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Cellular FLICE-like Inhibitory Protein (c-FLIP) and PS1-associated Protein (PSAP) Mediate Presenilin 1-induced γ-Secretase-dependent and -independent Apoptosis, Respectively*

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Linlin Zeng[‡], Chen Hu[‡§], Fuqiang Zhang[‡], Daniel C. Xu[¶], Mei-Zhen Cui[‡], and Xuemin Xu[‡1]

From the [‡]Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, and the [§]Department of Comparative and Experimental Medicine, University of Tennessee, Knoxville, Tennessee 37996 and the [¶]Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

Background: The PS1-induced apoptosis mechanism is not fully understood.
Results: PS1-induced apoptosis depends on PSAP-Bax complex formation and γ-catalyzed turnover of c-FLIP.
Conclusion: PS1 induces apoptosis through the γ-secretase-dependent PS1-c-FLIP-caspase-8-Bid cascade and γ-secretase-independent PS1-PSAP cascade, which converge on Bax to activate the mitochondrial apoptotic pathway. PS2 induced apoptosis through a different pathway.
Significance: This study provides new insight into the mechanism of PS1-induced apoptosis.

Presenilin 1 (PS1) has been implicated in apoptosis; however, its mechanism remains elusive. We report that PS1-induced apoptosis was associated with cellular FLICE-like inhibitory protein (c-FLIP) turnover and that γ-secretase inhibitor blocked c-FLIP turnover and also partially blocked PS1-induced apoptosis. A complete inhibition of PS1-induced apoptosis was achieved by knockdown of PS1-associated protein (PSAP), a mitochondrial proapoptotic protein that forms a complex with Bax upon induction of apoptosis, in the presence of γ-secretase inhibitor. PS1-induced apoptosis was partially inhibited by knockdown of caspase-8, Fas-associated protein with death domain (FADD), or Bid. However, knockdown of Bax or overexpression of Bcl-2 resulted in complete inhibition of PS1-induced apoptosis. These data suggest that PS1 induces apoptosis through two pathways: the γ-secretase-dependent pathway mediated by turnover of c-FLIP and the γ-secretase-independent pathway mediated by PSAP-Bax complex formation. These two pathways converge on Bax to activate mitochondria-dependent apoptosis. These findings provide new insight into the mechanisms by which PS1 is involved in apoptosis and the mechanism by which PS1 exerts its pathogenic effects. In addition, our results suggest that PS2 induces apoptosis through a pathway that is different from that of PS1.

Apoptosis, also known as programmed cell death, plays a pivotal role in development, cancer, normal aging, and neurological disorders, such as Alzheimer disease (AD)[2] and Parkinson disease (1). AD is characterized clinically by progressive loss of memory and other cognitive functions and pathologically by progressive neuronal degeneration and loss of synapses. The central issue in AD research is to understand the causes and mechanisms of neurodegeneration in the brain. Many studies have indicated that apoptosis might occur in and contribute to AD onset and progression (2). Cells undergo apoptosis through two major pathways: the extrinsic pathway (death receptor pathway) and the intrinsic pathway (mitochondrial pathway) (3). In the conventional death receptor pathway, ligand-activated death receptors, such as FAS, recruit the adaptor Fas-associated protein with death domain (FADD) and procaspase-8 through the death domain to form the death-inducing signaling complex (DISC), resulting in activation of caspase-8, which in turn activates caspase-3 and caspase-7, leading to apoptosis. In the conventional mitochondrial pathway, the effects of intracellular stimuli are mediated by Bcl-2 family proteins. Upon activation, Bax translocates to mitochondria and triggers the release of apoptogenic factors, such as cytochrome *c*, resulting in the formation of the cytochrome *c*/Apaf-1/caspase-9 apoptosome that leads to the activation of caspase-9, which in turn activates caspase-3. The receptor and the mitochondrial pathways can be interconnected by Bid, a BH3-only protein of the Bcl-2 family, upon activation by caspase-8 (4).

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[1] To whom correspondence should be addressed: Dept. of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, 2407 River Dr., Knoxville, TN 37996. Tel.: 865-974-8206; Fax: 865-974-5616; E-mail: xmx@utk.edu.

[2] The abbreviations used are: AD, Alzheimer disease; PS1 and PS2, presenilin 1 and 2, respectively; PSAP, presenilin-1-associated protein; PARP, poly(ADP-ribose) polymerase; Bcl-2, B-cell lymphoma 2; LacZ, β-galactosidase; Z, benzyloxycarbonyl; fmk, fluoromethylketone; IETD, Ile-Glu(OMe)-Thr-Asp(OMe); DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester; DISC, death-inducing signaling complex; FADD, Fas-associated protein with death domain; c-FLIP, cellular FLICE-like inhibitory protein; APP, amyloid precursor protein; CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid; PI, propidium iodide; DED, death effector domain.

Genetic studies show that mutations in genes encoding presenilin 1 (PS1) and presenilin 2 (PS2) account for most cases of familial AD (5). Since the discovery of the association of presenilin mutations with familial AD, the function of presenilin proteins has been the subject of intense investigation. Studies suggest that presenilins function as the catalytic subunit of the γ-secretase complex, which is a key enzyme catalyzing amyloid precursor protein (APP) processing to generate β-amyloid peptide, a hallmark of AD (6). Besides APP, many other cell surface receptor and adhesion molecules, such as Notch, ErbB4, CD44, and E-cadherin, have been identified as substrates of γ-secretase, suggesting that presenilins play important roles in regulating cell signaling mediated by these molecules (7). In addition to functioning as a catalytic component of γ-secretase, a role of presenilins in apoptosis has also been reported in many studies. The first clue that presenilins might regulate apoptosis emerged from the finding that the 103-amino acid C-terminal fragment of PS2 functions as an inhibitor of T-cell receptor-induced apoptosis (8). Subsequently, it was reported that overexpression of PS2 induces apoptosis in transfected cells (9, 10). Similarly, studies have shown that PS1 is also pro-apoptotic and regulates apoptotic cell death (including cells with neuronal origin) (11, 12). In addition, it has been observed that overexpression of AD-linked PS1 mutants directly induces apoptosis in PC12 cells (13). Specifically, massive apoptotic neuronal death was observed in transgenic mice expressing PS1 (14). However, the molecular cascades that mediate the apoptotic effect of PS1 are not fully understood (15).

Using a yeast two-hybrid system, our previous study identified PS1-associated protein (PSAP); this protein was subsequently found to be a mitochondrial proapoptotic molecule (16, 17). This finding provides a direct molecular link between PS1 and the apoptotic pathway and opens a new avenue for studying the molecular mechanism by which PS-1 regulates apoptosis. In the current study, using an siRNA approach and the γ-secretase inhibitor, we found that PS1-induced apoptosis is mitochondria-dependent and is mainly mediated by PSAP. Furthermore, our results also revealed that the cellular FLICE-like inhibitory protein (c-FLIP) is a new substrate of γ-secretase and that γ-secretase-catalyzed turnover of c-FLIP also contributes to PS1-induced apoptosis. These novel observations might be exploited to develop a novel drug approach to manage AD.

Experimental Procedures

Reagents—Lipofectamine 2000 transfection reagent, Lipofectamine RNAiMax reagent, and antibiotic G418 were purchased from Life Technologies, Inc. Specific siRNAs were purchased from Qiagen. Anti-β-actin antibody and streptolysin O were purchased from Sigma-Aldrich. Complete protease inhibitor mixture tablets were purchased from Roche Applied Science. The general caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), caspase-8 inhibitor Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (Z-IETD-fmk), and antibody against c-FLIP (NF6) were purchased from Enzo Life Sciences. The γ-secretase inhibitor L685,458 was purchased from Tocris Bioscience, and compound E and *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT) were purchased from EMD Chemicals. Antibodies against FADD and cytochrome *c* were purchased from BD Biosciences. Antibodies against poly(ADP-ribose)-polymerase (PARP); caspase-3, -8, and -9; C-terminal fragment of PS1 (PS1C); Bax; Bak; Bid; Bcl-2; and Smac/DIABLO were purchased from Cell Signaling. Anti-COX I and anti-c-Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-PS1N was raised against a peptide corresponding to residues 27–50 of human PS1 (24). Horseradish peroxidase-linked anti-rabbit IgG antibody (donkey), horseradish peroxidase-linked anti-mouse IgG antibody (sheep), and the developing reagent ECL Plus were purchased from GE Healthcare. The plasmid pcDNA3.1/LacZ-Myc-His, which expresses Myc-tagged LacZ protein, was provided in the vector packages by the vendor (Life Technologies). Annexin V-enhanced green fluorescent protein apoptosis detection kit was purchased from GenScript. Trypsin without EDTA was purchased from Lonza. Human wild type PS1 (PS1wt), PS1D385A, PS1D257A, and PS1D385A/D257A cDNA were generated as described previously (17). PSAP-specific antibody Ab1 was raised against an N-terminal peptide of PSAP as described in a previous study (18).

Cell Culture and Transfection—Human cervical cancer HeLa cells, human neuroglioma H4 cells, and human colon cancer HCT116 cells were cultured in DMEM (Lonza) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human prostate cancer DU145 cells were cultured in RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For transient transfection, cells were transfected with pcDNA3.1 expressing PS1 or LacZ using Lipofectamine 2000 following the manufacturer's instructions.

