p53-dependent Aph-1 and Pen-2 Anti-apoptotic Phenotype Requires the Integrity of the γ-Secrate Complex but Is Independent of Its Activity*

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The presenilin-dependent γ-secretase activity, which is responsible for the generation of amyloid β-peptide, is a high molecular weight complex composed of at least four components, namely, presenelin-1 (or presenelin-2), nicastrin, Aph-1, and Pen-2. Previous data indicated that presenilins, which are thought to harbor the catalytic core of the complex, also control p53-dependent cell death. Whether the other components of the γ-secretase complex could also modulate the cell death process in mammalian neurons remained to be established. Here, we examined the putative contribution of Aph-1 and Pen-2 in the control of apoptosis in TSM1 cells from a neuronal origin. We show by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and DNA fragmentation analyses that the overexpression of Aph-1, Aph-1b, or Pen-2 drastically lowered staurosporine-induced cellular toxicity. In support of an apoptosis rather than necrosis process, Aph-1 and Pen-2 also lowered staurosporine- and etoposide-induced caspase-3 expression and diminished caspase-3 activity and poly(ADP-ribose) polymerase inactivation. The Aph-1 and Pen-2 anti-apoptotic phenotype was associated with a drastic reduction of p53 expression and activity and lowered p53 mRNA transcription. Furthermore, the Aph-1- and Pen-2-associated reduction of staurosporine-induced caspase-3 activation was fully abolished by p53 deficiency. Conversely, Aph-1, Aph-1b, and Pen-2 gene inactivation increases both caspase-3 activity and p53 mRNA levels. Finally, we show that Aph-1 and Pen-2 did not trigger an anti-apoptotic response in cells devoid of presenilins or nicastrin, whereas the protective response was still observed in fibroblasts devoid of β-amyloid precursor protein and amyloid precursor protein like-protein 2. Furthermore, Aph-1- and Pen-2-associated protection against staurosporine-induced caspase-3 activation was not affected by the γ-secretase inhibitors N-(3,5-difluorophenacetyl)-l-Val-Ala-γ-secretase inhibitors N-(3,5-difluorophenacetyl)-l-Val-Ala-Tyr-phenylethylketone. Altogether, our study indicates that Aph-1 and Pen-2 trigger an anti-apoptotic response by lowering p53-dependent control of caspase-3. Our work also demonstrates that this phenotype is strictly dependent on the molecular integrity of the γ-secretase complex but remains independent of the γ-secretase catalytic activity.

Alzheimer disease (AD) is an age-associated neurodegenerative disorder that is characterized, at the histopathological level, by the occurrence of senile plaques that are due to the abnormal and exacerbated production of a set of hydrophilic peptides referred to as amyloid β-peptide (Aβ) (1). These peptides are generated by sequential cleavage of a type I transmembrane protein precursor, the β-amyloid precursor protein (βAPP), by two proteases called β- and γ-secretase (2). β-Secretase apparently corresponds to β-site APP cleaving enzyme β-secretase activity. Aph-1 and Pen-2 also lower staurosporine- and etoposide-induced caspase-3 expression and diminished caspase-3 activity and poly(ADP-ribose) polymerase inactivation. The Aph-1 and Pen-2 anti-apoptotic phenotype was associated with a drastic reduction of p53 expression and activity and lowered p53 mRNA transcription. Furthermore, the Aph-1- and Pen-2-associated reduction of staurosporine-induced caspase-3 activation was fully abolished by p53 deficiency. Conversely, Aph-1, Aph-1b, and Pen-2 gene inactivation increases both caspase-3 activity and p53 mRNA levels. Finally, we show that Aph-1 and Pen-2 did not trigger an anti-apoptotic response in cells devoid of presenilins or nicastrin, whereas the protective response was still observed in fibroblasts devoid of β-amyloid precursor protein and amyloid precursor protein like-protein 2. Furthermore, Aph-1- and Pen-2-associated protection against staurosporine-induced caspase-3 activation was not affected by the γ-secretase inhibitors N-(3,5-difluorophenacetyl)-l-Val-Ala-γ-secretase inhibitors N-(3,5-difluorophenacetyl)-l-Val-Ala-Tyr-phenylethylketone. Altogether, our study indicates that Aph-1 and Pen-2 trigger an anti-apoptotic response by lowering p53-dependent control of caspase-3. Our work also demonstrates that this phenotype is strictly dependent on the molecular integrity of the γ-secretase complex but remains independent of the γ-secretase catalytic activity.

