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Presenilin-1 Protects against Neuronal Apoptosis Caused by Its Interacting Protein PAG

Yan Zhou,* Wanjiang Zhang,* Rachael Easton,† James W. Ray,‡ Patricia Lampe,† Zhihong Jiang,* Anne L. Brunkan,‡ Alison Goate,‡ Eugene M. Johnson,†,§ and Jane Y. Wu*,†,[1]

**Department of Pediatrics, †Department of Molecular Biology and Pharmacology, §Department of Neurology, and ‡Department of Psychiatry, Washington University School of Medicine, St. Louis, Missouri 63110*

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Mutations in the presenilin-1 (PS-1) gene account for a significant fraction of familial Alzheimer's disease. The biological function of PS-1 is not well understood. We report here that the proliferation-associated gene (PAG) product, a protein of the thioredoxin peroxidase family, interacts with PS-1. Microinjection of a plasmid expressing PAG into superior cervical ganglion (SCG) sympathetic neurons in primary cultures led to apoptosis. Microinjection of plasmids expressing wild-type PS-1 or a PS-1 mutant with a deletion of exon 10 (PS1dE10) by themselves had no effect on the survival of primary SCG neurons. However, co-injection of wild-type PS-1 with PAG prevented neuronal death, whereas co-injection with the mutant PS-1 did not affect PAG-induced apoptosis. Furthermore, overexpression of PAG accelerated SCG neuronal death induced by nerve growth factor deprivation. This sensitizing effect was also blocked by wild-type PS-1, but not by PS1dE10. These results establish an assay for studying the function of PS-1 in primary neurons, reveal the neurotoxicity of a thioredoxin peroxidase, demonstrate a neuroprotective activity of the wild-type PS-1, and suggest possible involvement of defective neuroprotection by PS-1 mutants in neurodegeneration.

Alzheimer's disease (AD) is a neurodegenerative disease with progressive memory loss and intellectual decline. Neuropathological hallmarks of AD include the presence of a large number of senile plaques and neurofibrillary tangles in the brain accompanied by loss of neurons involved in learning and memory. Significant progress has been made in identifying genetic defects causing familial Alzheimer's disease (FAD) or predisposing individuals to FAD. Analysis of the FAD families with linkage to chromosome 21 revealed the first gene implicated in FAD, the β-amyloid precursor protein (APP) gene (St George-Hyslop *et al.*, 1987; Goate *et al.*, 1991; Mullan *et al.*, 1993). Genetic studies suggest that allelic isoforms of the ApoE gene on chromosome 19 influence risk for AD. Studies over the past several years have established that two transmembrane proteins, presenilin-1 and -2, encoded by the PS-1 and PS-2 genes on chromosome 14 and 1, respectively, play an important role in pathogenesis of FAD (reviewed in Selkoe, 1997; Czech *et al.*, 2000; St George-Hyslop, 2000). Studies of FAD-associated mutations revealed that mutations in the PS-1 gene may account for up to 50% of early onset FAD cases (Kim & Tanzi, 1997; Selkoe, 1997).

Presenilin genes are expressed in many tissues, including the brain, and they encode transmembrane proteins with a large hydrophilic loop between the sixth and the seventh hydrophobic domains (Doan *et al.*, 1996; De Strooper *et al.*, 1997; Lehmann *et al.*, 1997; Nakai *et al.*, 1999). The biological roles of PS-1 and PS-2 are still not clear, although they are implicated in the intramembranous cleavage of several transmembrane proteins such as APP, Notch, and Ire1p (Borch-

[1] To whom correspondence should be addressed. Fax: (314) 286-2892. E-mail: jwu@molecool.wustl.edu.

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elt *et al.*, 1996, 1997; Duff *et al.*, 1996; Scheuner *et al.*, 1996; De Strooper *et al.*, 1998, 1999; Annaert *et al.*, 1999; Katayama *et al.*, 1999; Ni *et al.*, 2001; Niwa *et al.*, 1999; Wolfe *et al.*, 1999). Presenilins have also been implicated in regulation of apoptosis (Vito *et al.*, 1996; Haass, 1997; Kim *et al.*, 1997; Kosik, 1998; Tani *et al.*, 1998; Wolozin *et al.*, 1998; Z. H. Zhang *et al.*, 1998). PS-1 expression is induced by transient ischemia in hippocampal neurons relatively resistant to ischemic stress, but not in neurons that are susceptible to ischemia (Tanimukai *et al.*, 1998), suggesting that the induction of PS-1 gene expression may be associated with protective responses in neurons. However, overexpression of wild-type PS-2, N141I mutant PS-2, or PS-1 L286V mutant in PC12 cells rendered the cells more susceptible to death induced by different insults, including Aβ peptide (Deng *et al.*, 1996; Guo *et al.*, 1996; Wolozin *et al.*, 1998). Absence of PS-1 in skin epithelium leads to uncontrolled growth and tumor formation (Xia *et al.*, 2001).

In this study, we identified a PS-1-interacting protein of the thioredoxin peroxidase (Tpx) family. This protein is the product of the proliferation-associated gene (PAG) (Prosperi *et al.*, 1993) and has been previously identified as an oxidative stress- and serum-induced protein with antioxidant properties. When overexpressed in primary neurons, PAG causes apoptosis. Wild-type, but not a mutant, PS-1 is capable of protecting neurons in primary cultures from the apoptosis induced by PAG overexpression.

METHODS

Cell Culture and Transient Transfection

HEK 293 cells were obtained from the ATCC and maintained in 10% FBS in DMEM (Gibco). For transfection, cells were seeded at 2×10^5 cells per 35-mm dish, allowed to grow 24 h, and then transiently transfected with a total of 5 μg supercoiled plasmid DNA by the calcium phosphate method for 16–18 h (Li *et al.*, 1999). GFP plasmid was cotransfected to monitor transfection efficiency.

Antibodies, Plasmids, and Chemical Reagents

Anti-PAG antisera were raised in rabbits against recombinant PAG protein purified from an *Escherichia coli* expression system. This antibody reacted with a single band of 26-kDa protein in the brain lysate as detected by a Western blotting assay. This band was not detectable if the antiserum was preabsorbed with the purified recombinant PAG protein. R-22, a polyclonal antiserum raised against the loop region of PS-1 protein, was used for immunoprecipitation as described previously (Ray *et al.*, 1999). A monoclonal anti-PS-1 antibody kindly provided by P. Seubert (Podlisny *et al.*, 1997; Malin *et al.*, 1998) was used for Western blotting. Anti-9E10 hybridoma culture supernatant was used for Western blotting to detect myc-tagged proteins. Expression of full-length PS-1 (containing the VSRQ alternative sequence) and PAG or control proteins was under the control of the CMV promoter. PAG and SC35 were expressed as myc-tagged proteins. The green fluorescent protein (GFP) expression plasmid was Green Lantern-1 (Gibco). Bisbenzimide and Boc-aspartyl(ome)fluoromethyl ketone (BAF) were purchased from Sigma (St. Louis, MO) and Enzyme Systems Products (Livermore, CA), respectively.