Establishment of a HeLa Cell Line Stably Expressing Bcl-2—HeLa cells were transfected with pcDNA3.1/Bcl-2 plasmid with Lipofectamine 2000 transfection reagent. The transfectants were cultured in DMEM supplemented with 10% fetal bovine serum, and the stable clones were selected by G418 (400 μg/ml).

Subcellular Fractionation and Cytochrome c Release—For examination of cytochrome *c* release, the cytosolic extracts and mitochondria-containing membrane fractions were prepared by permeabilization of cells with streptolysin O using the method described previously by Mosser *et al.* (19) with slight modification (17). Briefly, cells (10^6) were washed with phosphate-buffered saline (PBS), collected by centrifugation, and resuspended in 10 μl of streptolysin O buffer (20 mM HEPES, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture) containing 60 units of streptolysin O (Sigma). After incubation at 37 °C for 20 min, the permeabilization of cells was monitored by trypan blue staining. At the time when 95% cells were stained, permeabilized cells were pelleted by centrifugation at 16,000 × *g* for 15 min at 4 °C. The supernatant was collected as the cytosolic fraction, and the pellet was collected as a mitochondria-containing membrane fraction. Both the cytosolic and mitochondrial fractions were then subjected to SDS-PAGE (10–14%) followed by Western blot.

siRNA Treatment—siRNA duplexes specific to caspase-8, FADD, Bax, Bak, Bid, and PSAP were generated by Qiagen. A control siRNA duplex that does not target any sequence in the genome (by BLAST search) was also purchased from Qiagen. Cells were transfected with these siRNAs twice at 2-day intervals using Lipofectamine RNAiMAX reagent, following the instructions provided by the manufacturer. On day 5, cells were transfected with PS1 or LacZ using Lipofectamine 2000 reagent. Twenty-four h after transfection, cells were harvested and lysed for further analysis. In some cases, half of the cells were lysed and directly subjected to SDS-PAGE followed by Western blot analysis; the other half of the cells were used to prepare cytosolic and mitochondrial fractions.

Immunoprecipitation, SDS-PAGE, and Western Blotting—For immunoprecipitation, cells were lysed in immunoprecipitation lysis buffer (1% CHAPSO, 30 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 1× protease inhibitor mixture). After sonication for 20 s, the total cell lysates were centrifuged at 14,000 × *g* for 5 min at 4 °C to remove cell debris, and the supernatants were incubated with anti-PSAP together with protein A-Sepharose overnight at 4 °C. After washing five times with cold PBS, the immunoprecipitates were subjected to Western blot analysis. Cell lysates and the mitochondria-containing membrane fraction were lysed by sonication for 20 s on ice in Western blot lysis buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and 1× protease inhibitor mixture). For routine Western blot analysis, after the addition of 4× SDS sample buffer, cell lysates, immunoprecipitates, and the cytosolic fraction were boiled at 100 °C for 7 min, and samples were subjected to SDS-PAGE (8% for LacZ and PARP; 10–14% for cytochrome *c*, Bax, and Smac/DIABLO) and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membranes were probed with appropriate antibodies.

Flow Cytometric Analysis—Twenty-four h after transfection, both floating and attached cells were collected. After washing twice with ice-cold PBS, cells were stained with annexin V-enhanced green fluorescent protein/propidium iodide (PI), using an annexin V-enhanced green fluorescent protein apoptosis detection kit and analyzed using an EpicsXL-MCL flow cytometer (Beckman Instruments) following the manufacturer's protocol. Cells were exposed to carvacrol at concentrations of 0, 200, 400, or 600 μM for 24 h. Cells were harvested after incubation and washed in cold PBS. Cells were resuspended in 400 μl of solution with 10 mM HEPES, 140 mM NaC1, and 2.5 mM $CaCl_2$, pH 7.4. Alexa Fluor 488 annexin V/PI staining solution (Molecular Probes Invitrogen) was added in the dark. After incubation for 15 min, the cells were collected and analyzed in a FACScanflow cytometry analyzer. Excitation wavelength was at 488 nm, and the emitted green fluorescence of annexin V (FL1) and red fluorescence of PI (FL2) were collected using 530- and 575-nm band pass filters, respectively. Both PI- and annexin V-negative cells (quadrant P3) were considered normal intact cells, PI-negative and annexin V-positive cells were considered early apoptotic (quadrant P4) cells, cells that were both PI- and annexin V-positive (quadrant P2) were considered late apoptotic cells, and cells that were PI-positive and annexin V-negative were considered mechanically injured cells (quadrant P1) during the experiment.

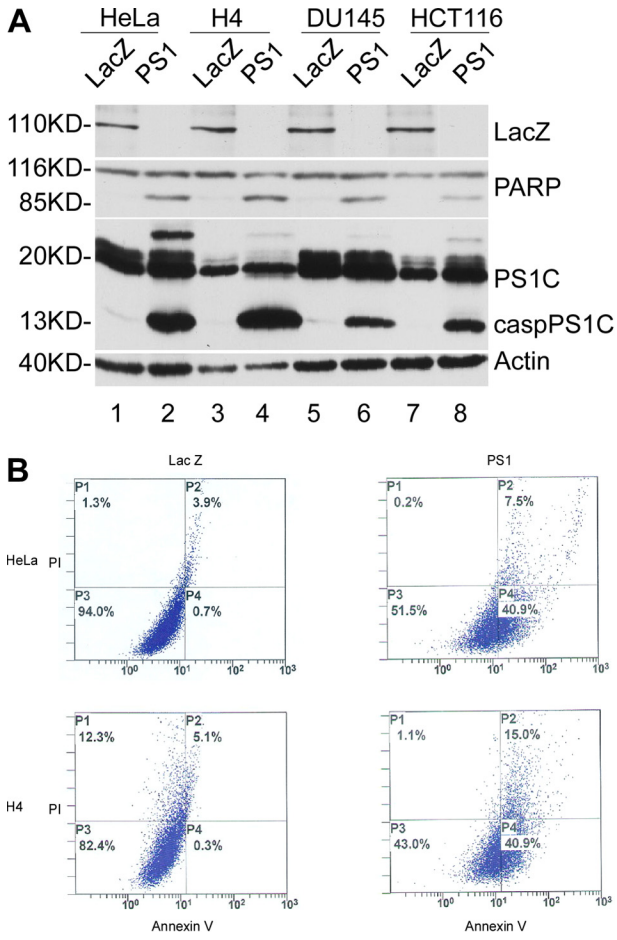

FIGURE 1. **Overexpression of PS1-induced apoptotic cell death in various types of cells.** *A*, HeLa, H4, DU145, and HCT116 cells were transiently transfected with either the PS1-expressing construct pcDNA3.1/PS1 (*PS1*) or a control plasmid, pcDNA3.1/Myc-His/LacZ (*LacZ*), which expresses Myc-tagged LacZ protein. Twenty-four h after transfection, cell lysates were separated by SDS-PAGE, followed by Western blot analysis. Expression of LacZ was detected as a 110-kDa peptide using anti-Myc antibody (*top panel*, *lanes 1*, *3*, *5*, and *7*). Using an anti-C-terminal antibody, PS1 was mainly detected as a 20-kDa C-terminal fragment (PS1C) produced by regular endoproteolytic processing (*panel 3*, *lanes 1–8*) and a 13-kDa C-terminal fragment (PS1caspCTF) produced by caspase activity in PS1-transfected cells (*panel 3*, *lanes 2*, *4*, *6*, and *8*). PARP cleavage was detected in the PS1-transfected cells (*lanes 2*, *4*, *6*, and *8* in *panel 2*) but not in the LacZ-transfected cells (*lanes 1*, *3*, *5*, and *7* in *panel 2*). This membrane was also reprobed with anti-actin antibody to indicate relative loading of samples (*bottom panel*). *B*, representative FACS scatter plots of three independent experiments. LacZ-transfected (*left column*) and PS1-transfected (*right column*) HeLa (*top row*) and H4 cells (*bottom row*) were double-stained with annexin-V-enhanced green fluorescent protein and PI. Fluorescence was detected using a fluorescence-activated Epics XL-MCL flow cytometer to analyze necrotic (PI-positive, *quadrant P1*), non-apoptotic (negative for both dyes, *quadrant P3*), early apoptotic (annexin-positive/PI-negative, *quadrant P4*), and late apoptotic cells (positive for dyes, *quadrant P2*).

