4-Nitrophenyl)-5-(2,4-Di Sulphophenyl)-2H-Tetrazolium.

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37 °C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine (Invitrogen), and 60 μg/ml kanamycin sulfate (Wako Chemical Co., Hiroshima, Japan). All culture dishes and chamber slides were purchased from BD Biosciences.

Hoechst 33342 Staining Assay—PC12 cells (1 × 10⁵ cells/cm²) or B65 cells (2.5 × 10⁶ cells/cm²) were plated on two-well collagen I-coated or noncoated chamber slides, respectively. After an overnight culture, cells were treated with 25–200 μM 6-OHDA for 8 h and stained with 10 μg/ml Hoechst 33342 (Molecular Probes). Nuclear morphology was examined by fluorescence microscopy using a mercury lamp through a 360–370 nm bandpass filter to excite Hoechst 33342.

Construction of Expression Vectors—To obtain rat PAG608 cDNA expression vector, a fragment of PAG608 cDNA was ligated between the EcoRI and KpnI sites in pcDNA1.1 (+) (Invitrogen). To generate a polynucleotide metal-binding (His)-tagged wild-type PAG608 construct, a cDNA fragment encoding the entire open reading frame of PAG608 was amplified by PCR using the antisense PAG608 expression vector as a template, lower primer uHis/PAG, 5'-gggaattctgatgattctttt-3', and upper primer 5'-ccgctcgagcggtcactgaacgtagccca-3'. The fragments were ligated independently between the EcoRI and XhoI sites in pcDNA3.1/HisA (Invitrogen). His-tagged mutant PAG608 constructs (ΔC582 and ΔC741) were generated by amplifying cDNA fragments encoding amino acids 1–194 and 1–174 by PCR using the antisense PAG608 expression vector as a template, with upper primer uHis/PAG and lower primers 5'-gttacatgctggccgaggtTG-3' and 5'-gttacatgctggccgaggtTG-3', respectively. The fragments were ligated independently between the EcoRI and EcoRV sites in pcDNA3.1/HisA. His-tagged mutant PAG608 constructs (ΔN207 and ΔN522) were constructed by amplifying cDNA fragments encoding amino acids 69–289 and 174–289 by PCR using the antisense PAG608 expression vector as a template, lower primer ΔN207 and ΔN522, and upper primers 5'-gggaattctgatgattctttt-3' and 5'-gggaattctgatgattctttt-3', respectively. The fragments were ligated independently between the EcoRI and EcoRV sites in pcDNA3.1/HisA. A green fluorescent protein (GFP)-tagged wild-type PAG608 construct was generated by amplifying a cDNA fragment encoding the PAG608 open reading frame by PCR using the antisense PAG608 expression vector as a template, upper primer 5'-tcgagggatccctttgcttgatgattc-3' and lower primer 5'-ccgaatttcggagtgccgttgagtccgtctg-3', and ligation the product between the XhoI and Smal sites in pEGFP-C2 (Clontech Laboratories, Palo Alto, CA), respectively.

Analysis of Cell Viability by a Modified Standard MTT Assay—PC12, PC12/PAG608AS and/or PC12/CTL cells (1 × 10⁵ cells/cm²) were plated on each well of 96-well collagen I-coated plates. After a 24-h attachment period, these cells were treated with 50–100 μM 6-OHDA or 50–200 μM hydrogen peroxide (H₂O₂) (Wako). At 24 h after treatment, the cell viability was assessed by quantitative colorimetric assay with WST-1 (DOJINDO, Kumamoto, Japan), a modification of the standard MTT assay (20).

Analysis of Cell Viability by Trypan Blue Exclusion Assay—PC12 or PC12/PAG608AS cells (5 × 10⁵ cells/ml) were plated on each well of six-well collagen I-coated plates as reported previously (21). After a 24-h attachment period, the cells were treated with 50 μM 6-OHDA or 50–200 μM hydrogen peroxide (H₂O₂) (Wako). At 48 h after treatment, the cell viability was assessed by quantitative colorimetric assay with WST-1 (DOJINDO, Kumamoto, Japan), a modification of the standard MTT assay (20).