Yeast Two-Hybrid Interaction cDNA Library Screening and Interaction Assay

The yeast two-hybrid interaction screening and pair-wise interaction were carried out as described in Wu and Maniatis (1993) and W. J. Zhang *et al.* (1998). Because the PS-1 loop region is rich in hydrophilic amino acid residues, we used control bait plasmids expressing proteins also rich in hydrophilic amino acid residues, such as the intracellular domain of APP [APP (ICD)] and SC35.

Coimmunoprecipitation and Western Blotting

For Western analysis of HEK 293 lysates, cells were collected 16–18 h posttransfection into 200 μl 2$\times$ Laemmli buffer including 2-mercaptoethanol, passed through a 20-gauge needle five times, and incubated at 50°C for 5 min. For co-immunoprecipitation, cells were lysed in lysis buffer containing 1% NP-40 at a protein concentration of approximately 0.4 mg/ml. Cell lysates were then cleared overnight at 4°C with 20 μl protein A–agarose beads (Repligen). Cleared lysates were then incubated at 4°C with R-22 for 1 h. Antibody–antigen complexes were captured with 40 μl protein A–agarose beads for 1 h to overnight, washed twice with Wash I (1% NP-40, 50 mM Tris, pH 7.5, 500 mM NaCl, 2 mM EDTA) and twice with Wash II (1% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA) at ambient temperature. Proteins were recovered into 35 μl 2$\times$ Laemmli buffer with DTT at 50°C

for 5 min. Cell lysates or immunoprecipitated proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes (Amersham). Western blotting was then carried out using anti-myc antibody, and the signals were detected by enhanced chemiluminescence following the manufacturer's instructions (Amersham).

Sympathetic Neuronal Culture and Microinjection

Primary sympathetic neuronal cultures were established from superior cervical ganglia (SCG) of embryonic day 21 rats using a previously described method (Easton *et al.*, 1997). Briefly, the dissected SCG were treated with 1 mg/ml collagenase for 30 min at 37°C followed by 2.5 mg/ml trypsin for 30 min at 37°C. The ganglia were then triturated with a flame-polished Pasteur pipette. About 4000 cells were plated in the center of a 35-mm dish. The SCG cells were maintained in AM 50: Eagle's minimal essential medium with Earle's salts (Life Technologies, Gaithersburg, MD), with the addition of 50 ng/ml mouse nerve growth factor (NGF) (Harlan Bioproducts, Indianapolis, IN), 10% fetal bovine serum (Sigma), 100 μg/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, and 20 μM uridine. When SCG neurons were deprived of NGF, they were rinsed once with the same medium but without NGF (AM0), followed by the addition of AM0 containing goat polyclonal anti-NGF antiserum.

Microinjection of plasmids expressing target genes was performed in SCG cells 5–7 days after the cells were plated. Plasmids were mixed in injection buffer (100 mM KCl, 10 mM potassium phosphate, pH 7.4). DNA concentrations of different plasmids in the injection solution were adjusted to the same amount (at less than 0.2 ng/μl) with vector DNA in all experiments. The vector plasmid was injected in the control group. Before injection, SCG culture medium was changed to L-15 (Life Technologies) containing 100 μg/ml penicillin and 100 μg/ml streptomycin. To mark injected cells, 4 μg/μl rhodamine dextran (Sigma) and/or a GFP-expressing plasmid (at 40 ng/μl final concentration) was added into injection solution. Femtotips (Eppendorf, Madison, WI) were used for all injections, and approximately 50 fl of injection mixture was injected into the nucleus of individual neurons. After microinjection, SCG cultures were given fresh AM50 medium. Survival of injected neurons was scored by an observer blinded to the experimental setup. The numbers of SCG neurons injected were determined 24 h after injection by monitoring under a fluorescence microscope the injected neurons that were marked with rhodamine dextran and/or GFP fluorescence. The number of viable injected neurons was determined longitudinally by counting the number of GFP-expressing or rhodamine-positive phase-bright cell bodies.

Immunocytochemistry

Two days after injection of the expression vectors, neurons were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, permeablized 10 min in PBS containing 1% Triton X-100, and then blocked with 20% fetal bovine serum, 0.05% Tween 20 in PBS. Primary monoclonal antibodies, anti-Myc (Babco) or anti-GFP (Boehringer Mannheim), were diluted 1:1000 in blocking buffer and incubated for 1.5 h at room temperature. Secondary antibodies were either Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove) diluted 1:400 from 1.5 mg/ml stock or Alexa 488-conjugated goat anti-mouse IgG diluted 1:400 from 1 mg/ml stock. After the incubation of secondary antibody for 1 h at room temperature, cells were stained for 15 min in 1 μg/ml bisbenzimide (Sigma) in PBS. Cultures were then rinsed twice with PBS and examined under a Zeiss fluorescence microscope.

RESULTS

PS-1 Interacts with PAG, a Protein of the Thioredoxin Peroxidase Family

Several proteins have been reported to interact with presenilins, including glycogen synthetase kinase-3β, tau (Takashima *et al.*, 1998), cadherin/catenin (Z. H. Zhang *et al.*, 1998; Georgakopoulos *et al.*, 1999; Kang *et al.*, 1999), calsenilin (Buxbaum *et al.*, 1998), Rab11 (Dumanchin *et al.*, 1999), G-protein G0 (Smine *et al.*, 1998), QM/Jif1 (Imafuku *et al.*, 1999), calpain (Shinozaki *et al.*, 1998), filamin (W. J. Zhang *et al.*, 1998), Bcl-2 (Alberici *et al.*, 1999), Bcl-xl (Passer *et al.*, 1999), APP (Xia *et al.*, 1997), Ire1p (Katayama *et al.*, 1999; Niwa *et al.*, 1999), and Notch (Ray *et al.*, 1999). Although the significance of these interacting proteins in neuronal function and in AD pathogenesis remains to be elucidated, these studies suggest that presenilin proteins may exert their biological effects through interactions with different proteins.