Unlike PS, relatively few works document the function of Aph-1, Pen-2, and nicastrin. It is clear however that in mammals, the interaction of the four members is necessary for the functionality of the PS-dependent γ-secretase activity. Aph-1 contributes largely to the stabilization of γ-secretase complex by forming a subcomplex with nicastrin (20–22), whereas

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4 The abbreviations used are: AD, Alzheimer disease; Aph-1, anterior pharynx defective-1; Pen-2, presenilin enhancer-2; NCT, nicastrin; TSM, telencephalon specific murine; PS, presenilin; APLP, amyloid precursor protein like-protein; MEF, mouse embryonic fibroblasts; STS, staurosporine; Eto, etoposide; Ac-DEVD-al, acetyl-Asp-Glu-Val-Asp-aldehyde; TUNEL, terminal dUTP nick-end labeling; PARP, poly(ADP-ribose) polymerase; Mdm2, mouse double minute 2; siRNA, small interference RNA; DAPT, N-[N-(3,5-difluorophenacyl)-l-alanyl]-S-phenylglycine t-butyl ester; DFK, difluoromethyl ketone; Aβ, amyloid β-peptide; βAPP, β-amyloid precursor protein; AICD, βAPP intracellular domain; E3, ubiquitin-protein isopeptide ligase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ALID2, APP-like intracellular domain 2.
Pen-2 is required for the maturation of both PS by endoproteolysis (18, 23) leading to the generation of two C- and N-terminal fragments, namely CTF-PS and NTF-PS. The stringency of the necessary presence of each of the components of the complex is illustrated by the fact that the absence of any of these proteins affects Aβ production (18, 19). Accordingly, the loss of function of Aph-1 and Pen-2 genes in Caenorhabditis elegans results in a Notch signaling deficiency, a phenotype that is directly linked to the ability of γ-secretase to process Notch (24).

The implication of programmed cell death in AD has been largely documented in the last 10 years (25–29). In agreement with these studies, several works have consistently documented a pro-apoptotic phenotype of wild-type PS2, the extent of which appeared exacerbated by mutations responsible for familial forms of AD (30–32), whereas, conversely, PS1 appeared biologically inert in relation to apoptosis (33). Recently, we demonstrated that PS2 and PS1 cross-talk to con-
provided by Dr. L. Mercken, Aventis), anti-Mdm2 (mouse monoclonal, kindly provided by Dr. J. C. Bourdon), anti-β-tubulin (mouse monoclonal, Sigma), and anti-actin (mouse monoclonal, Sigma). Immunological complexes were revealed by enhanced electrochemiluminescence (Roche Applied Science) with either an anti-rabbit peroxidase or with an anti-mouse peroxidase (Jackson ImmunoResearch, Cambridgeshire, UK) antibodies according to the nature of the primary antibodies listed above.

p53 Transcriptional Activity and p53 Promoter Activity Measurement—The activity of p53 is measured with a bioassay by transient transfection of the PG13-luciferase cDNA (kindly provided by Dr. B. Vogelstein, Baltimore, MD), which is based on the genomic DNA consensus sequence recognized by p53 on its target genes (40). The transcriptional activation of the p53 promoter was measured after transfections of cDNAs harboring the murine (41) p53 promoter sequences in-frame with luciferase (provided by Dr. M. Oren, Rehovot, Israel). TSM-1 cells were cultivated in 12-well plates until 60% confluence and then cotransfected with 1.0 μg of PG13-luciferase cDNA or 1.0 μg of p53-promoter-luciferase cDNA and 0.5 μg of a β-galactosidase transfection vector (to normalize transfection efficiency) by means of the SuperFect transfection reagent according to the manufacturer’s conditions (Qiagen). Forty-eight hours after transfection, luciferase and β-galactosidase activities were analyzed according to manufacturer’s conditions (Promega kit).

Real-time Quantitative PCR—Total RNA from HEK293 or TSM1 cells was extracted by the mean of the RNeasy kit (Qiagen) according to the manufacturer’s recommendations, then treated with DNase I, and 4 μg of RNA obtained was reverse transcribed as previously described then Real-time PCR was performed as extensively described (34) in an ABI PRISM 5700 sequence detector system (Applied Biosystems, Foster City, CA) with gene-specific primers for human Aph-1a, Aph-1b, Pen-2, p53, and human glyceraldehyde-3-phosphate dehydrogenase to control RNA concentrations.

siRNA Approach—siRNA duplexes (Qiagen) for Aph-1a (42), Aph-1b (42), and Pen-2 (43) were previously shown to drastically reduce endogenous levels of these proteins. A control non-silencing siRNA duplex (siCT) was used as a control. HEK293 cells were cultured until they reach 70% of confluency, and then transfected with the appropriate siRNA duplex for 24 h using HiPerfect transfection reagent (Qiagen). Cells were then harvested, and the levels of mRNA for Aph-1a, Aph-1b, and Pen-2 were analyzed by real-time quantitative PCR as described above. Then, caspase-3 activity and p53 mRNA levels were analyzed by fluorometry and real-time quantitative PCR as described above.

Statistical Analysis—Statistical analysis was performed with PRISM (GraphPad, San Diego, CA) using the Newman-Keuls multiple comparison test for one-way analysis of variance.