Analysis of Caspase-3 Activity—To determine caspase 3 activity in PC12 cells and PC12/PAG608AS cells treated with 100 μM 6-OHDA, a caspase-3 assay kit (Sigma) was used. PC12 or PC12/PAG608AS cells (1 × 10⁵ cells/cm²) were plated on six-well collagen I-coated plates. After a 24-h attachment period, the cells were treated with 50 μM 6-OHDA or 50–200 μM hydrogen peroxide (H₂O₂) (Wako). At 4 h after treatment, total cell lysates from PC12 cells and PC12/PAG608AS cells were prepared (50 μg/μl HEPE, pH 7.4, 5 μg CHAPS, and 5 μg dithiothreitol), and then were incubated with 0.2 μM 4-acetyl-Asp-Glu-Val-Asp-NA, a colorimetric substrate for caspase-3 (Ac-DEVD-pNA). A colorimetric substrate for caspase-3 at 37 °C for 90 min. The activity of caspase-3 was determined by measuring of absorption of paranitroanilide release from Ac-DEVD-pNA.

TUNEL Assay—To detect cells undergoing apoptosis, a colorimetric TUNEL system (Promega, Madison, WI) was used. PC12 or PC12/PAG608AS cells (1 × 10⁵ cells/cm²) were plated on six-well collagen I-coated plates. After a 24-h attachment period, the cells were treated with 50 μM 6-OHDA. At 4 h after treatment, total cell lysates from PC12 cells and PC12/PAG608AS cells were prepared (50 μg/μl HEPE, pH 7.4, 5 μg CHAPS, and 5 μg dithiothreitol), and then were incubated with 0.2 μM 4-acetyl-Asp-Glu-Val-p-NCA, a colorimetric substrate for caspase-3 at 37 °C for 90 min. The activity of caspase-3 was determined by measuring of absorption of paranitroanilide release from Ac-DEVD-pNA.

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Analysis of Membrane Potential by the TUNEL Assay—After overnight culture of PC12 cells (1 × 10⁵ cells/cm²) in a 60-mm collagen I-coated dish, 10 μM of empty or antisense PAG608 cDNA expression vector was cotransfected with 2 μg of pcDNA/Hygro/AscZ plasmid (Invitrogen) encoding the β-galactosidase gene into cultured cells using the calcium phosphate method (17). Cells were transfected with BglII-digested PAG608 plasmid (18). At 24 h after transfection, the cells were treated with 100 μM 6-OHDA for a further 24 h. GFP-positive cells were visualized by fluorescence microscopy using a mercury lamp through a 470–490 nm bandpass filter to excite GFP.

of Transfected Cells Stably Expressing Antisense PAG608—To establish the empty vector-transfected PC12 cell line (PC12/CTL) and antisense PAG608 cDNA-transfected PC12 cell line (PC12/PAG608AS), PC12 cells were seeded at a density of 6 × 10⁶ cells/60-mm dish. After overnight culture, 10 μg of empty or antisense PAG608 expression vector was transfected into cultured cells using the calcium phosphate method, and the medium was replaced with BSS. After 72 h in 5% CO₂ at 37 °C. The cells were trypsinized and replated at 4 × 10⁵ cells/100-mm dish and then incubated for 24 h. The culture medium was replaced with fresh culture medium containing 400 μg/ml Genetin and thereafter replaced every 3–4 days. Individual Geneticin-resistant colonies were isolated 2 weeks later and expanded into cell lines.