To understand the function of PS-1, we constructed a human brain cDNA library and used the PS-1 hy-

drophilic loop region as a bait to screen this library using yeast two-hybrid interaction cloning (Wu & Maniatis, 1993; W. J. Zhang *et al.*, 1998). Among the cDNA clones identified by this interaction screening, two groups of cDNAs encode members of the Tpx family: PAG and thiol-specific antioxidant protein (TSA). Detailed characterization of the interaction between TSA and presenilins will not be described here. In this paper, we will focus on the interaction between PS-1 and PAG. The gene coding for PAG was originally identified as one of the genes that were up-regulated by serum stimulation (Prosperi *et al.*, 1993). Similar to PS-1, PAG is expressed in many tissues, including the brain (Prosperi *et al.*, 1993). The PAG cDNA encodes a protein with significant sequence similarity to other members of the Tpx family. Another human Tpx protein was identified from erythroid cells as a natural killer-enhancing factor (NKEF) that augments NK cell-mediated cytotoxicity (Shau *et al.*, 1993). A murine Tpx was found to be expressed in the brain regions most susceptible to hypoxia and ischemia (Ichimiya *et al.*, 1997), and murine macrophage Tpx protein (MSP23) was shown to be induced by oxidative stress (Ishii *et al.*, 1993). The biological functions of these Tpx proteins are not clear.

After confirming the specific interaction of PAG with PS-1 by testing with other unrelated bait proteins using the yeast two-hybrid assay, we examined whether PS-1 interacted with PAG in mammalian cells. Human embryonic kidney (HEK) cells were transfected with either the vector control or a plasmid expressing PAG as a 6× myc-tagged protein. Immunoprecipitation was carried out using a previously characterized anti-PS1 antibody (Ray *et al.*, 1999), and the presence of myc-tagged PAG in the immunoprecipitates was detected by a monoclonal anti-myc antibody. As predicted from the sequence, the PAG protein tagged with the 6× myc tag migrated at a molecular weight of approximately 40 kDa (Fig. 1A, lane 1). The PAG protein was coprecipitated with PS-1 when cells were cotransfected PS-1 (Fig. 1A, lane 4). In the absence of PAG–myc transfection, this band was not detectable (Fig. 1A, lane 3), suggesting the specificity of the signal. To confirm that the observed PAG–PS1 interaction was specific, the PS-1 plasmid was cotransfected with an unrelated myc-tagged protein rich in hydrophilic amino acid residues, SC35 (Jiang *et al.*, 1998), and similar immunoprecipitation was carried out. Although the expression level of SC35 was comparable to that of PAG (lanes 1 and 5), SC35 was not detectable in the immunoprecipitates (Fig. 1A, lane 6). To test whether PAG interacted with endogenous PS-1, coimmunoprecipitation was carried out in cells

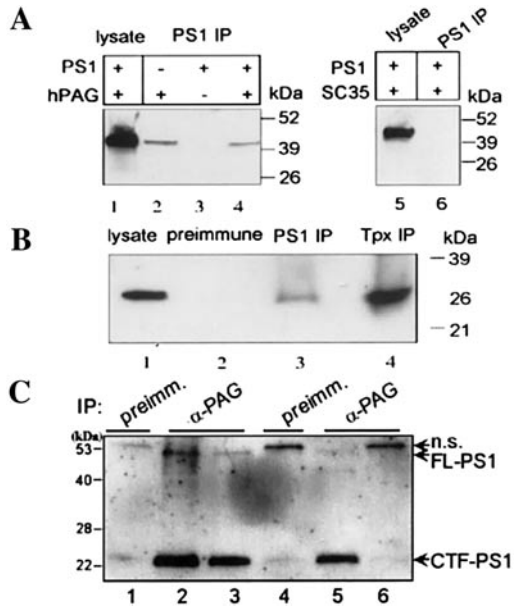

FIG. 1. PAG interacts with PS-1 in transfected cells and in murine brain extracts. (A) HEK cells were transfected with PS-1 or PAG–myc or control SC35–myc plasmids in different combinations as shown above the gel. Immunoprecipitation by anti-PS-1 (Ray, 1999) was carried out using cell lysates prepared from transfected cells. Western blotting was performed on the cell lysates or the immunoprecipitated proteins using a monoclonal anti-myc antibody. (B) Cell lysates were prepared from the murine brain tissue. Immunoprecipitates were obtained using preimmune serum or anti-PAG or anti-PS-1. Western blotting was then performed using anti-PAG antibody on the brain lysate (lane 1) and immunoprecipitates formed with preimmune serum (lane 2) or anti-PS-1 (lane 3) or anti-PAG (lane 4). (C) Coimmunoprecipitation experiment was performed using anti-PAG or preimmune sera with murine brain lysates (lanes 1 and 2) or HEK cell lysates (lanes 3 and 4) or cell lysates prepared from fibroblast cells obtained from wild-type (lane 5) or PS-1-deficient (lane 6) mice. Western blotting was then performed using the anti-PS1 monoclonal antibody as described under Methods. The position of a nonspecific band is marked (n.s.). The bands corresponding to full-length (FL-) or carboxy-terminal fragment (CTF-) of PS1 are also indicated.

transfected with PAG–myc without cotransfection of PS-1. PAG was detected in the proteins immunoprecipitated by the anti-PS-1 antibody (Fig. 1A, lane 2), suggesting that PAG may interact with endogenous PS-1.

We then investigated whether endogenous PAG interacted with PS-1 in the brain. A polyclonal antibody was generated against recombinant PAG protein. This antibody detected one predominant band of 25–26 kDa in the mouse brain extracts (Fig. 1B, lane 1) and in the total cell lysates of different cell lines (data not shown), consistent with the previous study (Jin *et al.*, 1997). PAG protein was coprecipitated from mouse

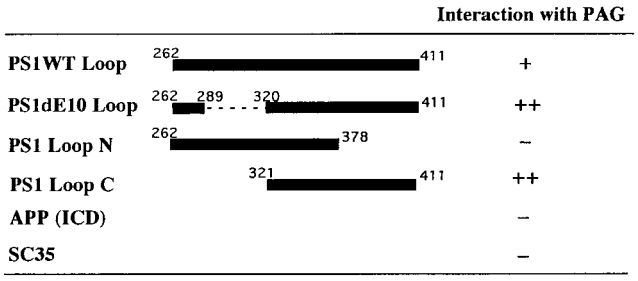

FIG. 2. Dissection of the PS-1 hydrophilic loop region for its interaction with PAG. The wild-type PS-1 hydrophilic loop region (amino acid residues 262–411), or the PS1dE10 loop region (with residues 290 to 319 removed), or the amino-terminal fragment of the loop containing residues 262–378 as well as the carboxyl fragment containing residues 321–411 was expressed as a fusion protein containing a LexA DNA-binding domain in yeast cells expressing PAG as a protein fused to a transcription activation domain. The yeast two-hybrid interaction assay was performed as described (Wu & Maniatis, 1993). The intracellular domain of APP and an unrelated protein, SC35, which are rich in hydrophilic amino acid residues, were included as controls in addition to the vector control.