RESULTS

Aph-1a, Aph-1b, and Pen-2 Overexpression Reduces Staurosporine-induced Toxicity in TSM1 Neuronal Cells—We took advantage of the recent acquisition and characterization (36) of stably transfected TSM-1 cells overexpressing Aph-1a, Aph-1b, or Pen-2 (Fig. 1A) to examine the influence of these proteins on the responsiveness of the TSM1 neuronal cell line (44) to staurosporine. Staurosporine drastically increased the number of TUNEL-positive, mock transfected TSM1 cells (Fig. 1, B and C). The number of apoptotic nuclei was significantly reduced by either Aph-1a, Aph-1b, or Pen-2 overexpression (Fig. 1C). In agreement with these observations, staurosporine drastically enhanced the DNA fragmentation of mock transfected TSM1 neurons (Fig. 2A), whereas a statistically significant reduction of DNA fragmentation was observed in Aph-1a-, Aph-1b-, and Pen-2-overexpressing TSM1 cells (Fig. 2B).

Aph-1a, Aph-1b, and Pen-2 Overexpression Reduces Staurosporine- and Etoposide-induced Caspase-3 Activation in TSM1 Cells—TSM1 cells respond to staurosporine by activating caspase-3-like activity in a dose- (Fig. 3A) and time-dependent manner (Fig. 3B). Although the Ac-DEVD-7-amido-4-methyl coumarin substrate used to monitor cellular caspase activity is not strictly specific of caspase-3, the full blockade of staurosporine-induced caspase activation by the inhibitor Ac-DEVD-al (Fig. 3C) together with the drastic increase of active caspase-3 immunoreactivity (p17 and p20, Fig. 3D) suggest that caspase-3 indeed accounts for most of the staurosporine-induced increase in caspase-like activity. Interestingly, Aph-1 and Pen-2 expression reduces both caspase-3 activity (Fig. 3, A–C) and expression (Fig. 3D). PARP is involved in DNA repair and is inactivated by caspase-3 cleavage in apoptotic conditions (45, 46). As expected, staurosporine-induced caspase-3 activation


**FIGURE 2.** Aph-1a, Aph-1b, and Pen-2 expression decreases staurosporine-induced DNA fragmentation in TSM1 cells. Mock transfected TSM1 neurons (A) or cells expressing Aph-1a, Aph-1b, and Pen-2 (B) were treated for 16 h without (CT) or with staurosporine (STS, 0.5 μM). Cells were then treated with propidium iodide, and nuclear propidium iodide incorporation was measured as described under “Materials and Methods.” Typical representative histograms obtained in mock cells are shown in A. In B, the percentage of apoptotic nuclei measured by FACScan flow cytometry (see “Materials and Methods”) corresponds to the mean ± S.E. of three to five independent analyses. *p* values indicate the statistical significance of the indicated cell lines compared with mock transfected cells.

**FIGURE 3.** Aph-1a, Aph-1b, and Pen-2 expression decreases caspase-3-like activity and expression in TSM1 cells. Mock transfected TSM1 neurons or cells expressing Aph-1a, Aph-1b, and Pen-2 (A and B) were treated for 2 h without (CT) or with various concentrations of staurosporine (STS, μM). In some experiments, cells were pretreated for 16 h with Ac-DEVD-al (100 μM) prior to exposure to staurosporine. Bars are the means of four to nine independent determinations. **C** and **D** depict the levels of caspase-3-like activity as described under “Materials and Methods.” Curve points are the mean values of two independent determinations. In C, neurons were treated for 2 h without (CT) or with staurosporine (1 μM). In some experiments, cells were pretreated for 16 h with Ac-DEVD-al (100 μM) prior to exposure to staurosporine. Bars are the means of four to nine independent determinations. Active caspase-3 fragments (D) and PARP (E) were monitored by Western blot in lysates of indicated stably transfected cell lines treated for 2 h in the absence (CT) or in the presence of staurosporine (STS, 1 μM) as extensively described under “Materials and Methods.” In F, TSM1 neurons stably transfected with empty vector (DNA4), Aph-1a, Aph-1b, or Pen-2 cDNA were treated for 16 h without (CT) or with etoposide (Eto, 100 μM), then analyzed for caspase-3 activity as described under “Materials and Methods.” Bars are the means ± S.E. of seven independent determinations. *p* values when compared with etoposide-treated mock transfected cells.