Results

Overexpression of PS1-induced Apoptotic Cell Death in Various Types of Cells—To determine the apoptotic activity of PS1, we transfected human cervical cancer HeLa cells, human neuroglioma H4 cells, human prostate cancer DU145 cells, and human colon cancer HCT116 cells with PS1wt. As shown in Fig. 1*A*, transfection with PS1wt for 24 h resulted in cleavage of PARP, an indicator of apoptosis (20), in all cells examined (*lanes*

2, *4*, *6*, and *8* of *panel 2*). No apoptotic signs were detected in cells transfected with LacZ protein (*lanes 1*, *3*, *5*, and *7* of *panel 2*). In PS1-transfected cells, in addition to the 20-kDa C-terminal fragment of PS1 (PS1C) produced by normal processing of PS1, a 13 kDa band (PS1caspCTF), which is believed to be produced by caspase activity from the C terminus of PS1 (21, 22), was also detected. Data presented in Fig. 1*B* are results of an annexin V/PI staining assay and confirm apoptosis induction by PS1. The amounts of early apoptosis (annexin V^+/PI^-, P4) and late apoptosis/necrosis (annexin V^+/PI^+, P2) cells were about 41 and 7%, respectively, in PS1-transfected HeLa cells (*top panel*, *right column*) and 41 and 15%, respectively, in PS1-transfected H4 cells (*bottom panel*, *right column*). The basal levels of P4 and P2 in the LacZ-transfected cells were about 1 and 4%, respectively (*left column*).

PS1 Stimulates γ-Secretase Activity-dependent Turnover of c-FLIP, and PS1-induced Apoptosis Was Partially Blocked by γ-Secretase Inhibitor—To investigate the mechanism of PS1-induced apoptosis, we cultured HeLa, H4, and DU145 cells in the presence or absence of the general caspase inhibitor Z-VAD (100 μM) or γ-secretase inhibitor L-685,458 for 30 min prior to transfection with PS1. As shown in Fig. 2*A*, 24 h after transfection with PS1, PARP cleavage, caspase activation, and the generation of PS1caspCTF were detected in all cells cultured in the absence of Z-VAD (*lanes 2*, *5*, and *8* in *panels 2–6*). When the cells were cultured in the presence of Z-VAD, all apoptotic events induced by PS1 were inhibited (*lanes 3*, *6*, and *9* in *panels 2–6*), indicating that PS1-induced apoptosis depends on caspase activation. Next, we examined the effect of γ-secretase inhibitor on PS1-induced apoptosis. As shown in Fig. 2*B*, when cells were cultured in the presence of L-685,458 (a transition state analog inhibitor of γ-secretase), PS1-induced apoptosis was partially inhibited, as indicated by reduction of PARP cleavage, caspase activation, and formation of PS1caspCTF (compare *lane 3* with *lane 2*, *lane 8* with *lane 7*, and *lane 11* with *lane 10* in *panels 2–6*). These results suggest that PS1-induced apoptosis is partially dependent on γ-secretase activity.

It was noted that expression of PS1 also led to dramatic reduction in the levels of c-FLIP (both long form $c\text{-}FLIP_L$ and short form $c\text{-}FLIP_S$), which is a well characterized inhibitor of caspase-8 activation at the DISC (23). Interestingly, it was found that the decrease in the levels of $c\text{-}FLIP_L$ and $c\text{-}FLIP_S$ was not significantly affected by caspase inhibitor Z-VAD (Fig. 2*A*, compare *lanes 3*, *6*, and *9* with *lanes 2*, *5*, and *8* in *panel 7*) but was completely inhibited by γ-secretase inhibitor L-685,458 (Fig. 2*B*, compare *lanes 3*, *8*, and *11* with *lanes 2*, *7*, and *10* in *panel 7*). These results strongly suggest that the reduction in c-FLIP protein level caused by PS1 is a γ-secretase activity-dependent event.

It has been reported that γ-secretase-catalyzed APP processing can be differentially inhibited by transition state and non-transition state analog inhibitors (24). Next, we explored the specificity of the inhibitory effect of γ-secretase inhibitors on PS1-induced apoptosis and c-FLIP turnover. When the cells were treated with transition state analog γ-secretase inhibitor L-685,458 or non-transition state analog inhibitor compound E or DAPT, as shown in the first column of Fig. 2*B*, it was observed that, in contrast with L-685,458, compound E and DAPT had almost no effect on PS1-induced apoptotic events, including PARP cleavage, caspase activation, and the formation of PS1caspCTF (compare *lanes 4* and *5* with *lane 3* in *panels 2–6*). Moreover, in contrast to L-685,458, which strongly inhibited PS1-induced cFLIP turnover (compare *lane 3* with *lane 2* in *panel 7*), compound E and DAPT had almost no effect on the PS1-induced decrease in the levels of $c\text{-}FLIP_L$ and $c\text{-}FLIP_S$ (compare *lanes 4* and *5* with *lane 2* of *panel 7*).

To further determine whether PS1-induced turnover of c-FLIP is a γ-secretase-dependent event, we examined the effect of L-685,458 on the level of c-FLIP in intact cells. As shown in Fig. 2*C*, L-685,458 caused a dose-dependent increase in the level of c-FLIP, and c-FLIP reached a maximum level at 0.5 μM inhibitor. We also performed a co-immunoprecipitation experiment. As shown in Fig. 2*D*, an increased amount of c-FLIP was co-immunoprecipitated with PS1 in cells transfected with either PS1wt or inactive mutant PS1 variants, suggesting that both wild type and inactive mutant PS1 are capable of interacting with c-FLIP.

PS1-induced Apoptosis Was Partially Blocked by Inhibition of Caspase-8 and Knockdown of FADD—The finding that a γ-secretase inhibitor, such as L-685,458, that inhibited c-FLIP turnover also partially inhibited PS1-induced apoptosis and, conversely, that a γ-secretase inhibitor, such as compound E and DAPT, that did not inhibit c-FLIP turnover also had no effect on PS1-induced apoptosis suggests that the turnover of c-FLIP plays a role in PS1-induced apoptosis. C-FLIP is believed to regulate apoptosis by inhibiting caspase-8 activation (25). We then examined the effects of caspase-8-specific inhibitor Z-IETD on PS1-induced apoptosis. As shown in Fig. 3*A*, similar to γ-secretase inhibitor L-685,458, Z-IETD partially inhibited PS1-induced apoptosis (compare *lane 4* with *lane 2*). As shown in *panel 7*, similar to Z-VAD, Z-IETD had negligible effects on PS1-induced turnover of c-FLIP. Data presented in Fig. 3*A* were obtained using HeLa cells. Similar results were also obtained when H4 and DU145 cells were used (data not shown). From this point forward, unless otherwise indicated, all data presented herein are representative results obtained when HeLa cells were used.

The finding that caspase-8 inhibitor partially inhibited PS1-induced apoptosis suggests that caspase-8 plays an important role in initiating PS1-induced apoptosis. To substantiate this finding, we next determined the effect of caspase-8 knockdown on PS1-induced apoptosis. Prior to transfection with PS1, cells were treated with caspase-8-specific siRNA or non-silencing control siRNA. As shown in Fig. 3*B*, efficient knockdown of caspase-8 was achieved after 4 days' treatment with siRNA (*lanes 3* and *4* in *panel 2*). Similar to the addition of caspase-8 inhibitor Z-IETD, knockdown of caspase-8 partially inhibited PARP cleavage, caspase-3 and caspase-9 activation, and the generation of PS1caspCTF induced by PS1 (compare *lane 4* with *lane 2* in *panels 3–6*). However, c-FLIP turnover was not affected by knockdown of caspase-8 (compare *lane 4* with *lane 2* in *panel 7*). Next, we examined the effect of knockdown of FADD, which is an important regulator in caspase-8 activation (26), on PS1-induced apoptosis. As shown in Fig. 3*C*, similar to knockdown of caspase-8, ablation of FADD also partially inhibited PS1-induced apoptotic events (compare *lane 4* with *lane 2*