Western Blot Analyses—Total cell lysates from PC12 cells and transformant PC12 cells (PC12/CTL and PC12/PAG608AS) treated with 100 μM 6-OHDA for various times were prepared with 10 μg/ml phenylmethylsulfonyl fluoride (Sigma) in ice-cold RIPA buffer (PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). To overexpress PAG608, 2 μg of empty vector, pcDNA3.1/HisA, or His-tagged wild-type PAG608 construct was transfected into PC12 cells (1 × 10⁵ cells/cm²) and B65 cells (2.5 × 10⁵ cells/cm²) by lipofection. Total cell lysates from these PAG608 transiently transfected cells were also prepared 24 or 48 h after the treatment. Western blot analysis was performed as described previously (19). Blots were incubated with rabbit anti-rat PAG (Novagen, Madison, WI, UK, 1:300 dilution), rabbit anti-rat Bax (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution), rabbit anti-rat Bcl-2 (Santa Cruz Biotechnology; 1:150 dilution), or goat anti-rat PAG608 (Santa Cruz Biotechnology; 1:150 dilution) polyclonal antibody and then reacted with donkey anti-rabbit or rabbit anti-goat secondary antibody conjugated to horseradish peroxidase (Chemicon, Temecula, CA). After washing with 20 mM Tris-buffered saline containing 0.1% Tween 20, blots were developed using the ECL Western blotting detection system (Amersham Biosciences) according to the protocol provided by the manufacturer. Sample loading and transfer were normalized by using 2 mg/ml Pronase S solution or goat anti-action polyclonal antibody (Santa Cruz Biotechnology; 1:150 dilution).
were used to excite the green fluorescent JC-1 monomer. The 543 nm line of a helium-neon laser without attenuation was used to excite red fluorescent J-aggregates formed by JC-1. Light emitted from the green fluorescent JC-1 monomer was collected through a 560 nm longpass filter. Filtered beams were detected by photomultiplier tubes. Images were recorded with a 20 x objective (Plan NeoFluar, 0.50 NA or 0.30 NA, Zeiss) at 2 x zoom (0.9 μm/pixel).

Redistribution of GFP-tagged Wild-type PAG608—PC12 cells (1 x 10^5 cells/cm^2) were plated on each well of two-well collagen I-coated chamber slides. After a 24 h attachment period, 2 μg of empty or His-tagged wild-type or mutant PAG608 expression vectors were cotransfected with 1 μg of GFP-tagged wild-type PAG608 cDNA expression vector into cultured cells by lipofection. At 24 h after transfection, the cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer and then stained with 10 μg/ml Hoechst 33342. GFP-tagged wild-type PAG608 transfornant cells and nuclei stained with Hoechst 33342 were visualized by fluorescence microscopy using a mercury lamp through a 470–490 nm bandpass filter and 360–370 nm bandpass filter to excite GFP and Hoechst 33342, respectively.

Statistical Analyses—All data were expressed as the mean ± S.E. Differences between groups were examined for statistical significance using one- or two-way ANOVA, followed by a post hoc Fisher’s PLSD test.

RESULTS

6-OHDA Induces PAG608 in Catecholaminergic Neuronal Cell Lines—In rat catecholaminergic neuronal PC12 cells and B65 cells, small but significant increases in the proportions of apoptotic cells appeared after exposure to 6-OHDA (PC12 cells, 50–100 μM; B65 cells, 25–50 μM) for 8 h (Fig. 1). Therefore, 6-OHDA was used in the following experiments at concentrations of 100 μM for PC12 cells and 50 μM for B65 cells. p53 protein expression was induced in PC12 cells at 3–12 h after treatment with 100 μM 6-OHDA (Fig. 2, A and C), coinciding with the 6-OHDA-induced p53 expression described in previous studies (12, 22). Changes in PAG608 expression were examined during 6-OHDA-induced cell death of PC12 cells. An increase in PAG608 expression in PC12 cells was detected at 6–12 h after 6-OHDA treatment (Fig. 2, A and C). In B65 cells, 50 μM 6-OHDA treatment also led to induction of PAG608 expression after an increase in p53 (Fig. 2B).

6-OHDA-induced Apoptotic Cell Death Is Inhibited by Transient Transfection of Antisense PAG608 cDNA—Fig. 3 shows the effects of inhibition of PAG608 expression (by transient transfection of an antisense PAG608 cDNA) on 6-OHDA-induced apoptotic cell death in PC12 cells. There were no morphological differences between β-galactosidase-positive PC12 cells transfected with the antisense PAG608 cDNA expression vector and empty vector (Fig. 3, A and B). A recent study reported that apoptosis-related morphological changes in PC12 cells were detectable 8 h after treatment with 6-OHDA (23). Therefore, we exposed transfected PC12 cells to 100 μM 6-OHDA for 8 h and examined cell morphology. Nearly 11% of β-galactosidase-positive cells transfected with an empty vector exhibited shrunken cell size and condensed chromatin after treatment with 6-OHDA (Fig. 3, C and E). In contrast, cells transfected with antisense PAG608 cDNA remained predominantly normal and healthy after the addition of 6-OHDA, although they were β-galactosidase-positive (Fig. 3, D and E).