Additional text:

*p53*-Dependent Aph-1 and Pen-2 Anti-apoptotic Phenotype

leads to the cleavage of PARP as evidenced by the accumulation of the inactive 89-kDa PARP product in mock transfected TSM1 neurons (Fig. 3E), whereas Aph-1a, Aph-1b, and Pen-2 overexpression reduces the production of this 89-kDa product (Fig. 3E). To examine whether the Aph-1- and Pen-2-protective effect was restricted to staurosporine-stimulated caspase-3 activation, we studied the influence of Aph-1a, Aph-1b, and Pen-2 on etoposide-stimulated caspase-3. As expected, etoposide potently increases caspase-3 activity (Fig. 3F). This phenotype was fully prevented by Aph-1a, Aph-1b, and Pen-2 overexpression, indicating that these proteins exert their protective effect toward various pro-apoptotic stimuli in neuronal cells. Importantly, the overexpression of TMP21, a regulator of the γ-secretase complex (47), did not modify staurosporine-induced caspase-3 activation in TSM1 cells (data not shown).

Aph-1a, Aph-1b, and Pen-2 Overexpression Interferes with the p53-dependent Cell Death Pathway—Because p53 is directly involved in the transcriptional control of caspases (48, 49), we examined whether Aph-1a, Aph-1b, and Pen-2 could modulate this tumor suppressor, thereby leading to decreased caspase-3 activation. First, we show that both proteins drastically reduce p53-like immunoreactivity (Fig. 4A). This mimics the PS1-associated decrease observed in PS1-transfected cells (Fig. 4E). By means of a PG13-luciferase construct harboring a consensus sequence targeted by p53 (40), we were able to show that p53 activity was drastically reduced by Aph-1a, Aph-1b, and Pen-2 expression (Fig. 4B). We then examined whether Aph-1 and Pen-2 could regulate p53 at a transcriptional or post-transcriptional level. By means of a construct corresponding to the promoter of murine p53 in-frame with luciferase, we observed that Aph-1a, Aph-1b, and Pen-2 all reduce p53 promoter transactivation (Fig. 4C) as does PS1 (Fig. 4E), in agreement with our observations on p53 protein levels. Interestingly, Mdm2, an E3 ubiquitin ligase that triggers p53 proteasomal degradation by increasing p53 ubiquitination, is drastically enhanced by Aph-1a,b and Pen-2 (Fig. 4D). Altogether, this indicates that Aph-1 and Pen-2 likely modulate p53 at both transcriptional and post-transcriptional levels.

Aph-1a, Aph-1b, and Pen-2 Anti-apoptotic Phenotype Is Fully p53-dependent in TSM1 Neurons—To assess whether Aph-1a, Aph-1b, and Pen-2 anti-apoptotic phenotype was strictly dependent on the presence of p53, we took advantage of a fibroblast cell line devoid of p53 (37). We transiently transfected the cDNA coding for the three proteins in p19Aref null fibroblasts (p19Aref/−/−) or fibroblasts lacking p19Aref and p53.
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(p19Arf−/−p53−/−). It should be noted that p19Arf−/− is a tumor suppressor that needs p53 to regulate senescence and to induce G1 phase arrest (37). However, p53 can still regulate apoptotic function in p19Arf−/− (37). The comparison of both p19Arf−/− and p19Arf−/− p53−/− cell systems therefore allows to study the influence of Aph-1 and Pen-2 on p53-dependent apoptosis unrelated to the role of p53 in cell cycle progression. As expected from the above statements, p19Arf−/− fibroblasts still responded, to apoptotic stimulation as evidenced by the staurosporine-induced caspase-3 activation (Fig. 5A). Caspase-3 activity in p19Arf−/−p53−/− fibroblasts is still stimulated, although to a lesser extent (Fig. 5A), by staurosporine, indicating that part of the staurosporine-stimulated caspase-3 activation was p53-independent in these cells. Aph-1a, Aph-1b, and Pen-2 expression lowered caspase-3 activity observed in p19Arf−/−p53−/− cells. Aph-1a, Aph-1b, and Pen-2 expression lowered caspase-3 activity (Fig. 5B) in p19Arf−/−, but this reduction was totally abolished by p53 deficiency (Fig. 5C). This data indicate that Aph-1- and Pen-2-associated anti-apoptotic phenotype is fully p53-dependent and that these protein did not interfere with the p53-independent STS-stimulated caspase-3 activation observed in p19Arf−/−p53−/− cells.

Depletion of Endogenous Aph-1a and Pen-2 Increases Caspase-3 Activity and Potentiates the p53-dependent Cell Death Pathway—To examine whether the phenotype observed after overexpression of Aph-1 and Pen-2 could be physiologically relevant, we inactivated Aph-1a, Aph-1b, and Pen-2 genes by an siRNA approach. As expected, siRNA significantly lowered Aph-1a, Aph-1b, and Pen-2 mRNA levels, whereas control siRNA remain ineffective (Fig. 6A). Interestingly, proteins depletion increased the susceptibility of HEK293 cells to staurosporine, leading to enhanced caspase-3 activation (Fig. 6B). In agreement with these data, silencing of Aph-1a, Aph-1b, and Pen-2 increased p53 mRNA levels (Fig. 6C). Therefore, depletion of endogenous proteins triggers a fully opposite phenotype than the one observed when the corresponding proteins are overexpressed. The same observations stood for fibroblasts devoid of PS1 (Fig. 6D and E), depletion of TMP21 by an siRNA