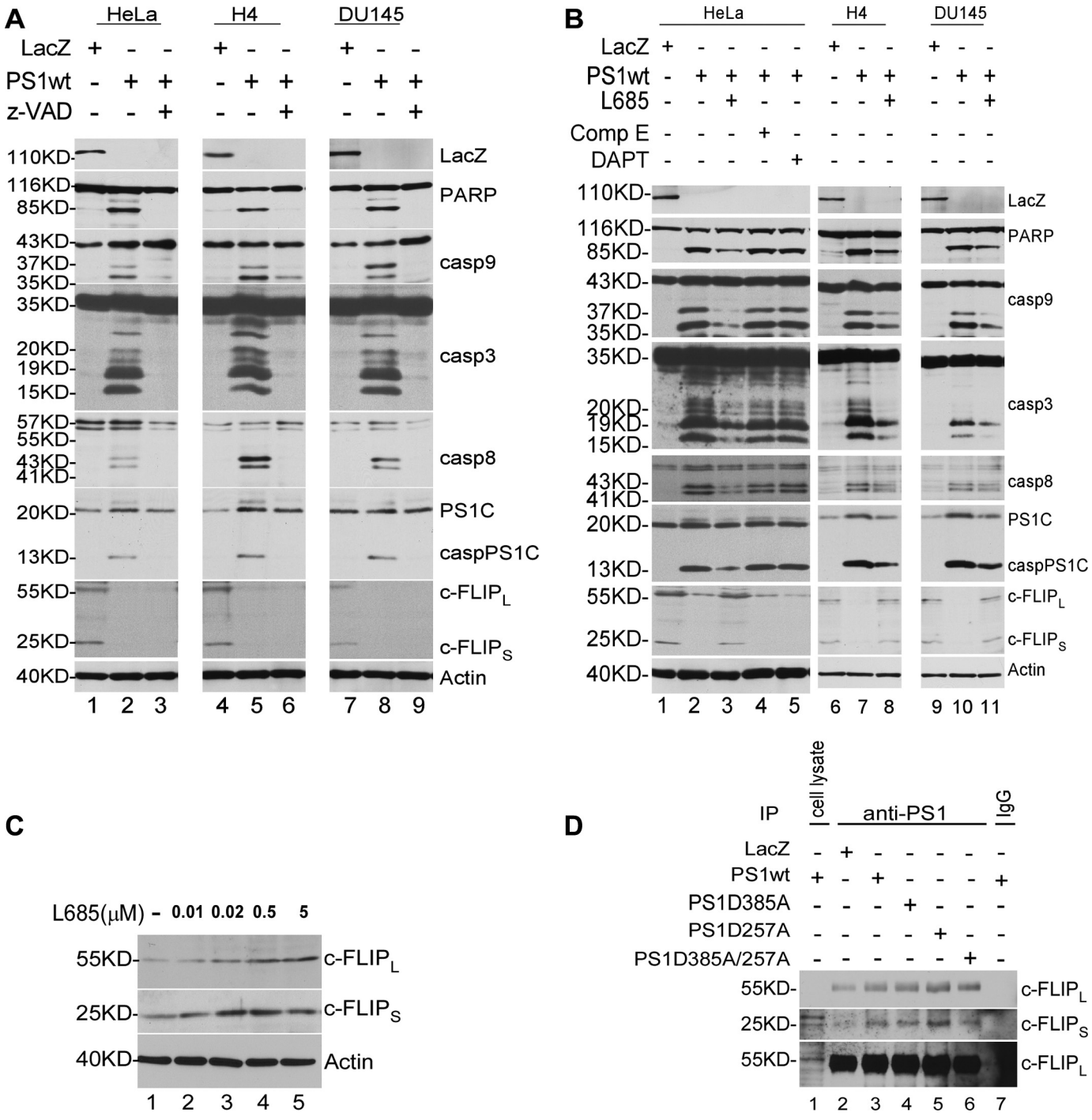

FIGURE 2. **PS1 stimulates γ-secretase-dependent turnover of c-FLIP, and PS1-induced apoptosis was partially blocked by γ-secretase Inhibitor.** *A*, HeLa, H4, and DU145 cells were transfected with either PS1 or LacZ in the presence or absence of the general caspase inhibitor Z-VAD (100 μM) for 24 h. The *top panel* shows expression of LacZ. *Panel 2* shows PARP cleavage. *Panels 3–5* show activation of caspase-9, caspase-3, and caspase-8, respectively. *Panel 6* was probed for PS1. *Panel 7* was probed for c-FLIP$_L$ and c-FLIP$_S$. The *bottom panel* was probed for actin as a loading control. *B*, PS1-induced apoptosis was inhibited by transition state γ-secretase inhibitor but not affected by non-transition state inhibitor. Cells were transiently transfected with either PS1 or LacZ in the presence or absence of γ-secretase inhibitors for 24 h. L685,458 (*lanes 3*, *8*, and *11*), compound E (*lane 4*), and DAPT (*lane 5*) were used at 0.5 μM, 5 nM, and 0.5 μM, respectively. *C*, intact HeLa cells were treated with L-685,458 at different concentrations. *D*, cells were transfected with different PS1 variants for 16 h, and cell lysates were subjected to immunoprecipitation (*IP*) using anti-PS1N antibody and probed with anti-c-FLIP. The *third panel* is a longer exposure of the *top panel* for visualizing c-FLIP$_L$ in the cell lysate.

in *panels 3–7*). These data indicate that caspase-8 activation partially accounts for PS1-induced apoptosis.

PS1-induced Apoptosis Involves Cytochrome c and Smac/DIABLO Release and Bax Translocation—In addition to the roles of caspase-8 and the factors that regulate caspase-8 activation, we also determined the possible roles of mitochondria and the Bcl-2 family of apoptotic regulatory molecules in PS1-induced apoptosis. First, we determined whether PS1 induces the release of proapoptotic mitochondrial proteins, such as cytochrome *c* and Smac/DIABLO. As shown in Fig. 4*A*, an

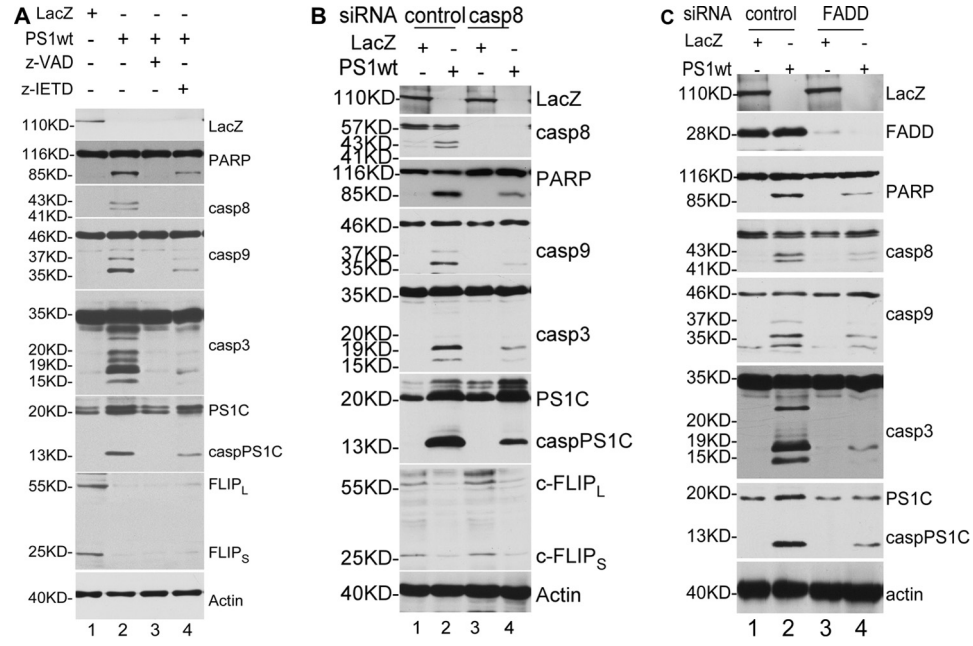

FIGURE 3. **PS1-induced apoptosis was partially inhibited by inhibition of caspase-8 activation.** *A*, caspase-8-specific inhibitor Z-IETD partially blocked PS1-induced apoptosis. HeLa cells were transiently transfected with either PS1 or LacZ in the presence or absence of caspase inhibitors for 24 h. The general caspase inhibitor Z-VAD (*lane 3*) and the caspase-8-specific inhibitor Z-IETD (*lane 4*) were used at 100 μM. *Lanes 1* and *2* are samples from cells treated with vehicle only. The *bottom panel* is the reprobe of the membrane of *panel 4* with anti-actin antibody to indicate relative loading of samples. *B* and *C*, PS1-induced apoptosis was partially blocked by knockdown of caspase-8 and FADD. Cells were transiently transfected with either LacZ (*lanes 1* and *3*) or PS1 (*lanes 2* and *4*) after treatment with control non-silencing siRNA (*lanes 1* and *2*), caspase-8-specific siRNA (*lanes 3* and *4*) (*A*), or FADD-specific siRNA (*lanes 3* and *4*) (*B*) for 24 h. LacZ, PS1, c-FLIP, actin, cleavage of PARP, and activation of caspases were detected by Western blot as described above.

increase in the levels of cytochrome *c* and Smac/DIABLO in the cytosol fraction (*lane 2*) with a concomitant decrease of these proteins in the mitochondria-containing membrane fraction (*lane 4*) was detected in cells transfected with PS1. On the other hand, a decrease in the level of Bax in the cytosol fraction (*lane 2*) and a concomitant increase in the level of Bax in the membrane fraction (*lane 4*) were detected in PS1-transfected cells.

PS1-induced Apoptosis Was Strongly Inhibited by Overexpression of Bcl-2 or Knockdown of Bid or Bax but Not Bak—Bax translocation and cytochrome *c* release can be triggered by caspase-8-activated tBid (27). We next determined the effect of knockdown of Bid on PS1-induced apoptosis using an siRNA approach. Cells were treated with Bid-specific siRNA or control non-silencing siRNA prior to transfection with PS1-expressing plasmid. As shown in the *first column* of Fig. 4*B*, Bid was almost completely ablated by treatment with Bid-specific siRNA (*lanes 3* and *4* in *panel 2*). As a result, PS1-induced PARP cleavage, caspase activation, and PS1caspCTF generation were partially blocked by knockdown of Bid (compare *lane 4* with *lane 2* in *panels 3–7*) to a similar degree as that observed by knockdown of caspase-8, which cleaves Bid to generate the active form of truncated Bid or tBid.

It is well established that activated tBid further activates downstream targets, such as Bax and Bak, resulting in the release of cytochrome *c* (28). We next determined the roles of Bax and Bak in PS1-induced apoptosis. As shown in the *second column* of Fig. 4*B*, PS1-induced apoptotic events were completely inhibited when the cells were treated with Bax-specific siRNA (*lane 8*, *panels 3–7*). In contrast, *a*s shown in the *third column* of Fig. 4*B*, knockdown of Bak had only a negligible effect on PS1-induced apoptosis. These results clearly indicate that Bax, but not Bak, is specifically required for PS1-induced apoptosis.