brain extract by anti-PS-1 antibody (Fig. 1B, lane 3), but not by the preimmune serum (Fig. 1B, lane 2). To further confirm the specificity of the PS-1–PAG interaction observed, the coimmunoprecipitation experiment was also carried out using anti-PAG antibody for immunoprecipitation and anti-PS-1 antibody for Western blotting (Fig. 1C). Anti-PAG antibody brought down endogenous PS-1 in the mouse brain lysates (Fig. 1C, lane 2) or HEK cell lysates (Fig. 1C, lane 3), mainly the carboxy-terminal fragment (CTF-PS1) and also a smaller amount of the full-length (FL-PS1), whereas the preimmune serum did not precipitate the PS-1 protein in the corresponding lysates (Fig. 1C, lanes 1 and 4). The identity of the PS-1 protein band detected was further confirmed using the lysates prepared from the wild-type or PS-1-deficient mouse fibroblast cells (Fig. 1C, lanes 5 and 6, respectively). These results demonstrate that PAG interacts with PS-1 in the brain.

Using the yeast two-hybrid interaction assay, we further characterized the interaction between PS-1 and PAG. The loop region of both the wild-type PS-1 and the FAD mutant with exon 10 deletion (PS1dE10) interacted with PAG (Fig. 2), whereas control proteins such as the intracellular domain of APP (APP-ICD) and SC35 had no detectable interaction with PAG. Therefore, the interaction of PAG with PS-1 appears specific and not due to nonspecific interaction with proteins rich in hydrophilic amino acid residues, because both APP-ICD and SC35 are rich in hydrophilic residues, similar to the PS-1 loop region. We then tested the amino-terminal (PS-1 loop N) and carboxyl-terminal (PS-1 loop C) fragments of the PS-1 loop to further define the region responsible for the interaction with PAG. The PS-1 loop C fragment containing amino acid residues 321–411 was sufficient for the interaction with PAG, whereas the PS-1 loop N fragment did not interact with PAG. This is consistent with the observation that PS1dE10, which lacks amino acid residues 290–319, retains the ability to interact with PAG. Thus, the region of the PS-1 loop that mediates the interaction with PAG resides in the carboxyl terminus of the loop. This region is highly conserved in PS-1 throughout evolution and is also conserved between PS-1 and PS-2. Consistent with this, this region of PS-2 also interacts with PAG (data not shown).

Overexpression of Wild-Type PS-1 or PS-1 Exon 10 Deletion Mutant in SCG Neurons Did Not Affect Neuronal Viability

To begin to understand the functional significance of the interaction between PAG and PS-1, we examined potential effects of PAG and PS-1 proteins on the viability of neurons in primary neuronal culture. The SCG sympathetic neuron culture system was used because it has several advantages over cultured cell lines. First, SCG neurons are primary neurons, not an immortalized cell line. Second, similar to the forebrain cholinergic neurons, which are severely affected in AD, SCG neurons depend on NGF for survival. Therefore, the cell death pathway(s) in SCG neurons may share common features with that of neurons involved in AD pathogenesis. Third, neuronal cell death has been well characterized in the SCG model (Greenlund *et al.*, 1995; Easton *et al.*, 1997; Deshmukh & Johnson, 1998). Fourth, a protocol for the introduction of exogenous genes into SCG neurons by microinjection has been established (Greenlund *et al.*, 1995; Easton *et al.*, 1997). We microinjected plasmids expressing proteins of interest together with one expressing the GFP, which allowed longitudinal tracing and monitoring of injected cells. Injected neurons were counted 24 h after injection and the survival of neurons was scored for up to 4 days after injection according to an established protocol described previously (Greenlund *et al.*, 1995; Easton *et al.*, 1997). Expression of corresponding proteins from injected plasmids was detected by immunofluorescent staining with anti-PS-1 or anti-myc tag present on the fusion protein (data not shown; see Fig. 5).

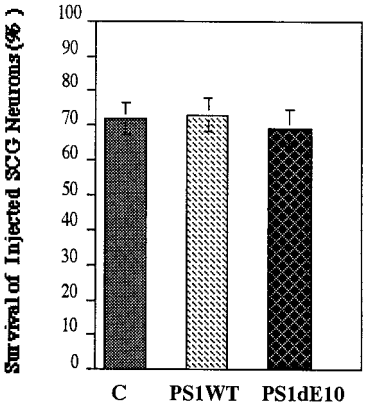

FIG. 3. Microinjection of wild-type PS-1 or FAD mutant PS1dE10 does not affect neuronal viability. SCG neurons were microinjected with 100 ng / μl wild-type PS-1 (n = 189) or 100 ng / μl PS1dE10 (n = 189). The controls were injected with 100 ng / μl pcDNA3 vector (n = 198) alone. Injected neurons were marked by expression of GFP from co-injected GFP plasmid. The viability of the injected neurons was shown as the percentage of live neurons at the different time points compared to GFP expressing neurons 24 h after injection. The survival of injected neurons 96 h after injection is shown. In four independent experiments, there was no significant difference in neuronal viability among these groups ($P > 0.5$, ANOVA).

Microinjection of wild-type PS-1 (PS1WT) or the FAD mutant PS-1 with an exon 10 deletion (PS1dE10) did not affect the survival of SCG neurons (Fig. 3). These results from primary neurons do not support the idea that the PS1dE10 mutant causes AD by a gain of a neurotoxic function.