FIGURE 4. Aph-1a, Aph-1b, and Pen-2 interfere with p53-dependent pathway in TSM1 cells. Mock transfected TSM1 neurons or cells expressing Aph-1a, Aph-1b, and Pen-2 were treated for 2 h without (A) or with staurosporine (STS, 1 µM) then harvested, lysed in Tris-HCl 10 mmo (pH 7.3), and analyzed for their endogenous p53 immunoreactivity by Western blot, as described under “Materials and Methods” (A). p53 activity (B) and p53-promoter transactivation (C) were monitored after transfection of a PG13-luciferase or p53-promoter-luciferase reporter gene construct, respectively, together with a β-galactosidase reporter gene construct to normalize the transfection efficiencies as described under “Materials and Methods.” Bars are the means ± S.E. of six to ten independent determinations. p values indicate statistical significance of indicated cell lines compared with mock transfected cells. In D, the expressions of Mdm2 and actin (loading control) were analyzed by Western blot as described under “Materials and Methods.” In E and F, HEK293 cells overexpressing either empty vector (Mock) or PS1 were analyzed for their endogenous p53 expression (E) or p53 activity (E) and promoter transactivation (F) as described under “Materials and Methods.”

FIGURE 5. Aph-1a-, Aph-1b-, and Pen-2-associated decrease of caspase-3 activity is p53-dependent. A, p19Arf−/− and p19Arf−/− p53−/− fibroblasts were treated for 2 h without (CT) or with staurosporine (STS, 1 µM) then assayed for caspase-3 activity as described under “Materials and Methods.” p19Arf−/− (B) and p19Arf−/− p53−/− (C) fibroblasts were transiently transfected with either empty pcDNA4 vector (CT) or Aph-1a, Aph-1b, or Pen-2 cDNA. 48 h after transfection, cells were treated for 2 h without (CT) or with staurosporine (STS, 1 µM) then caspase-3 activity was monitored. Bars correspond to caspase-3 activity expressed in percentage of activity obtained in STS-stimulated mock transfected cells and are the means ± S.E. of four to seven independent determinations. p values compare with mock transfected cells. ns, not statistically significant.
Aph-1a, Aph-1b, and Pen-2 Anti-apoptotic Phenotype Is Fully Dependent on the Integrity of the γ-Secretase Complex in TSM1 Neurons—To determine the importance of the molecular integrity of the γ-secretase complex in the Aph-1- and Pen-2-associated modulation of p53-dependent cell death, we examined whether Aph-1a, Aph-1b, or Pen-2 still modulate caspase-3 activity in PS1 and PS2 (PS−/−) or nicastrin (NCT)-deficient fibroblasts. As previously described [34], PS−/−-deficient fibroblasts exhibit lower staurosporine-stimulated caspase-3 activation (Fig. 7A), in agreement with the dominant pro-apoptotic function of PS2 over PS1 in the control of cell death. Although transient transfection of Aph-1a, Aph-1b, or Pen-2 cDNA statistically significantly reduces caspase-3 activity in wild-type fibroblasts (Fig. 7B and C), this phenotype was abolished by presephin deficiency (Fig. 7, D and E). Similar experiments carried out with NCT-deficient fibroblasts (Fig. 8) indicate first that NCT deletion increased staurosporine-induced caspase-3 activation (Fig. 8A), mimicking the phenotype triggered by Aph-1a, Aph-1b, and Pen-2 deficiency. Here again, nicastrin deficiency abolished Aph-1a-/, Aph-1b-/, and Pen-2-associated modulation of caspase-3 activity. Altogether, our experiments show that the function of Aph-1a, Aph-1b, and Pen-2 in the control of cell death is strictly dependent on the integrity of the γ-secretase complex.

Aph-1a, Aph-1b, and Pen-2 Anti-apoptotic Phenotype Is Independent of the γ-Secretase Activity—We previously established that the PS-dependent control of cell death was tightly associated with the γ-secretase complex activity [34]. Thus, we demonstrated that PS-associated...
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FIGURE 8. Aph-1α-, Aph-1β-, and Pen-2-associated decrease of caspase-3 activity is nicastrin-dependent. Wild-type NCT(+/−) and nicastrin-deficient (NCT−/−) cells (A) were treated for 4 h without (CT) or with staurosporine (STS, 1 μM), and then caspase-3 activity was measured as indicated under “Materials and Methods.” Wild-type (B) or nicastrin-deficient (D) fibroblasts were transiently transfected with empty vector (DNA4) or with the cDNA coding for Aph-1α, Aph-1β, or Pen-2 as described under “Materials and Methods.” Cells were then treated with staurosporine (1 μM) for 2 h and assayed for their endogenous caspase-3 activity. Values are expressed as the percentage of caspase-3 activity present in STS-stimulated mock transfected cells. Analyses of Aph-1, Pen-2, and β-tubulin immunoreactivities (C and E) were performed by Western blot as described under “Materials and Methods.” Bars are the means ± S.E. of three to five independent experiments. p values indicate statistical significance of indicated cell lines compared with mock transfected cells. ns, not statistically significant.