To further determine the roles of Bcl-2 family proteins in regulating PS1-induced apoptosis, we determined the effect of overexpression of Bcl-2 on PS1-induced apoptosis. As shown in the *fourth column* of Fig. 4*B*, we established a stable cell line expressing Bcl-2 protein in HeLa cells (*lanes 15* and *16* in *panel 2*). As a control, we also stably transfected HeLa cells with an empty vector (*lanes 13* and *14*). Upon expression of PS1, apoptosis was detected in HeLa cells bearing an empty vector, as determined by the cleavage of PARP, activation of caspases, and the formation of PS1caspCTF (*lane 14* in *panels 3–7*). However, these apoptotic events were strongly inhibited in cells stably expressing Bcl-2 (*lane 16* in *panels 3–7*). These findings strongly suggest that PS1-induced apoptosis is largely mediated by the mitochondrial pathway and is regulated by Bcl-2 protein.

γ-Secretase-inactive PS1 Mutants Induced γ-secretase Activity-independent Apoptosis—The above results demonstrated that γ-secretase inhibitor only partially blocked PS1wt-induced apoptosis, suggesting that PS1 could induce apoptosis independent of γ-secretase activity. To address this issue, next we determined whether γ-secretase-inactive PS1 mutants are capable of inducing apoptotic cell death. As shown in Fig. 5*A*, when cells were transfected with PS1 mutants PS1D385A and PS1D257A (*lanes 3* and *4*), which have been reported to be dominant negative γ-secretase-inactive mutants of PS1 (29, 30), strong apoptotic signals (PARP cleavage, caspase activation, and PS1caspCTF formation) were detected, similar to PS1wt-transfected cells (*lane 2*). However, when cells were transfected with a PS1 variant bearing double aspartate mutations (PS1D257A/D385A), which is also γ-secretase-inactive

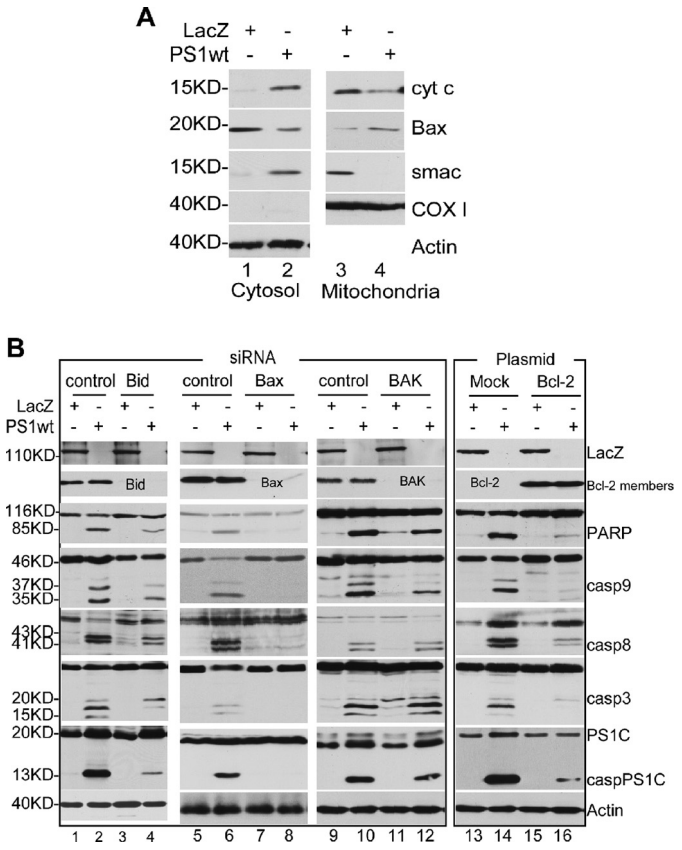

FIGURE 4. **PS1-induced apoptosis involved cytochrome *c* and Smac/DIABLO release and was regulated by Bcl-2 family proteins.** *A*, PS1-induced apoptosis involved cytochrome *c* and Smac/DIABLO release. Cells were transiently transfected with either PS1 or LacZ. Twenty-four h after transfection, cells were harvested for preparation of cytosolic extracts (*left column*) and mitochondria-containing membrane fraction (*right column*) as described under "Experimental Procedures." *Top panel*, release of cytochrome *c* from mitochondria to cytosol; *panel 2*, translocation of Bax from cytosol to mitochondria; *panel 3*, release of Smac/DIABLO from mitochondria to cytosol; *panel 4*, reprobe of the *top panel* with anti-COX I antibody, confirming the mitochondria remained intact during preparation; *bottom panel*, reprobe for actin of the membrane of *panel 2*. *B*, as indicated, 24 h after treatment with non-silencing siRNA (*lanes 1*, *2*, *5*, *6*, *9*, and *10*) or with siRNA specific to Bid, Bax, and Bak (*lanes 3*, *4*, *7*, *8*, *11*, and *12*), cells were transiently transfected with either LacZ (*lanes 1*, *3*, *5*, *7*, *9*, and *11*) or PS1 (*lanes 2*, *4*, *6*, *8*, *10*, and *12*). Knockdown of Bid strongly inhibited PS1-induced apoptosis (*lane 4*). Knockdown of Bax almost completely inhibited PS1-induced apoptosis (*lane 8*). Knockdown of Bak had only a negligible effect on PS1-induced apoptosis (*lane 12*). In the *fourth column*, cells bearing an empty vector (*lanes 13* and *14*) or stably expressing Bcl-2 (*lanes 15* and *16*) were further transfected with either LacZ (*lanes 13* and *15*) or PS1 (*lanes 14* and *16*). LacZ (*top panel*), Bcl-2 proteins (*second panel*), PARP cleavage (*third panel*), caspase activation (*panels 4–6*), PS1 (*panel 7*), and actin (*panel 8*) were determined by Western blot as described above. Overexpression of Bcl-2 strongly inhibited PS1-induced apoptosis.

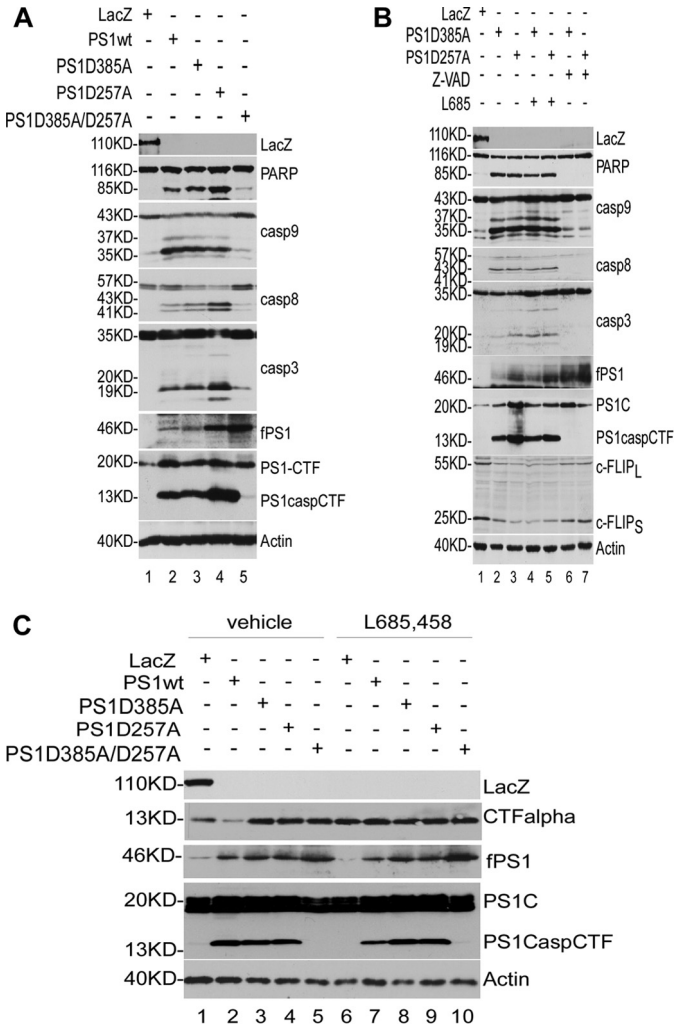

FIGURE 5. **γ-Secretase-inactive PS1 mutants induced γ-secretase activity-independent apoptosis.** *A*, γ-secretase-inactive PS1 mutants PS1D257A and PS1D385A, but not PS1D385A/D257A, induced apoptotic cell death. HeLa cells were transiently transfected with LacZ (*lane 1*), PS1wt (*lane 2*), or γ-secretase inactive mutants of PS1, PS1D385A (*lane 3*), PS1D257A (*lane 4*), and PS1D385A/D257A (*lane 5*) for 24 h. *B*, γ-secretase inhibitors had no effect on γ-secretase-inactive PS1 mutant-induced apoptosis. HeLa cells were transiently transfected with LacZ or γ-secretase inactive mutants of PS1 (PS1D385A and PS1D257A) in the presence of γ-secretase inhibitor L685,458 (0.5 μM; *lanes 4* and *5*) or caspase inhibitor Z-VAD (100 μM; *lanes 6* and *7*) for 24 h. *C*, PS1wt, but not inactive mutant PS1 variants, induced significant reduction in the level of CTFα (*lane 2*), the C-terminal fragment of APP produced by α-cleavage of APP, which is the primary substrate of γ-secretase. The decrease of CTFα caused by overexpression of PS1wt was completely inhibited by γ-secretase inhibitor L-685,458 (*lane 7*). It is notable that in the presence of L-685,458, the basal level of CTFα was higher than in controls (compare *lane 6* with *lane 1*). LacZ expression (*top panel*), PARP cleavage, caspase activation, and c-FLIP turnover were determined by Western blot analysis, as described above. Full-length PS1 (*fPS1*) and PS1C were detected with anti-PS1N and anti-PS1C, respectively. The blot in the *bottom panel* was reprobed with anti-actin antibody as a loading control.