Overexpression of PAG Led to Neuronal Cell Death That Was Inhibited by Coexpression of Wild-Type PS-1 But Not PS-1 Exon 10 Deletion Mutant

When the PAG-expressing plasmid was microinjected into SCG neurons, the number of surviving neurons was significantly decreased (Fig. 4), compared to that seen when control vector or PS-1 plasmids were injected. This effect on SCG neuronal survival was dose-dependent: with the increase of DNA dose injected, from 25, 75, to 100 ng / μl, the neuronal survival rate at 96 h postinjection declined from 60 $\pm$ 4.3 to 41 $\pm$ 5.1 to 28 $\pm$ 5.2%, respectively. Whereas the neurons injected with the vector control plasmid (75 ng / μl) showed a survival rat of 72 $\pm$ 1.5% 4 days after injection, the survival of SCG neurons injected with the PAG plasmid at the same dose and the same time point decreased to 42.5 $\pm$ 2.5% (mean $\pm$ SD, $P < 0.001$, ANOVA) (Fig. 4). We then tested whether coexpression of PS-1 had any effect on neuronal cell death induced by PAG overexpression. Co-injection of wild-type PS-1 significantly increased the survival of the injected SCG neurons from 42.5 $\pm$ 2.5 to 65.7 $\pm$ 4.4% ($P < 0.001$, ANOVA), a level similar to that in the control group (72 $\pm$ 1.5%). However, co-injection of mutant PS1dE10 did not significantly change the percentage of surviving SCG neurons, with the neuronal survival rate 44.6 $\pm$ 7%, similar to that in neurons injected with PAG alone (Fig. 4). Due to the nature of mixed neuronal / glial cells in the SCG primary culture and the technical difficulty in injecting a large number of neurons sufficient for Western detection, we used immunofluorescent staining of injected neurons to show that in these microinjection experiments, wild-type PS-1 was expressed at a level similar to that of mutant PS-1 (Fig. 5, top). Taken together with the finding that wild-type PS-1 or PS1dE10 alone did not affect the survival of SCG neurons (Fig. 3), these results indicate that PS-1, but not the FAD mutant PS1dE10, has neuroprotective activity against neuronal cell death induced by PAG.

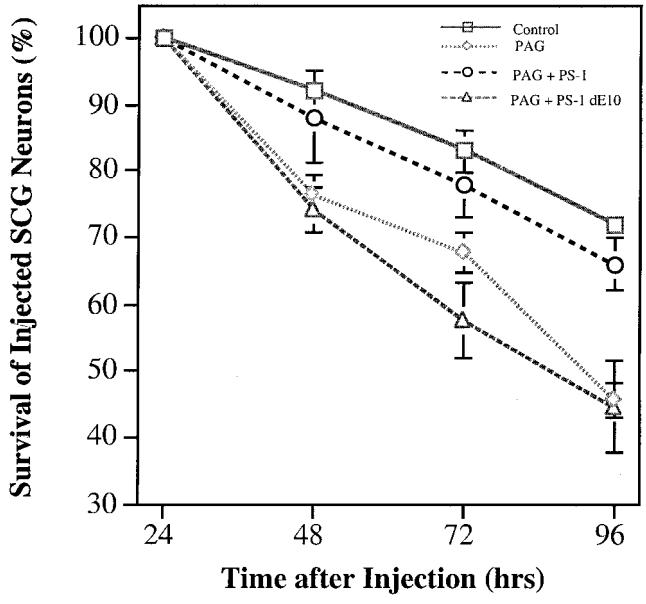

FIG. 4. SCG neuronal viability following microinjection with different plasmids. The viability of injected SCG neurons is shown as a percentage of surviving neurons at different time points compared to that seen 24 h after injection. Control vector or the plasmid expressing PAG was injected alone or with PS-1 (WT or dE10 mutant). Injection of PAG plasmid (75 ng / μl) into the nuclei of SCG neurons increased neuronal cell death (n = 368) compared to controls injected with the same concentration of pcDNA3 vector DNA (n = 336) ($P < 0.0001$, ANOVA). SCG neuronal cell death caused by PAG overexpression was protected against by co-injection with 60 ng / μl wild-type PS-1 (n = 330) ($P < 0.0001$, ANOVA), but not by co-injection with 60 ng / μl PS1dE10 (n = 319) (P = 0.94, ANOVA). Data from four independent experiments are shown.