control of p53 was linked to the production of the γ-secretase-derived BAPP and APLP2 fragments, AICD and APP-like intracellular domain 2 (ALID2), respectively, and fully prevented by γ-secretase inhibitors. We therefore examined whether Aph-1α, Aph-1β, and Pen-2 remained protective in BAPP/APLP2-deficient fibroblasts, which do not harbor AICD/ALIF fragments. Fig. 9 (B–E) indicates that Aph-1- and Pen-2-induced decrease in caspase-3 activity remained similar in wild-type and APP/APLP2-deficient fibroblasts. Therefore, Aph-1 and Pen-2, unlike PS, appear to control cell death in a γ-secretase activity-independent manner. To further confirm this conclusion, we examined the direct effect of γ-secretase inhibitors on Aph-1- and Pen-2-associated phenotypes. Fig. 10 shows that DAPT (Fig. 10A) and DFK167 (Fig. 10B) did not affect the inhibition of staurosporine-stimulated caspase-3 activation triggered by Aph-1α, Aph-1β, and Pen-2 in TSM1 neurons in conditions where Aβ production is fully prevented (not shown).

DISCUSSION

Two of the key histological stigmata in AD-affected brains are the senile plaques and neuronal loss (50). Senile plaques are mainly due to the exacerbated production of a set of hydrophobic Aβ-like peptides, whereas several histochemical clues indicate that cellular loss could be associated with increased apoptotic cell death (25–27). Thus, AD pathology selectively affects a set of proteins involved in cell death (51–53), including caspase-8 (54).

Several previous studies also indicate that the tumor suppressor p53 could participate in neuronal cell death in AD. First p53-like immunoreactivity was enhanced in a subpopulation of degenerative cortical neurons in sporadic AD brains (55–58). Second, p21, which is transcriptionally activated by p53, was increased in AD brains (52). Whether Aβ overproduction and exacerbated cell death are linked is still a matter of discussion, but it should be noted that several studies indicated that Aβ could trigger apoptosis in neuronal cell cultures (59) and that this Aβ-induced neuronal cell death appeared caspase-8-mediated (60) and associated with a transcriptional activation of p53 (58).

The possible link between Aβ production and control of cell death immediately suggested that γ-secretase, the proteolytic activity involved in Aβ formation, could control p53-dependent cell death. Although both PS-dependent and PS-independent γ-secretase activities exist (9–12), the major contribution in Aβ production is due to a high molecular weight complex that includes PS1 or PS2, nicastrin, Pen-2, and Aph-1 (13–19). Interestingly, both PSs appear to modulate cell death. Thus, although wild-type PS1 appeared biologically inert (33), AD-associated PS1 mutants increased neuronal vulnerability and activated caspase-3 (61), and PS1 antisense treatment enhanced Bcl-2-sensitive cell death (62). Conversely, wild-type and mutated PS2 clearly trigger a pro-apoptotic phenotype (30–32, 63). Of most interest is the observation that both PS1- and PS2-associated control of cell death appear linked to p53. Thus, we previously demonstrated that PS2-induced caspase-3 activation was strictly p53-dependent (30). More recently, we showed that, in opposition to PS2, PS1 lowered the p53-dependent pathway (34). These opposite phenotypes agreed well with the obser-
vation that p53 directly down-regulates PS1 expression (64) likely by directly repressing the transcription of PS1 human gene (65). Therefore, PS2-induced augmentation of p53 indirectly lowers PS1 levels and thereby potentiates PS2-associated pro-apoptotic phenotype.

It was interesting to note that both phenotype appeared kinked to the catalytic activity of the γ-secretase complex. Thus, we demonstrated that both PS2 and PS1 contributed to control the intracellular level of AICD and ALIDs, the C-terminal fragments of βAPP and APLPs from which production is derived by the γ- and ε-secretase cleavages (34). In agreement, γ-secretase inhibitors fully prevent AICD-mediated control of p53 in wild-type but not in PS-deficient fibroblasts. Overall, it was concluded that PS1- and PS2-mediated control of p53-dependent cell death was directly linked to their associated γ-secretase activity (34).

Very little is known concerning the contribution of the other components of the PS-dependent γ-secretase complex in the control of cell death in mammalian cells and more particularly in neurons, but two studies suggested that they could have a role. Thus, selective apoptosis in the neuronal tube was observed in Aph-1a knock-out mice (66). Furthermore, while the present work was being completed, an elegant study demonstrated that the depletion of Pen-2 led to neuronal loss and apoptosis in zebrafish (67). We therefore examined the influence of overexpressed and endogenous Aph-1a and Pen-2 expression in the TSM1 neuronal cell line.