Attenuation of 6-OHDA and H2O2-induced Apoptotic Cell Death in Cells Stably Expressing Antisense PAG608—to confirm the association of PAG608 with apoptotic cell death, we generated a clone of stable transformant PC12 cells, which were transfected with an empty vector (PC12/CTL) or an antisense PAG608 cDNA expression vector (PC12/PAG608AS) and then selected with Geneticin. Western blot analyses using anti-PAG608 antibody revealed that the expression of PAG608 decreased in PC12/PAG608AS cells (Fig. 4A). At 24 h after treatment with 100 μM 6-OHDA, the numbers of surviving naive

![FIG. 1](image1.png)

Fig. 1. Appearance of 6-OHDA-induced apoptotic morphological changes in PC12 cells or B65 cells. Apoptotic morphological changes in PC12 cells and B65 cells were examined after treatment with 0–200 μM 6-OHDA for 8 h and Hoechst nuclear staining. Data are the mean number of apoptotic cells ± S.E. (n = 4) and represent the ratio of the number of 6-OHDA-induced apoptotic cells relative to the number in each control group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the respective control without 6-OHDA (one-way ANOVA followed by Fisher’s PLSD post hoc test).

![FIG. 2](image2.png)

Fig. 2. Immunoblot analysis of p53 and PAG608 in PC12 and B65 cells treated with 6-OHDA. Total cell lysates from PC12 cells (A) or B65 cells (B) which were treated with 6-OHDA (100 μM for PC12 cells or 50 μM for B65 cells) for 3–24 h were analyzed by Western blot using anti-p53 or anti-PAG608 antibodies. Anti-actin antibody was used to normalize for loading and transfer artifacts. C, changes in p53 and PAG608 in PC12 cells treated with 100 μM 6-OHDA. The ratio of band intensity (each protein/actin protein) was calculated, and values represent mean percentages of values in each nontreated control group ± S.E. (n = 3). *p < 0.05 compared with the respective control without 6-OHDA (one-way ANOVA followed by post hoc Fisher’s PLSD test).
and empty vector-transfected PC12 cells diminished to 52.6 ± 5.0% and 31.8 ± 2.7%, respectively, of the control (Fig. 4B). In contrast to these control cells, cell death induced by 6-OHDA was prevented in PC12/PAG608AS cells (Fig. 4B), consistent with the results after transient transfection of antisense PAG608 cDNA shown in Fig. 3. PC12/PAG608AS cells were also resistant to 200 mM H2O2-induced cell death, whereas the viability of naive PC12 cells was reduced by 42.6 ± 5.0% 24 h after the 200 mM H2O2 treatment (Fig. 5, A and B).

Because it has been reported that MPP+ causes inhibition of cell proliferation and cell death of PC12 cells, we examined the viability of PC12/PAG608AS cells treated with 250 μM MPP+ using trypan blue exclusion as reported previously (21). Treatment with MPP+ inhibited cell proliferation and led to cell death of PC12/PAG608AS cells at 48 and 72 h as well as naive PC12 cells (Fig. 6).

**Antisense PAG608 Rescued PC12 Cells from 6-OHDA-induced Apoptotic Events**—It has been reported that caspase-3 mediates 6-OHDA-induced apoptosis in PC12 cells (22). Fig. 7 shows the inhibitory effects of PAG608 expression on caspase-3 activity and DNA fragmentation in PC12 cells after treatment with 6-OHDA. At 4 h after 6-OHDA treatment, no significant increase in the activity of caspase-3 was observed in PC12/PAG608AS cells in contrast to the increase in naive PC12 cells (Fig. 7A). Furthermore, no appearance of DNA fragmentation in PC12/PAG608 cells treated with 6-OHDA for 8 h was detected by TUNEL assay, although the TUNEL-positive signals were increased in 6-OHDA-treated naive PC12 cells (Fig. 7B).