(31), only negligible apoptotic signs were detected (*lane 5*). As shown in Fig. 5*B*, when the cells were pretreated with γ-secretase inhibitors, it was interestingly found that, in contrast to PS1wt, PS1D257A- and PS1D385A-induced apoptosis was not affected by γ-secretase inhibitor L-685,458 (*lanes 4* and *5*). All PS1 variant-induced apoptosis was inhibited by caspase inhibitor Z-VAD (*lanes 6* and *7*). These results clearly indicate that PS1 could induce apoptosis independent of γ-secretase activity. A slight decrease in the level of c-FLIP was also observed in γ-secretase-inactive mutant PS1D257A- and PS1D385A-transfected cells (*lanes 2* and *3* in *panel 8*). However, the decrease of c-FLIP caused by PS1D257A and PS1D385A was not inhibited by γ-secretase inhibitor L-685,458 but was inhibited by caspase inhibitor Z-VAD. This result indicates that the caspase activity accounts for the slight decrease of c-FLIP under these conditions.

To further determine whether PS1D385A and PS1D257A have any γ-secretase activity, we determined the effects of overexpression of these mutants on the level of CTFα, the C-terminal fragment of APP produced by α-cleavage, which is the pri-

mary substrate of γ-secretase. As shown in Fig. 5*C*, PS1wt enhanced the turnover of CTFα (*lane 2*), and the decrease of CTFα caused by overexpression of PS1wt was completely inhibited by the addition of γ-secretase inhibitor L-685,458 (*lane 7*). However, in contrast to PS1wt, overexpression of the enzymatically inactive mutant PS1 variants did not cause a reduction but rather resulted in a slight increase in the level of CTFα (compare *lanes 3–5* with *lane 1*). This result is in agreement with previous studies that showed that these PS1 mutations, PS1(D385A) and PS1(D257A), completely abolish the γ-secretase activity of PS1 protein (29). Thus, our data confirmed that the single mutant, as well as the double mutant, has no γ-secretase activity, as determined by the turnover of CTFα. These data provide further support for the notion that PS1D385A- and PS1D257A-induced apoptosis is independent of γ-secretase activity.

PS1-induced Apoptosis Was Strongly Blocked by Knockdown of PSAP and Completely Blocked by Knockdown of PSAP in the Presence of γ-Secretase Inhibitor—The above results clearly indicate that γ-secretase activity contributes to only a portion of PS1-induced apoptosis. In other words, the majority of PS1-induced apoptosis probably occurred in a γ-secretase activity-independent manner. In a previous study, we identified PSAP as a PS1-associated protein by using the yeast two-hybrid system. This PSAP protein was subsequently recognized as a mitochondrial proapoptotic molecule. The interaction of PS1 with PSAP suggests a possible role for PSAP in PS1-induced apoptosis. We then examined the effect of knockdown of PSAP on PS1-induced apoptosis. As shown in Fig. 6*A*, knockdown of PSAP resulted in a strong inhibition of PS1wt- and AD-linked mutant PS1L286V-induced apoptosis (*lanes 5* and *11*) and completely inhibited PS1D385A- and PS1D257A-induced apoptosis (*lanes 17* and *18*). It was also noted that the effect of PSAP knockdown is much greater than that of γ-secretase inhibitor in inhibiting PS1wt-induced apoptosis (compare *lane 5* with *lane 3* in the *third panel*). Next, we examined the effects of the combination of PSAP knockdown and γ-secretase inhibitor on PS1-induced apoptosis. As shown in *lanes 6* and *12* of Fig. 6*A*, knockdown of PSAP in the presence of γ-secretase inhibitor L-685,458 resulted in a complete inhibition of PS1wt- and PS1L286V-induced apoptosis. These results strongly suggest that PS1-induced apoptosis is mediated by both γ-secretase activity and interaction with PSAP and that interaction with PSAP accounts for the majority of PS1-induced apoptosis.

PS1wt, PS1D257A, and PS1D385A, but not PS1D257A/D585A Double Mutant, Induced PSAP-Bax Complex Formation, and This Complex Formation Was Blocked by Bcl-2—To further explore the mechanism by which PSAP mediates PS1-induced apoptosis, we determined the possible interaction between PSAP and Bax upon PS1 expression. As shown in Fig. 6*B*, upon transfection with wild type PS1, Bax was co-immunoprecipitated with PSAP by anti-PSAP antibody (*lane 3*) but not by IgG (*lane 7*). This complex was not detected in LacZ-transfected cells (*lane 2*). As shown in *lanes 4* and *5*, the PSAP-Bax complex was also detected in cells transfected with γ-secretase-inactive mutants PS1D257A and PS1D385A. However, this PSAP-Bax complex was not detected in cells transfected with double aspartate mutations PS1D257A/D385A (*lane 6*). Interestingly, PS1wt, PS1D257A, and PS1D385A (*lanes 2–5*), but not PS1D257A/D385A (*lane 6*), were found to complex with PSAP. When the Bcl-2-stably expressing cells were transfected with these PS1, as shown in Fig. 6*C*, the PSAP-Bax complex was not detected (compare *lane 5* with *lane 3*), indicating that Bcl-2 blocks the interaction of Bax with PSAP.

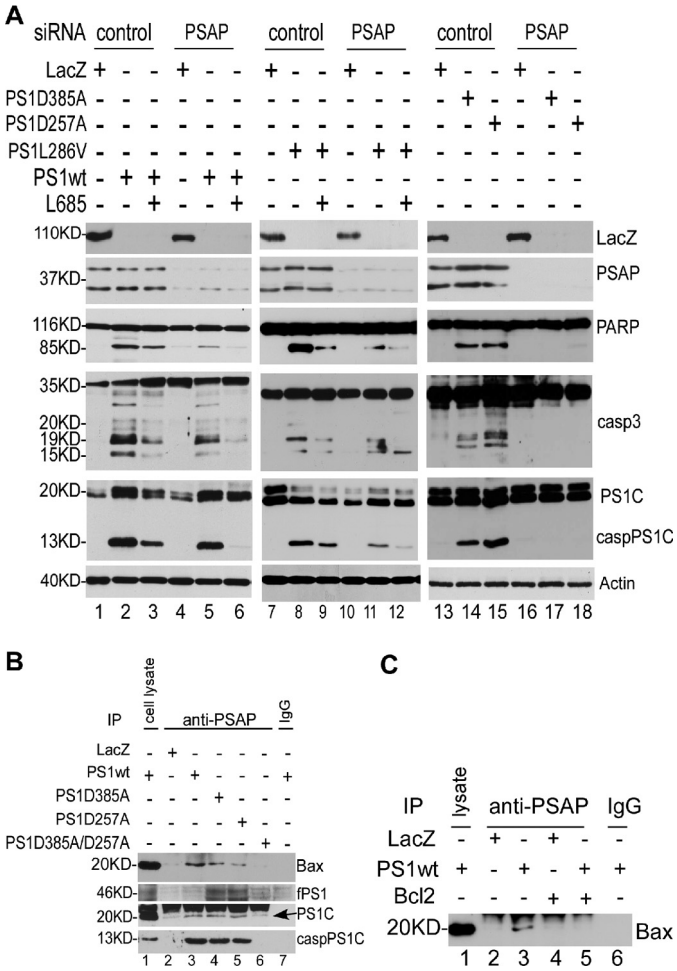

FIGURE 6. **PSAP is required for PS1-induced γ-secretase-independent apoptosis.** *A*, PS1-induced apoptosis was strongly blocked by knockdown of PSAP and completely blocked by knockdown of PSAP in the presence of γ-secretase inhibitor. Cells treated with non-silencing siRNA (*lanes 1*, *2*, *3*, *7*, *8*, *9*, *13*, *14*, and *15*) or with PSAP-specific siRNA (*lanes 4*, *5*, *6*, *10*, *11*, *12*, *16*, *17*, and *18*) were transfected with LacZ (*lanes 1*, *4*, *7*, *10*, *13*, and *16*), PS1wt (*lanes 2*, *3*, *5*, and *6*), PS1L286V (*lanes 8*, *9*, *11*, and *12*), PS1D385A (*lanes 14* and *17*), or PS1D257A (*lanes 15* and *18*) for 24 h in the absence (*lanes 1*, *2*, *4*, *5*, *7*, *8*, *10*, and *11*) or presence (*lanes 3*, *6*, *9*, and *12*) of γ-secretase inhibitor L685,458 (0.5 μM). *B*, both PS1wt and γ-secretase-inactive PS1 mutants complexed with PSAP and induced complex formation between PSAP and Bax. Cell lysates transfected with LacZ (*lane 2*), PS1wt (*lane 3*), PS1D385A (*lane 4*), PS1D257A (*lane 5*), and PS1D385A/PS1D257A (*lane 6*) were immunoprecipitated (*IP*) using anti-PSAP antibody, and the immunoprecipitants were probed with anti-Bax and anti-PS1C antibodies. *Lane 1*, positive control of cells transfected with PS1wt. PS1wt-transfected cells were also immunoprecipitated using nonspecific IgG as a negative control (*lane 7*). PS1C is indicated by the *arrow*, and the band *above* is nonspecific. *C*, PS1-stimulated PSAP-Bax complex was blocked by Bcl-2. Cell lysates transfected with LacZ (*lane 2*), PS1wt (*lane 3*), LacZ and Bcl-2 (*lane 4*), and PS1wt and Bcl-2 (*lane 5*) were immunoprecipitated using anti-PSAP antibody, and the immunoprecipitants were probed with anti-Bax antibody. *Lanes 1* and *6*, positive and negative control, respectively.