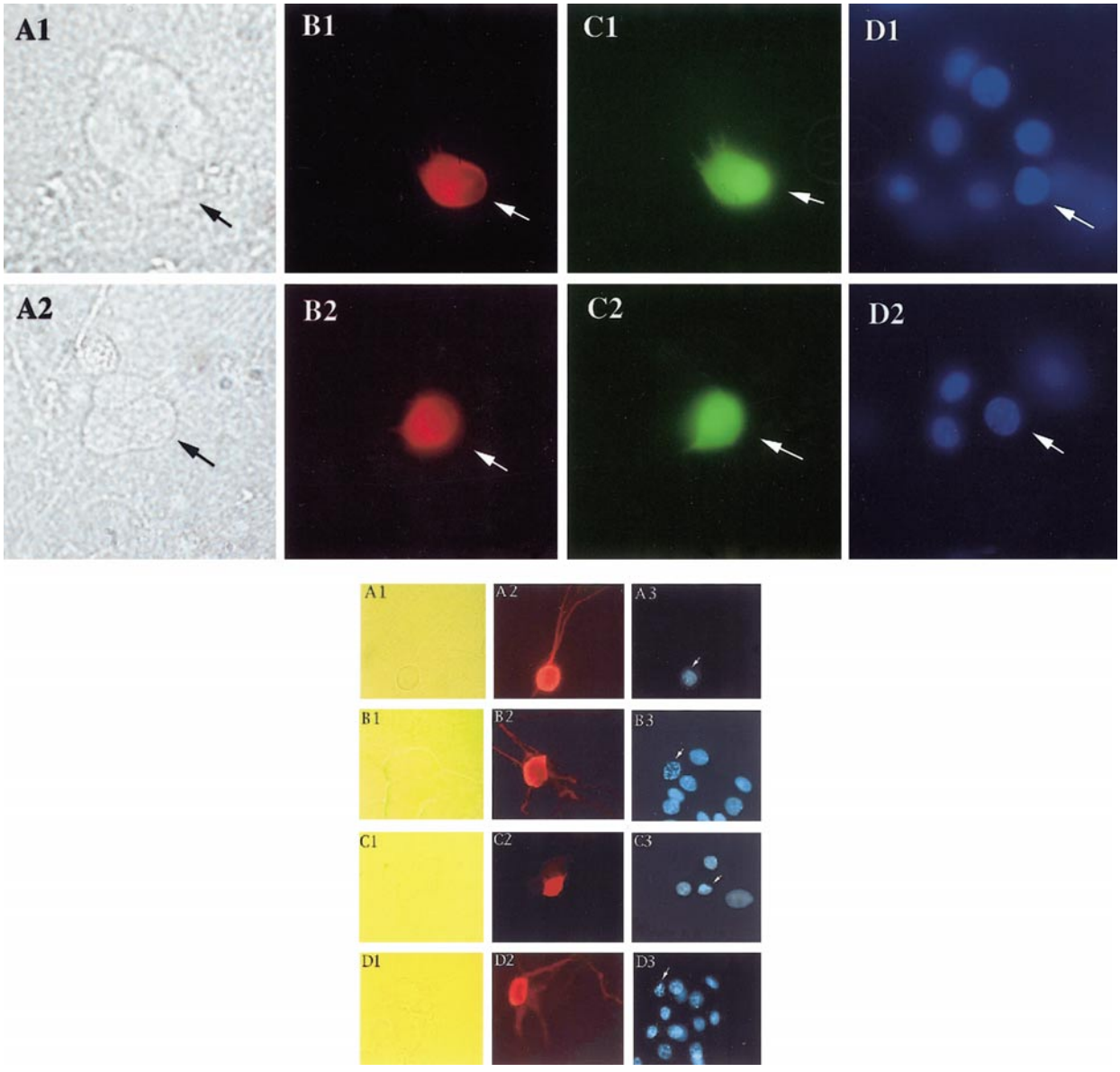

FIG. 5. PAG-induced SCG neuronal death showed morphological features of apoptosis. (Top) The overexpression of wild-type PS-1 or mutant PS1dE10 in SCG cells did not cause cell death. SCG neurons were injected with either wild-type PS-1 (A1–D1) or PS1dE10 (A2–D2) plasmids to express PS-1 proteins (Ray *et al.*, 1999). Seventy-two hours after injection, cells were fixed and immunostained with anti-PS-1 monoclonal antibody followed by a goat anti-mouse Alexa 594-conjugated secondary antibody. Cells were then stained with bis-benzimide for nuclear morphology. (A1 and A2) Cells under a phase-contrast microscope. (B1 and B2) Expression of injected plasmids as detected by immunostaining using anti-PS-1 antibody. (C1 and C2) Expression of co-injected GFP plasmid. (D1 and D2) Nuclear images following bis-benzimide staining of the cultured cells, showing that cells expressing either wild-type PS-1 or PS1dE10 had normal nuclear morphology. (Bottom) SCG neurons were injected with expression plasmids encoding a control myc-epitope-tagged cytoplasmic protein (A) or myc-tagged PAG (B) or myc-tagged PAG together with wild-type PS-1 (C) or myc-tagged PAG together with PS1dE10 (D). Seventy-two hours after injection, cells were fixed and immunostained with anti-myc monoclonal antibody followed by Cy3-conjugated secondary antibody. Cells were then stained with bis-benzimide for nuclear morphology. (A1, B1, C1, and D1) Cells under the phase-contrast microscope. (A2, B2, C2, and D2) Expression of injected plasmids as detected by immunostaining using anti-myc antibody. (A3, B3, C3, and D3) Nuclear images following bis-benzimide staining of the cultured cells. The injected cells corresponding to those shown in the middle are indicated with an arrow. The injected cells in B3 and D3 show condensed chromatin and fragmented nuclei with features of apoptosis, whereas the injected cells in A3 and C3 show normal nuclear morphology.

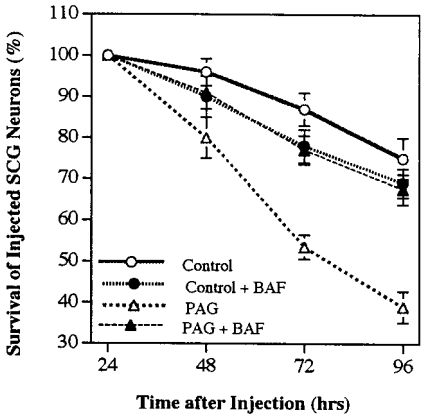

FIG. 6. BAF blocks SCG neuronal death induced by PAG overexpression. SCG neurons were injected with 75 ng/μl vector control or PAG expression plasmid. BAF (at 30 μM final concentration) was added to the culture medium 24 h after injection. Addition of BAF blocked neuronal death induced by PAG overexpression ($P < 0.0001$, ANOVA).

PAG-Induced Neuronal Death Had Features of Apoptosis

To understand the nature of PAG neurotoxicity, neuronal morphology was examined after microinjection of SCG neurons. Staining of the nuclei with bisbenzimide showed that PAG-injected SCG neurons had fragmented or condensed nuclei, characteristic of apoptosis (Fig. 5, bottom, B), whereas neurons injected with control vector DNA appeared healthy with normal nuclear staining (Fig. 5, bottom, A). In the majority of SCG neurons co-injected with wild-type PS-1 and PAG-expressing plasmids, nuclear staining showed healthy nuclear morphology, while those co-injected with PS1dE10 and PAG plasmids showed nuclei that were fragmented or condensed, with typical morphology of apoptosis (Fig. 5, bottom, C and D, respectively). These observations suggest that PAG overexpression decreased neuronal survival by inducing apoptosis. To confirm this possibility, we treated SCG sympathetic neurons after the microinjection of PAG-expressing plasmid with a caspase inhibitor known to block apoptosis in SCG neurons, BAF (Deshmukh & Johnson, 1998). BAF treatment did not change the viability of neurons injected with the control plasmid alone, and the survival of the control vector plasmid-injected neurons 4 days after injection was 69 $\pm$ 3.5% compared to 75 $\pm$ 5% in the absence of BAF (Fig. 6). In the PAG-injected SCG neurons, BAF treatment significantly enhanced neuronal viability, increasing the survival rate to 67.5 $\pm$ 3.7%, compared to 39 $\pm$ 3.8% without BAF treatment (Fig. 6), indicating BAF treatment protected SCG sympathetic neurons against neuronal death caused by PAG overexpression. Bisbenzimide staining showed that apoptotic changes in nuclei of injected neurons caused by PAG were prevented by BAF treatment (Fig. 7). These results demonstrate that PAG causes apoptosis of SCG sympathetic neurons in a caspase-dependent manner.