PAG608 could potentially mediate 6-OHDA-induced apoptotic cell death via a collapse in mitochondrial membrane potential because the mitochondrial membrane potential is reportedly decreased during p53-induced apoptosis, and 6-OHDA is known to alter mitochondrial membrane potential and membrane permeability in PC12 cells (24–26). Therefore, using JC-1, which is an indicator of mitochondrial membrane potential, we examined the viability of PC12/PAG608AS cells treated with 250 μM MPP+ using trypan blue exclusion as previously reported (21). Treatment with MPP+ inhibited cell proliferation and led to cell death of PC12/PAG608AS cells at 48 and 72 h as well as naive PC12 cells (Fig. 6).
potential, we examined changes in the mitochondrial membrane potential in PC12/PAG608AS cells after treatment with 6-OHDA (Fig. 8). JC-1 exhibits potential-dependent accumulation in mitochondria, as indicated by a fluorescence emission shift from green (488 nm) for the reduced form of JC-1 to red (543 nm) for the oxidized form. Consequently, loss of mitochondrial membrane potential is indicated by green fluorescence. The intensity of green fluorescence was maintained in PC12 cells treated with 100 \( \mu \text{M} \) 6-OHDA for 4 h (Fig. 8B). Untreated PC12/PAG608AS cells exhibited red fluorescence, which was similar to naive PC12 cells (Fig. 8A and C). In contrast to 6-OHDA-treated PC12 cells, the intensity of red fluorescence in PC12/PAG608AS cells treated with 100 \( \mu \text{M} \) 6-OHDA was similar to that in untreated PC12 cells (Fig. 8D).

**Inhibition of 6-OHDA-induced p53 and Bax Expression in Antisense PAG608-expressing Cells**—Because the mitochondrial membrane potential in PC12/PAG608AS cells was not disrupted by 6-OHDA (Fig. 8D), we investigated the effects of inhibition of PAG608 expression on several apoptosis-related molecules that are particularly associated with a collapse in mitochondrial membrane potential. Western blot analyses

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**Fig. 6. Effect of MPP⁺ on growth and survival of PC12/PAG608AS cells.** PC12 cells or PC12/PAG608AS cells were untreated or treated with 250 \( \mu \text{M} \) MPP⁺ for 24–72 h, and cell viability was measured by trypan blue exclusion. Data are the mean numbers of live cells (trypan blue-negative) ± S.E. (n = 3). *p < 0.05, **p < 0.001 compared with each control (0 h); +p < 0.05, ++p < 0.01, +++p < 0.005 compared with the respective time-matched untreated group (two-way ANOVA followed by Fisher’s PLSD post hoc test).

**Fig. 7. Analyses of 6-OHDA-induced apoptotic events, caspase-3 activity, and DNA fragmentation in PC12/PAG608AS cells.** Total cell lysates from naive PC12 cells or PC12/PAG608AS cells that were treated with 100 \( \mu \text{M} \) 6-OHDA for 4 h were assayed for caspase-3 activity using a colorimetric substrate, Ac-DEVD-pNA (A). Data are the mean ± S.E. (n = 3). *p < 0.001 between the two indicated groups (one-way ANOVA followed by Fisher’s PLSD post hoc test). The appearance of DNA fragmentation in naive PC12 (B and C) and PC12/PAG608AS cells (D and E) untreated (B and D) or treated with (C and E) 100 \( \mu \text{M} \) 6-OHDA for 8 h was detected by TUNEL assay. Arrows indicate TUNEL-positive cells. Bar = 50 \( \mu \text{m} \).
showed that expression of both p53 and Bax was increased by 100 μM 6-OHDA in naive PC12 cells. In contrast, neither p53 nor Bax expression was induced by 100 μM 6-OHDA in PC12/PAG608AS cells (Fig. 9, A and B). Treatment of both naive PC12 and PC12/PAG608AS cells with 100 μM 6-OHDA induced no changes in the expression of Bcl-2 (Fig. 9), which prevents oxidative stress-induced loss of mitochondrial membrane potential and consequent apoptosis (27, 28).