We establish that the expression of either Aph-1a, Aph-1b, or Pen-2 led to reduced susceptibility to staurosporine-induced toxicity. Thus, Aph-1 and Pen-2 lower the number of TUNEL-positive cells and DNA fragmentation in TSM1 neurons. This was accompanied by a reduction of caspase-3 activity and expression and concomitant lowered PARP cleavage. Here again, the influence of Aph-1 and Pen-2 on cell death appears to involve the control of p53. First, Aph-1 and Pen-2 reduce p53 expression and activity and diminish p53 promoter transactivation. Second, the Aph-1- and Pen-2-associated anti-apoptotic phenotypes appear fully p53-dependent, because they were both abolished by p53 depletion. Most importantly, the depletion of both Aph-1 and Pen-2 by the siRNA approach led to a drastic increase in both STS-induced caspase-3 activation and p53 mRNA levels. Therefore, Aph-1- and Pen-2-associated phenotypes were not the result of an artifact due to the overexpression of these proteins.

Several types of γ-secretase complex could occur, because there exists two different PSs, three Aph-1 homologs, and even two splice isoforms of Aph-1a. Whether selective functions could be specifically associated to a subset of complex remains to be established. A recent study indicates that this could indeed be the case, because Serneels and colleagues (66) reported differential participation of the Aph-1 proteins to PS-
p53-dependent Aph-1 and Pen-2 Anti-apoptotic Phenotype

![Graph A](attachment:graph_a.png)

![Graph B](attachment:graph_b.png)

**FIGURE 10.** γ-Secretase inhibitors do not abolish Aph-1- and Pen-2-associated anti-apoptotic phenotype. Mock transfected, Aph-1a-, Aph-1b-, or Pen-2-expressing TSM1 neurons were treated for 16 h without (black bars) or with (gray bars) DAPT (50 μM, A) or DFK167 (50 μM, B), and then treated with 1 μM STS. Cells were then harvested and analyzed for their caspase-3-like activity as described under "Materials and Methods." Bars are the mean ± S.E. of four to seven independent experiments. ***, p < 0.001, compares to DAPT or DFK167-treated mock transfected.

Our data indicate that PS, Aph-1, and Pen-2 all control p53-dependent cell death. However, the question remained as to whether Aph-1 and Pen-2 modulate p53 within the γ-secretase complex and, if so, whether Aph-1- or Pen-2-associated phenotypes could be strictly dependent of the catalytic activity linked to the γ-secretase complex. Our study clearly shows that the integrity of the PS-dependent γ-secretase complex is a strict requirement for Aph-1- and Pen-2-associated apoptosis-related phenotypes. Thus, Aph-1 and Pen-2 expression in fibroblasts devoid of both PS or nicasrin did not modulate staurosporine-induced caspase-3 activation. However, βAPP and APLP2 depletion did not abolish Aph-1- and Pen-2-associated phenotypes. Therefore, unlike for PS, the control of p53 by Aph-1 and Pen-2 was not mediated by the γ-secretase-derived fragments, AICD and ALIDs. Another possibility would have been the γ-secretase cleavage of another protein unrelated to βAPP and APLPs but also able to affect p53 could be controlled by Aph-1 and Pen-2. However, two well characterized γ-secretase inhibitors DAPT and DFK167 did not modify Aph-1 and Pen-2 anti-apoptotic phenotype. Therefore, one can conclude that Aph-1- and Pen-2-protective function was dependent on the structural integrity of the γ-secretase complex but remained unrelated to γ-secretase activity.

The physiology of the proteins contributing to the PS-dependent γ-secretase complex is likely much more complicated than previously anticipated. These proteins could display a large spectrum of functions either inside or outside of the complex. When complex-dependent, some of the functions could appear fully independent of the γ-secretase activity as is the case for the control of cell death by Aph-1 and Pen-2.