PS2 Induced Apoptosis via a Pathway That Is Different from That of PS1—In our previous study, we found that PSAP specifically interacts with PS1 but not PS2. To determine whether PSAP specifically mediates PS1-induced apoptosis, we exam-

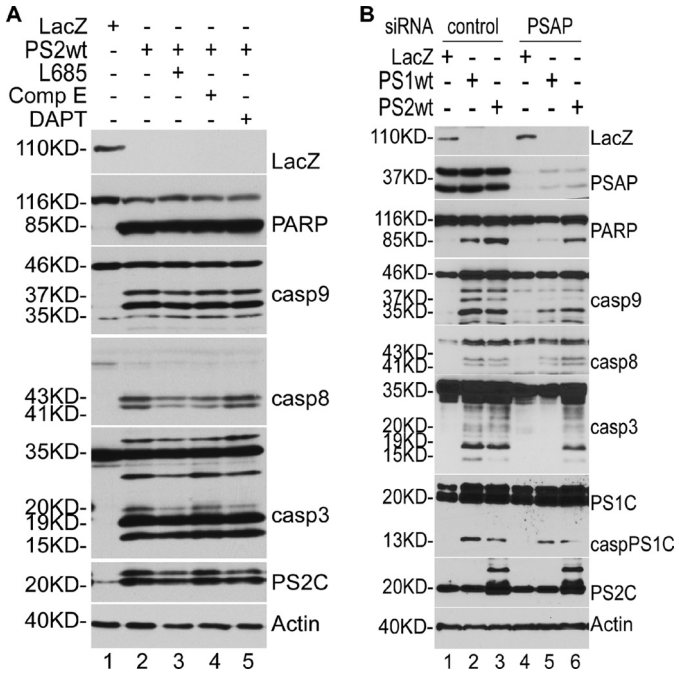

FIGURE 7. **PS2wt-induced apoptosis was inhibited neither by γ-secretase inhibitor nor by knockdown of PSAP.** *A*, cells were transfected with PS2 in the absence (*lane 2*) or presence (*lanes 3–5*) of γ-secretase inhibitor. *B*, cells treated with control siRNA (*lanes 1–3*) or with PSAP-specific siRNA (*lanes 4–6*) were transfected with PS1wt (*lanes 2* and *5*) or with PS2wt (*lanes 3* and *6*).

ined the effect of knockdown of PSAP on PS2-induced apoptosis. As shown in Fig. 7, PS2-induced apoptosis was inhibited neither by γ-secretase inhibitors (Fig. 7*A*) nor by knockdown of PSAP (Fig. 7*B*). These results clearly indicate that PS2 induced apoptosis via a pathway that is different from that of PS1.

Discussion

Through the use of γ-secretase inhibitors and siRNA approaches, our results strongly suggest that PS1 induces apoptosis through two pathways: the γ-secretase-dependent pathway, which is mediated by the γ-secretase-catalyzed turnover of c-FLIP, and the γ-secretase-independent pathway, which is mediated by PS1-stimulated PSAP-Bax complex formation. Specifically, our results suggest that the mitochondrial proapoptotic protein PSAP plays a major role in mediating PS1-induced apoptosis. These findings provided new insight into the role of PS1 in neurodegeneration in AD and may lead to the identification of novel therapeutic targets for treatment and prevention of AD.

Previous studies have shown that PS1-regulated apoptosis involves increasing oxidative stress (32), disturbance of cellular calcium homeostasis and elevation of p53 expression (33), and increasing vulnerability to ER stress by altering the unfolded-protein response signaling pathway (34). In this study, we conducted a series of experiments and found that overexpression of PS1 induced apoptosis in various types of cells, including neuroblastoma H4 and cervical cancer HeLa cells. In determining the mechanism by which PS1 induces apoptosis, our data revealed that PS1-induced apoptosis was associated with a decrease in the levels of c-FLIP. Interestingly, the decrease of c-FLIP in PS1-expressing cells was inhibited by the γ-secretase inhibitor, which also partially inhibited PS1-induced apoptosis. Although the degradation of c-FLIP may not be the only factor that mediates PS1-induced γ-secretase activity-dependent apoptosis, this finding strongly suggests that the turnover of c-FLIP catalyzed by γ-secretase activity contributes to PS1-induced apoptosis. c-FLIP is a well described inhibitor of death receptor-mediated apoptosis. Two major isoforms, c-FLIP$_L$ and c-FLIP$_S$, and a minor form, c-FLIP$_R$, are detected at the protein level in human cells. c-FLIP$_L$ is structurally similar to caspase-8 with two N-terminal death effector domains (DEDs) and a C-terminal caspase-like domain, which lacks the catalytic cysteine residue and, therefore, is enzymatically inactive. The two short forms, c-FLIP$_S$ and c-FLIP$_R$, are composed of mainly the two N-terminal DEDs, so they function solely as inhibitors of caspase-8 activation by competing with caspase-8 for binding to the adaptor protein FADD via DED-DED interaction during DISC formation. At high concentrations, c-FLIP$_L$ acts as an anti-apoptotic inhibitor; however, at low concentrations, c-FLIP$_L$ may complex with caspase-8, leading to activation of non-apoptotic signaling (for a review, see Refs. 35 and 36). Many previous studies have shown that knockdown or down-regulation of c-FLIP results in ligand-independent, but caspase-8-dependent, spontaneous apoptosis (37–39). Thus, our observation that γ-secretase inhibitor, which inhibited the turnover of both c-FLIP$_L$ and c-FLIP$_S$, also partially blocked PS1-induced apoptosis strongly suggests that PS1-induced apoptosis is at least partially mediated by γ-secretase-catalyzed turnover of c-FLIP. This speculation is supported by the finding that inhibition or knockdown of caspase-8, which is modulated by c-FLIP, inhibited PS1-induced apoptosis to a similar extent. This speculation is further supported by the finding that knockdown of FADD, which is a crucial adaptor for recruiting caspase-8 into DISC and its subsequent activation, resulted in a similar degree of inhibition of PS1-induced apoptosis. This observation is in agreement with previous studies that show that FADD is required for c-FLIP knockdown-triggered caspase-8 activation and apoptosis (38, 40).

Upon activation, caspase-8 can directly activate executioner caspase-3, leading to apoptosis. On the other hand, activated caspase-8 may trigger the mitochondrial apoptotic pathway through activation of Bid (41). In this regard, it is notable that cytochrome *c* and Smac/DIABLO release and Bax translocation were detected in PS1-induced apoptosis, suggesting a possibility that the mitochondrial pathway is involved in PS1-induced apoptosis. This possibility is strongly supported by our data demonstrating that knockdown of Bid inhibited PS1-induced apoptosis to the same degree as knockdown of caspase-8. The notion that PS1-induced apoptosis is mediated by the mitochondrial pathway is further supported by the observation that PS1-induced apoptosis was nearly completely inhibited by either knockdown of Bax or overexpression of the anti-apoptotic protein Bcl-2. These observations strongly indicate that PS1-induced apoptosis is mediated by the Bcl-2 protein-controlled mitochondrial pathway. However, it was noted that, in contrast to complete inhibition by knockdown of Bax or by overexpression of Bcl-2, PS1-induced apoptosis was only partially inhibited by knockdown of caspase-8 and Bid. These findings indicate that factors other than caspase-8-activated Bid