Effect of PAG608 Overexpression on p53 Expression in Catecholaminergic Neuronal Cells—Because 6-OHDA-induced p53 expression was completely inhibited in PC12/PAG608AS cells (Fig. 9 A), the effects of overexpression of PAG608 on p53 expression in PC12 cells were examined using transfection of expression vectors encoding His-tagged PAG608 or His alone (control). At 24–48 h after transient transfection with the His-tagged PAG608 expression vector, Western blot analyses confirmed expression of His-tagged PAG608 in PC12 cells. Moreover, p53 protein levels increased in His-tagged PAG608-overexpressing PC12 cells at the same time points that His-tagged PAG608 expression was observed, but this was not seen in PC12 cells overexpressing His alone (Fig. 10A). To provide additional evidence that PAG608 regulates p53 expression in other catecholaminergic neuronal cells, the His-tagged PAG608 expression vector was transfected into B65 cells. p53 protein levels increased in His-tagged PAG608-overexpressing PC12 cells at the same time points that His-tagged PAG608 expression was observed, but this was not seen in PC12 cells overexpressing His alone (Fig. 10A). To provide additional evidence that PAG608 regulates p53 expression in other catecholaminergic neuronal cells, the His-tagged PAG608 expression vector was transfected into B65 cells. p53 expression was increased in B65 cells transfected with His-tagged PAG608, in temporal association with His-tagged PAG608 expression (Fig. 10B). In contrast, transient transfection of His-tagged PAG608 expression vector failed to cause an increase in p53 expression in mouse glialoma C6 cells (data not shown).

Increased expression of p53 in response to several stresses is the result of inhibition of MDM2 activity via direct interaction of partner proteins (29). To investigate whether PAG608 and MDM2 can engage in a protein-protein interaction, PC12 cells were cultured for 48 h after the transfection with His-tagged PAG608 expression vector. Then, cell extracts were subjected to immunoprecipitation using anti-His or anti-PAG608 antibody followed by Western blot analysis using anti-MDM2 antibodies. The ratio of band intensity (each protein/actin protein) was calculated, and values represent the mean percentages relative to values of each untreated control group ± S.E. (n = 3). *p < 0.05 compared with the respective control without 6-OHDA. +p < 0.01, +++p < 0.005 compared with the dose-matched naive PC12 cells (two-way ANOVA followed by post hoc Fisher’s PLSD test).

FIG. 8. Effect of down-regulation of PAG608 expression on 6-OHDA-induced loss of mitochondrial membrane potential. Naive PC12 cells (A and B) or antisense PAG608 cDNA-expressing transformant PC12/PAG608AS cells (C and D) were untreated (A and C) or treated with (B and D) 100 μM 6-OHDA. Cells were stained with JC-1 after 4-h 6-OHDA treatment, and then fluorescent signals were observed using confocal laser scanning microscopy. Bar = 50 μm.

FIG. 9. Immunoblot analyses of p53, Bax, and Bcl-2 in PC12/PAG608AS cells exposed to 6-OHDA. Total cell extracts from naive PC12 cells and PC12/PAG608AS cells that were treated with 100 μM 6-OHDA for 6, 12, and 24 h were analyzed by Western blot using anti-p53 (A), anti-Bax (B), and anti-Bcl-2 (C) antibodies. The ratio of band intensity (each protein/actin protein) was calculated, and values represent the mean percentages relative to values of each untreated control group ± S.E. (n = 3). *p < 0.05 compared with the respective control without 6-OHDA. +p < 0.01, +++p < 0.005 compared with the dose-matched naive PC12 cells (two-way ANOVA followed by post hoc Fisher’s PLSD test).

N-terminal Truncation of PAG608 Prevents 6-OHDA-induced Cell Death—PAG608 contains three C2H2-type zinc finger domains and a putative nuclear localization signal (2), and these domains appear to be essential for 6-OHDA-induced cell death. To clarify the roles of the zinc finger domains of PAG608 in 6-OHDA-induced cell death, we generated a His-tagged mutant PAG608 expression vector, His-ΔN522, which encoded the first zinc finger domain-truncated PAG608 (Fig. 11). An expression vector encoding GFP was cotransfected with His-ΔN522 or the control vector into PC12 cells. There was no difference between the number of GFP-positive PC12 cells transfected
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Fig. 10. PAG608 cDNA transfection induces p53 expression in catecholaminergic neuronal cells. His-tagged PAG608 or control vector was transiently transfected into PC12 cells (A) or B65 cells (B). At 24 and 48 h after transfection, total cell extracts were prepared and then analyzed by Western blot using anti-PAG608, anti-p53, and anti-actin antibodies.