NGF Deprivation Exacerbated SCG Neuronal Death Induced by PAG Overexpression

Since SCG neurons rely on trophic factors for survival, we asked whether the effects of PS-1 or PAG on SCG neuronal survival were influenced by NGF. In the presence of NGF, 95 $\pm$ 5% SCG neurons survived 48 h after microinjection with control vectors (Fig. 4 and 6). When NGF was removed from the culture media 24 h after the microinjection of the control plasmid, 64 $\pm$ 2.6% of SCG neurons remained viable after another 24 h in culture (Fig. 8). Microinjection of either wild-type PS-1 or PS1dE10 plasmid did not change the neuronal death induced by NGF deprivation (Fig. 8). However, PAG injection further accelerated neuronal death in the absence of NGF because

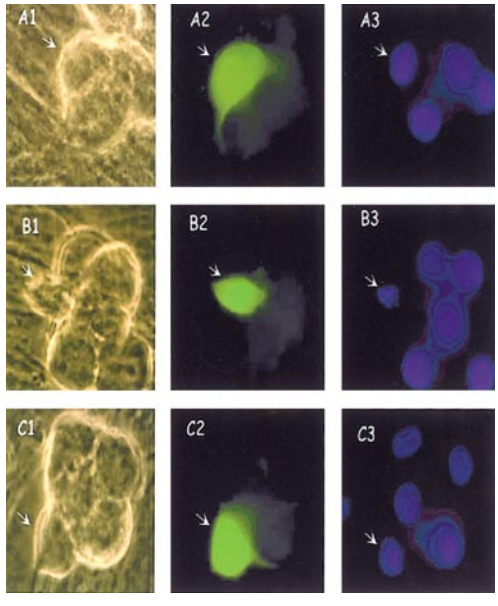

FIG. 7. PAG-injected neurons show normal morphology after BAF treatment. (A) Injection with vector control. (B) SCG neurons after PAG plasmid injection. (C) SCG neurons injected with PAG following BAF treatment. (A1, B1, and C1) The phase-contrast views of SCG neurons. (A2, B2, and C2) The fluorescence microscopic views of injected cells as marked by GFP expression. (A3, B3, and C3) Microscopic pictures of nuclei of the SCG neurons as revealed by bis-benzimide staining.

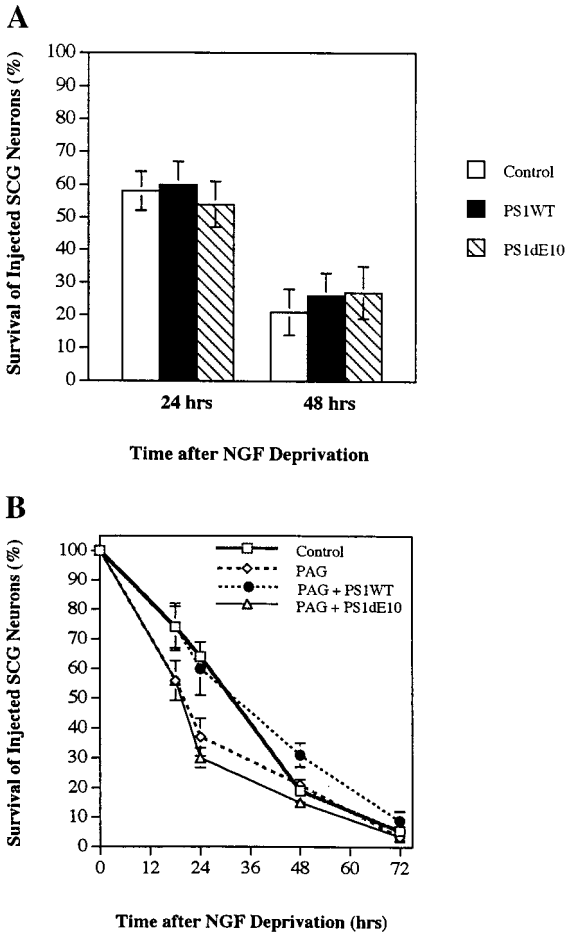

FIG. 8. Effects of microinjection with different plasmids on SCG neuronal death in the absence of NGF. (A) Injection of PS-1 expression plasmids (either WT or dE10 mutant) did not affect SCG neuronal death caused by NGF deprivation. SCG neurons were injected with control vector or PS-1 plasmids (100 ng/μl) and were then deprived of NGF 24 h after microinjection. Neither wild-type PS-1 nor PS1dE10 mutant significantly changed the neuronal viability after NGF deprivation. (B) NGF was depleted 24 h after injection of the control plasmid or PAG plasmid or co-injection of PAG with PS-1 plasmids (WT or dE10) into the cultured SCG neurons. The neurons were then maintained in the NGF-free medium containing polyclonal anti-NGF antibody. The survival of injected neurons was determined at different time points after NGF withdrawal.

only 37.4 ± 6.3% of PAG-injected neurons survived in the absence of NGF compared to the survival rate of 76.5 ± 3% in PAG-injected neurons in the presence of NGF at this time point (Figs. 4 and 8). Thus, in the presence of NGF, PAG injection caused the neuronal viability to decrease from 95 ± 5 to 76.5 ± 3%, whereas in the absence of NGF, PAG overexpression led to a further decrease in the survival rate from 64 ± 2.6 to 37.4 ± 6.3% 48 h after microinjection (24 h after NGF removal). That is, the ratio of survival in the PAG-injected to that in the control-injected neurons in the presence of NGF was 0.81, but this ratio was decreased to 0.58 when NGF was removed. Therefore, NGF depletion exacerbated SCG neuronal death induced by PAG overexpression.

In the absence of NGF, wild-type PS-1 plasmid co-injection also slowed down the neuronal death induced by PAG overexpression, whereas injection of the mutant PS-1 did not show significant protective activity. As shown in Fig. 8, this effect is most obvious 48 h after injection (24 h after NGF depletion). At this time point, 60 ± 9% of neurons co-injected with wild-type PS-1 survived compared to 37.4 ± 6.3% of neurons injected with PAG and 64 ± 2.6% of the control-injected neurons. In neurons co-injected with PS1dE10 and PAG, the survival was only 30 ± 3.3%, similar to that in the PAG-injected neurons (37.4 ± 6.3%). By 72–96 h after injection (48–72 h after NGF deprivation), the neuroprotective activity was less obvious, as the majority of neurons were dead (Fig. 8). Therefore, it appears that wild-type PS-1 but not PS1dE10 mutant is capable of delaying the neuronal death by approximately 24 h in the PAG-injected cells in the absence of NGF.

DISCUSSION

Our results show that PAG interacts with PS-1 in yeast, in cultured mammalian cells, and in mouse brain cell extracts. Consistent with the results in mammalian cells, recent genetic studies demonstrate that PS-1 and PAG interact in *Drosophila* (E. Bier, personal communication). When introduced into rat SCG neurons, PAG reduces the neuronal viability by causing apoptosis in a caspase-dependent manner. This neurotoxic effect is exacerbated by removal of NGF. Overexpression of the wild-type PS-1 but not the mutant PS1dE10 can protect against the neuronal death caused by PAG overexpression. These results suggest that a defect in PS-1 mutants in protecting neurons against apoptotic insults may contribute to the pathogenesis of AD.