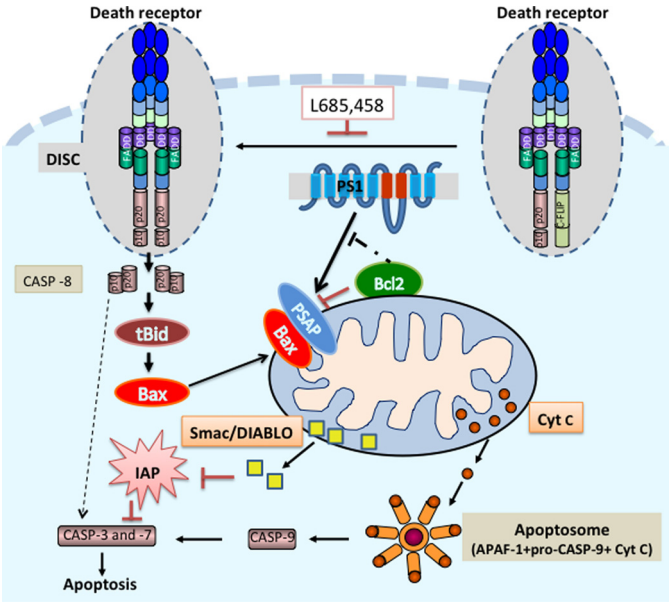

FIGURE 8. **Proposed signaling pathways of PS1-induced apoptosis.** Data produced in this study support the hypothesis that PS1 could induce apoptosis through two pathways, γ-secretase-dependent and γ-secretase-independent pathways. In the γ-secretase-dependent pathway, PS1 first catalyzes the turnover of c-FLIP, leading to the activation of caspase-8, which in turn activates tBid, resulting in Bax translocation and the release of cytochrome *c* and Smac/DIABLO. PS1-induced apoptosis through this pathway can be inhibited by γ-secretase inhibitor. In the γ-secretase-independent pathway, PS1 stimulates PSAP-Bax complex formation and subsequent mitochondrial dysfunction. Activated caspase-8 may directly activate caspase-3 and -7, leading to apoptosis. However, the finding that knockdown of Bax almost completely blocked PS1-induced apoptosis indicates that the contribution of this direct pathway is negligible.

may contribute to PS1-induced mitochondrial dysfunction. The fact that PS1 interacts with the mitochondrial proapoptotic molecule PSAP prompted us to determine whether PSAP plays a role in mediating PS1-induced apoptosis. Our data demonstrated that knockdown of PSAP strongly inhibited PS1-induced apoptosis. Moreover, the fact that the combination of PSAP knockdown with γ-secretase inhibitor completely inhibited PS1-induced apoptosis strongly suggests that, in addition to the γ-secretase activity-dependent pathway, PSAP is the major mediator in PS1-induced apoptosis. This conclusion is strongly supported by the finding that γ-secretase-inactive PS1 mutant-induced apoptosis was completely blocked by knockdown of PSAP but not affected by γ-secretase inhibitor. In determining the mechanism by which PSAP mediated PS1-induced apoptosis, our data demonstrated that both wild type PS1 and the γ-secretase-inactive PS1 mutants stimulated the formation of the PSAP-Bax complex, and this complex formation was blocked by overexpression of Bcl-2. These observations clearly suggest that PSAP-Bax complex formation is a crucial event in PS1-induced apoptosis. In a previous study, it was reported that PS1-induced cytochrome *c* release was blocked by expression of Bcl-2 (42). Our results suggest that this is probably due to a blockage effect of Bcl-2 on PS1-induced PSAP-Bax complex formation. Taken together, as shown in Fig. 8, our data reveal that PS1 activates two signaling cascades: the γ-secretase-dependent PS1-c-FLIP-caspase-8-Bid cascade and the γ-secretase-independent PS1-PSAP cascade, which converge on Bax to activate the mitochondrial apoptotic pathway. Thus, our findings revealed for the first time the direct molecular links between PS1 and the apoptotic cascades.

In addition to the pathways by which PS1 induces apoptosis, this study has revealed several interesting novel findings. First, the results of this study demonstrated that PS1 promotes c-FLIP degradation in a γ-secretase-dependent manner. γ-Secretase is a multiprotein complex composed of PS, nicastrin, Aph-1, and Pen-2, with PS functioning as the catalytic subunit with a loose substrate specificity (43, 44). The most studied among γ-secretase substrates are APP and Notch. Apart from them, a growing number of proteins have been reported to be processed by γ-secretase, and most of these proteins are type I transmembrane proteins (7). PS1 has been reported to promote the degradation of β-catenin by proteasome (45). However, PS1-promoted degradation of β-catenin was independent of γ-secretase activity. In this regard, it is notable that PS1-catalyzed c-FLIP turnover was not affected by proteasome inhibitor (data not shown). Therefore, the finding that γ-secretase inhibitor caused an increase in the level of c-FLIP in intact cells and that c-FLIP was co-immunoprecipitated with PS1 strongly suggests that the cytosolic protein c-FLIP was processed in a γ-secretase-dependent manner. However, whether the effect of PS1 on c-FLIP turnover is direct or indirect may require further clarification in a future study. Nevertheless, it is interesting to note that by evaluating the expression levels of caspases or apoptosis-related proteins in human postmortem brain cerebellum or frontal cortex tissues of patients with AD, studies have shown that c-FLIP expression levels were indeed decreased in AD patients compared with controls (46, 47). These observations provide additional support for the notion that the decrease of c-FLIP caused by PS1 may contribute to its pathogenic effects of PS1 in AD development.

The second interesting finding is the differential effects of γ-secretase inhibitor on PS1-catalyzed c-FLIP turnover. Previous studies, including ours, revealed that APP is processed by γ-secretase at multiple cleavage sites in a sequential manner (48). We further found that these cleavage sites were differentially inhibited by different inhibitors (*i.e.* the transition state analog inhibitor L-685,458 specifically inhibits ϵ-cleavage but has no effect on γ-and ζ-cleavages; on the other hand, the non-transition state analog inhibitors, such as compound E and DAPT, inhibit γ-cleavage but have no effect on ϵ- and ζ-cleavages) (24). More interestingly, our studies further revealed that inhibition of ϵ-cleavage by L-685,458 prevented γ- and ζ-cleavages from occurring, indicating that γ- and ζ-cleavages are dependent on ϵ-cleavage occurring first (24). In this study, our data clearly demonstrated that PS1-catalyzed c-FLIP turnover was specifically inhibited by L-685,458 but not affected by compound E and DAPT, suggesting that c-FLIP is processed by PS1 in a way similar to how PS1 processes APP. Similar sequential cleavage has also been shown to take place in Notch and presenilin endoproteolytic processing (49, 50). Thus, these observations strongly suggest that γ-secretase-catalyzed protein processing may follow a common sequential cleavage mechanism.

Third, our study also revealed that the enzymatically inactive PS1 mutants PS1D257A and PS1D385A possess apoptotic functions. Although the most characterized function of PS1 is to serve as the catalytic component of the γ-secretase complex,

there is intriguing evidence to suggest that PS1 also acts through γ-secretase-independent mechanisms to affect many cellular events (51). These include cell signaling, cell adhesion, and protein trafficking and degradation. Thus, our finding that the PS1D257A and PS1D385A induce apoptosis provides further evidence supporting the notion that PS1 can functionally regulate certain cellular events in a γ-secretase-independent manner.

Fourth, our results suggest that PSAP functions as a receptor or anchor for Bax in the outer membrane of mitochondria under certain apoptotic conditions. The finding that both wild type PS1 and enzymatically inactive mutant PS1 stimulate PSAP-Bax complex formation suggests that the γ-secretase-independent apoptotic signal induced by PS1 is transduced through PSAP-mediated translocation of Bax to mitochondria. PSAP, also known as MTCH1 (mitochondrial carrier homolog 1) is localized in the outer membrane of mitochondria (18). Thus, one possible way in which PSAP mediates PS1-induced apoptosis is that PSAP functions as an anchor to recruit Bax to mitochondria upon overexpression of PS1. In this regard, it is notable that MTCH2 (mitochondrial carrier homolog 2), a homologue of PSAP, has been reported to function as an anchor or receptor for tBid in the outer membrane of mitochondria in Fas-induced apoptosis (52, 53). Thus, the results of this study and previous studies suggest that PSAP and MTCH2 form a unique family of mitochondrial carrier homolog proteins that are localized in the outer membrane of mitochondria and play an important role in the mitochondrial pathway of apoptosis by functioning as receptors or anchors for Bax and tBid, respectively. Notably, previous studies have shown that overexpression of PSAP caused apoptosis in a Bcl-2 protein-independent manner (54, 55). One possibility for this result is that because PSAP is a six-transmembrane protein localized in the outer membrane of mitochondria (18), when it is overexpressed, PSAP may cause damage on the outer membrane of mitochondria or even form a channel by itself, leading to the release of apoptotic factors. However, at the normal endogenous level, PSAP may function as an anchor to recruit Bax to the mitochondria and form a complex with Bax upon activation of apoptosis by PS1, as observed in the current study.

Finally, our results revealed that PS2-induced apoptosis was inhibited neither by γ-secretase inhibitor nor by PSAP knockdown, strongly suggesting that PS2 induces apoptosis through a pathway that is different from that of PS1. This may be largely because, in contrast to PS1, PS2 does not interact with PSAP, as reported in our previous study (16).

Author Contributions—L. Z. performed and analyzed most of the experiments presented. C. H. performed and analyzed the experiment shown in Fig. 5*C*. F. Z. and D. C. X. contributed to Fig. 1*B* and Fig. 8. M. Z. C. and X. X. designed the study, analyzed the data, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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