Fig. 11. Schematic construction of His-tagged truncation mutants of PAG608. PAG608 contains three zinc finger domains. The cDNAs encoding wild-type and four different deletion mutants of PAG608 were fused with the C-terminal of His tag in the pcDNA3.1/HisA vector.

with ΔN522 and empty control vector (Fig. 12, A, B, and E). After treatment with 100 μM 6-OHDA, the number of GFP-positive cells transfected with control vector was reduced significantly to 25.8% of control (Fig. 12, C and E). In contrast, ΔN522-transfected PC12 cells were resistant to 6-OHDA-induced cell death (Fig. 12, D and E). Furthermore, three other His-tagged mutant PAG608 expression vectors, ΔN207, ΔC582, and ΔC741, were generated (Fig. 11) to evaluate the roles of other domains of PAG608 in 6-OHDA-induced cell death. After treatment with 6-OHDA, the number of GFP-positive cells transfected with these three mutant constructs decreased to levels similar to those noted after transfection with the control vector (data not shown).

Nuclear Localization of GFP-tagged Wild-type PAG608 Is Disrupted by a Truncated Mutant, ΔN522—PAG608 is reportedly localized in the nucleus and nucleolus of human tumor cells (1). We first confirmed PAG608 accumulation in the nucleus and nucleolus of PC12 cells, using transfection of a GFP-tagged wild-type PAG608 expression vector (data not shown). We then examined the effects of expression of a truncated PAG608 on the nuclear and nucleolar localization of PAG608 in PC12 cells. Fig. 13 shows changes in the subcellular localization of GFP-tagged wild-type PAG608 in PC12 cells after cotransfection with either a construct encoding His-tagged wild-type PAG608 or truncated mutant PAG608 (design described in Fig. 11) and nuclear staining with Hoechst 33342. The nuclear and nucleolar localization of GFP-tagged PAG608 in PC12 cells was not affected by cotransfection with the control vector or His-tagged wild-type PAG608 expression vector (Fig. 13 A, B, G, and H). In contrast to these vectors, the signal of GFP-tagged PAG608 in PC12 cells was distributed diffusely throughout the whole cell when His-ΔN522 was cotransfected (Fig. 13, C and I). Cotransfection of the other three His-tagged mutant PAG608 expression vectors (except His-ΔN522) had no effect on nuclear and nucleolar localization of GFP-tagged PAG608 (Fig. 13, D–F and J–L).

DISCUSSION

PAG608 expression is dependent on p53 (1, 5), which is increased and activated in response to various stimuli, such as reactive oxygen species and DNA-damaging reagents, to induce apoptosis and/or cell cycle arrest (30–32). Catecholaminergic neurotoxin 6-OHDA has been reported to induce an increase in p53 expression and caspase-mediated apoptosis in the PC12 cell line (12, 22). In the present study, the addition of 6-OHDA to PC12 cells resulted in elevation of PAG608 expression (6–24 h) after an increase in p53 expression (3–12 h) (Fig. 2). Furthermore, transient or permanent inhibition of PAG608 expression prevented cell death induced by 6-OHDA and H₂O₂ in PC12 cells (Figs. 3 and 4). Recently, it has been reported that addition and overexpression of catalase, which metabolizes H₂O₂, can rescue cells from 6-OHDA-induced toxicity (31, 34),
implying that the toxic effect of 6-OHDA is caused by the generation of \( \text{H}_2\text{O}_2 \), through autooxidation of the toxin.

It has been reported that generation of reactive oxygen species and inhibition of oxidative phosphorylation are considered important mediators of neuronal cell death in Parkinson’s disease (35) and that treatment with \( \text{MPP}^+ \) produces the same biochemical defect as detected in the substantia nigra of Parkinson’s disease because \( \text{MPP}^+ \) inhibits complex I of the mitochondrial electron transport chain (36). However, the effect of \( \text{MPP}^+ \) on mitochondrial membrane potential, release of cytochrome \( c \), and caspase-3 activity is less than that of 6-OHDA (37). Furthermore, \( \text{MPP}^+ \) induces cell death and inhibits cell proliferation without induction of p53 expression (21). Taken together with these reports, it is considered that \( \text{MPP}^+ \) induces cell death through action mechanism different from 6-OHDA. Indeed, the present study shows that inhibition of PAG608 expression had no effects on cell death and prevention of cell proliferation in PC12 cells by induced by \( \text{MPP}^+ \) (Fig. 6). Therefore, these results suggest that PAG608 is associated with the p53-mediated apoptotic pathway induced by 6-OHDA and \( \text{H}_2\text{O}_2 \), which generates reactive oxygen species in PC12 cells.