Presenilin proteins interact with a number of proteins, including transmembrane proteins [APP (Xia *et al.*, 1997; Pradier *et al.*, 1999) and Notch (Ray *et al.*, 1999)], intracellular proteins involved in signal transduction [G-protein G0 (Smine *et al.*, 1998), glycogen synthetase kinase-3β (Takashima *et al.*, 1998), armadillo/β-catenin (Murayama *et al.*, 1998; Tesco *et al.*, 1998; Z. H. Zhang *et al.*, 1998; Levesque *et al.*, 1999; Nishimura *et al.*, 1999; Stahl *et al.*, 1999; Tanahashi & Tabira,

1999), calsenilin (Buxbaum *et al.,* 1998), Rab11 (Dumanchin *et al.,* 1999), G-protein G0 (Smine *et al.,* 1998), QM/Jif1 (Imafuku *et al.,* 1999)], and cytoskeletal proteins [tau (Takashima *et al.,* 1998) and filamin (W. J. Zhang *et al.,* 1998; Johnsingh *et al.,* 2000)]. Cell death gene products, Bcl-2 and Bcl-xl, have also been found to interact with PS-1 (Alberici *et al.,* 1999; Passer *et al.,* 1999). Our study reveals members of the thioredoxin peroxidase family as proteins interacting with PS-1 and describes conditions under which a neuroprotective activity of wild-type PS-1 can be demonstrated in neurons. Taken together, these data strongly suggest that presenilins have multiple functional activities. Our results from the primary neuronal culture suggest that PS-1 may have neuroprotective activity and that certain PS1 mutants may be defective in such neuroprotective function.

Although PS-1 is involved in Aβ production, an extremely important event in AD pathogenesis, the gain of function of PS-1 mutation in Aβ production may not be the only mechanism involved in increasing neuronal death. Loss of protective function of wild-type PS-1 may also contribute to the neuronal loss in AD. Although it is still controversial whether neuronal loss in AD pathogenesis is due to apoptosis, accumulating evidence indicates that presenilins play a role in neuronal death. PS-1 knockout mice show significant loss of neurons, suggesting impaired neurogenesis or increased neuronal death in these animals (Shen *et al.,* 1997). Overexpression of PS-1 has been shown to protect against apoptosis of cortical neurons induced by etopside or staurosporine (Bursztajn *et al.,* 1998), whereas inhibition of PS-1 expression by an antisense strategy increases apoptosis in NT2 cells during retinoic acid induction and renders them unable to differentiate into neuronal cells (Hong *et al.,* 1999). PS-1 is also induced by transient ischemia in hippocampal neurons relatively resistant to ischemic stress, but not in susceptible hippocampal neurons (Tanimukai *et al.,* 1998). Also supporting the protective role of PS-1 are recent reports that PS-1 can suppress c-Jun-associated apoptosis (Imafuku *et al.,* 1999) and modulate mitochondria-dependent apoptosis events (Alberici *et al.,* 1999; Passer *et al.,* 1999).

Several additional lines of evidence support the hypothesis that wild-type presenilin may have neuroprotective activities and that presenilin mutations may lead to a deficiency in such neuroprotective mechanisms. PS-1 mutations increased neuronal vulnerability to excitotoxic agents in knock-in mice (Guo *et al.,* 1999). Hypoxia-induced neuronal death was enhanced by a PS-1 mutation (Mattson *et al.,* 2000). Increased sensitivity to mitochondrial toxin-induced apoptosis in cells expressing mutant PS-1 is associated with enhanced free radical production (Keller *et al.,* 1998). Furthermore, inhibition of PS-1 expression resulted in apoptosis (Roperch *et al.,* 1998).

Our results showing a physical interaction between PAG and PS-1 and showing neurotoxicity induced by PAG overexpression provide new information on the potential function of Tpx proteins. The first member of Tpx family was identified and cloned from *Saccharomyces cerevisiae* as a protein involved in antioxidant response (Kim *et al.,* 1988; Chae *et al.,* 1993). Further work led to the discovery of a large number of proteins in this family (Chae *et al.,* 1994b; Shau *et al.,* 1994). Biochemical studies suggested that Tpx proteins have thiol peroxidase activity that is involved in removal of peroxide (Chae *et al.,* 1994a; Netto *et al.,* 1996). PAG was cloned as a proliferation-associated gene (Prosperi *et al.,* 1993). Similar to PS-1, PAG is expressed in a number of tissues (Prosperi *et al.,* 1993). Sequence analysis indicates that PAG belongs to the thioredoxin peroxidase family. However, the function of PAG is not well established, although our results show that PAG has peroxidase activity *in vitro* (Y. Zhou & J. Y. Wu, data not shown). Another human Tpx protein, NKEF, was originally identified as a cytoplasmic factor from red blood cells, and NKEF enhances natural killer cell-mediated cytotoxicity (Shau *et al.,* 1993, 1994). A murine Tpx gene was expressed in all of the tissues, with high levels in brain, heart, kidney, bone marrow, and skeletal muscle (Ichimiya *et al.,* 1997). *In situ* hybridization reveals that this murine Tpx gene is expressed in most neurons, with highest levels detected in pyramidal neurons in cerebral cortex and in the CA1 region of the hippocampus as well as in Purkinje cells in the cerebellum. It was noted that the brain regions that showed high levels of Tpx expression are the areas that are especially susceptible to ischemic and hypoxic injury (Ichimiya *et al.,* 1997). Recent studies suggest that PC12 cells transfected with murine Tpx or Molt-4 leukemic cells transfected with human TpxII appeared to have delayed or reduced cell death after serum or NGF withdrawal (Ichimiya *et al.,* 1997; Zhang *et al.,* 1997). The mechanisms underlying these cytotoxic or protective effects of Tpx proteins in neuronal or nonneuronal cells are not clear. One simple prediction from the activity of Tpx on oxidation would be that Tpx proteins should decrease cell death. This is clearly not the case in SCG neurons. An alternative possibility is that PAG can affect expression and/or functional activities of specific proteins that are regulated by oxidation and that the resulting functional changes cause neuronal apoptosis. More experiments are needed in the future to

examine whether there are regional specificity and dynamic changes under different stress conditions in PAG expression, and the study from Ichimiya *et al.* (1997) suggests that this may be the case. Although PAG interacts with PS-1, it is clear that simple physical binding alone is not sufficient to explain the functional difference between the wild-type and the mutant PS-1 proteins. Both the wild-type and the mutant PS-1 can bind PAG, but only the wild-type PS-1 can reduce apoptosis caused by PAG. This observation suggests that wild-type and mutant PS-1 proteins may differentially regulate PAG activity. It will be interesting to investigate why PAG causes apoptosis in SCG neurons and how PS-1 protects neurons from apoptosis. The establishment of a neuronal survival assay in primary neurons may help further functional studies of PS-1 and its mutants.

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