The toxins 6-OHDA and \( \text{H}_2\text{O}_2 \) are known to reduce the mitochondrial membrane potential, increase caspase-3 activity, and produce DNA fragmentation, which are indicators of apoptotic cell damage (22, 27, 38). In this study, an increase in caspase-3 activity and DNA fragmentation in antisense PAG608-expressing PC12 cells (PC12/PAG608AS) after 6-OHDA treatment was significantly less than that in naive PC12 cells (Fig. 7). Furthermore, the mitochondrial membrane potential in PC12/PAG608AS cells was not disturbed by treatment with 6-OHDA, whereas that in naive PC12 cells was reduced (Fig. 8), indicating that PAG608 is associated with 6-OHDA-induced apoptosis upstream of the collapse in mitochondrial membrane potential. Translocation of Bax from the cytosol to mitochondrial membranes has been reported in some apoptotic events (39, 40). Recently it was demonstrated that Bax interacts with a voltage-dependent anion channel and adenine nucleotide translocator, components of the permeability transition pore complex, which precedes loss of mitochondrial membrane potential (41, 42). Moreover, Pastorino et al. (43) suggested that cell death resulting from overexpression of Bax is mediated by mitochondrial permeability transition. Conversely, a Bax-independent p53-related pathway was also reported, whereby overexpression of p53 (by infection with p53-containing adenovirus) induced apoptotic cell death through a Bax-independent decrease in mitochondrial membrane potential (24), and the accumulation of p53 in the mitochondria preceded the change in mitochondrial membrane potential (25). These reports, taken together, suggested that alternation of the mitochondria membrane potential in the p53-induced apoptosis cascade may or may not require Bax induction. In the present study, 6-OHDA failed to increase the expression of either p53 or Bax in PC12/PAG608AS cells (Fig. 9, A and B). Although it is not clear whether p53 can induce Bax expression in PC12 cells, less induction of p53 and Bax after 6-OHDA treatment may have prevented reduction of mitochondrial membrane potential in antisense PAG608-expressing PC12 cells. In other words, PAG608 may mediate 6-OHDA-induced p53 and Bax induction in catecholaminergic cells.

Although PAG608 expression has been reported to be induced by p53 \textit{in vitro} and \textit{in vivo} (1), to our knowledge there are no studies showing that PAG608 can regulate p53 expression. The present results showed that overexpression of PAG608 by transient transfection with His-tagged PAG608 cDNA caused a marked increase in p53 expression in both PC12 cells and B65 cells (Fig. 10) but not in C6 cells. Thus, the present study is the first to show that PAG608-induced p53 expression is neuronal cell-specific. p53 expression and function are controlled by several mechanisms. One well known mechanism is regulation of protein stability. Recently, several reports showed that p53 is regulated by various molecules such as MDM2, ARF, and c-Abl. MDM2 plays a role in exporting p53 from the nucleus to the cytoplasm and promotes degeneration of p53 in the proteasome (44). In contrast, both ARF and c-Abl directly bind to MDM2 and inhibit MDM2 activity, which lead to an increase in p53 protein levels (29, 45, 46). In the present study, however, a direct interplay between PAG608 and MDM2 was not unexpectedly observed using immunoprecipitation. Although further studies are needed to clarify the mechanism underlying PAG608-induced p53 expression, the present results suggest that PAG608 induced by p53 could positively regulate p53 expression during reactive oxygen species-induced apoptosis of neuronal cells.

To our knowledge, only two zinc finger proteins, PAG608 and JAZ, are reported to be located in the nucleus and nucleolus and to be involved in apoptosis. Interestingly, the amino acid sequences of the first and second zinc finger domains of PAG608 are homologous to those of JAZ (47). The zinc finger domains of JAZ are required for nuclear and nucleolar localization as well as for apoptosis (47). In this study, 6-OHDA-induced cell death was prevented by transfection of a mutant PAG608 expression vector, \( \Delta \text{N}522 \), which lacks the first zinc finger domain (Fig. 12, D and E). Moreover, overexpression of this truncated PAG608 mutant led to altered localization of
GFP-tagged wild-type PAG608 (Fig. 13C). These results suggest that PAG608 interacts with itself or other molecules via the first zinc finger domain to facilitate translocation into the nucleus and nucleolus and that this event is an important process for reactive oxygen species-induced apoptosis.

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