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**REFERENCES**

1. Selkoe, D. J. (1997) *Science* **275**, 630–631
2. Checler, F. (1995) *J. Neurochem*. **65**, 1431–1444
3. Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C., and Christie, G. (1999) *Mol. Cell. Neurosci.* **14**, 419–427
4. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberberg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S. M., Wang, S., Walker, D., Zhao, J., McConlogue, L., and John, V. (1999) *Nature* **402**, 537–540
5. Vassar, R., Bennett, B. D., Babu-Khan, S., Khan, S., Mendoza, E. A., Denis, P., Teplov, D., Ross, S., Amaranthe, P., Loelof, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J.-C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) *Science* **286**, 735–741
6. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brasher, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, I. A., Heinrikson, R. L., and Gurney, M. E. (1999) *Nature* **402**, 533–537
7. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1456–1460
8. De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Gahde, G., Von Figura, K., and Van Leuven, F. (1998) *Nature* **391**, 387–390
9. Armogida, M., Petit, A., Vincent, B., Scarzello, S., Alves da Costa, C., and Checler, F. (2001) *Nat. Cell Biol.* **3**, 1030–1033
10. Wilson, C. A., Doms, R. W., Zheng, H., and Lee, V. M.-Y. (2002) *Science* **296**, 118–125
11. Herrenman, A., Serneels, L., Annaert, W., Collen, D., Schoonjans, L., and De Strooper, B. (2000) *Nat. Cell Biol.* **2**, 461–462
12. Zhang, Z., Nadeau, P., Song, W., Donoviel, D., Yuan, M., Bernstein, A., and Aukerman, B. A. (2000) *Nat. Cell Biol.* **2**, 463–465
13. Francis, R., McGrath, G., Zhang, J., Rudy, D. A., Sym, M., Apfeld, J., Nicolini, M., Maxwell, M., Haji, B., Ellis, M. C., Parks, A. L., Xu, W., Li, H., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R., Ruble, C., Nye, J. S., and Curtis, D. (2002) *Dev. Cell* **3**, 85–97
16. Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y.-Q., Rogaeve, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D.-S., Holmes, E., Milman, P., Liang, Y., Zhang, D.-M., Xu, D.-H., Sato, C., Rogaeve, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L., Sorbi, S., Bruni, A., Fraser, P., and St. George-Hyslop, P. (2000) Nature 407, 48–54

17. Goutte, C., Tsnozaki, M., Hale, V. A., and Pries, J. R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 775–779

18. Takasugi, N., Tomita, T., Hayashi, I., Tsuuroka, M., Niimura, M., Takeda, S., Yamanaka, H., Gomi, K., Chihara, Y., Seiki, M., and Tanaka, H. (2005) Nature 432, 438–441

19. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2006) J. Neurosci. 26, 1161–1168

20. Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y.-Q., Rogaeve, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D.-S., Holmes, E., Milman, P., Liang, Y., Zhang, D.-M., Xu, D.-H., Sato, C., Rogaeve, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L., Sorbi, S., Bruni, A., Fraser, P., and St. George-Hyslop, P. (2000) Nature 407, 48–54

21. Cotman, C. W. (1998) Neuron 22, 91–97

22. Cotman, C. W. (1998) Neurobiol. Aging 19, S29–S32

23. Shimohama, S. (2000) Apoptosis 5, 9–16

24. Kopan, R., and Goate, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 19–45

25. Su, J. H., Anderson, A. J., Cummings, B. J., and Cotman, C. W. (1994) Neuroreport 5, 2529–2533

26. Cotman, C. W., and Anderson, A. J. (1995) Mol. Neurobiol. 10, 19–45

27. Cotman, C. W. (1998) Neurobiol. Aging 19, S29–S32

28. Shimohama, S. (2000) Apoptosis 5, 9–16

29. Marx, J. (2001) Science 293, 2192–2194

30. Alves da Costa, C., Paitel, E., Mattson, M. P., Amson, R., Telerman, A., Ancolio, K., and Checler, F. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4043–4048

31. Alves da Costa, C., Mattson, M., Ancolio, K., and Checler, F. (2003) J. Biol. Chem. 278, 12064–12069

32. Wolozin, B., Iwasaki, K., Vito, P., Ganjei, J. K., Lacana, E., Sunderland, T., Zhao, B., Kusiak, J. W., Wasco, W., and D’Adamo, L. (1996) Science 274, 1710–1713

33. Rursztajn, S., DeSouza, R., McPhie, D. L., Berman, S. A., Shioi, J., Robakis, N. K., and Neve, R. L. (1998) J. Neurosci. 18, 9790–9799

34. Alves da Costa, C., Sunyach, C., Pardossi-Piquard, R., Sevalle, J., Vincent, B., Boyer, N., Kawarai, T., Girardot, N., St. George Hyslop, P., and Checler, F. (2006) J. Neurosci. 26, 6377–6385

35. Xie, Z., Romano, D. M., Kovacs, D. M., and Tanzi, R. E. (2004) J. Biol. Chem. 279, 34130–34137

36. Dunys, J., Kawarai, T., Wilk, S., St. George-Hyslop, P., Alves da Costa, C., and Checler, F. (2006) Biochem. J. 394, 501–509

37. Kamijo, T., Zindy, F., Roussel, M., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. (1997) Cell 91, 649–659

38. Li, J., Fici, G. J., Mao, C.-A., Myers, R. L., Shuang, R., Donoho, G. P., Pauley, A. M., Himes, C. S., Qin, W., Kola, I., Merchant, K., and Nye, J. S. (2003) J. Biol. Chem. 278, 33445–33449

39. Heber, S., Herms, J., Gajic, V., Hainfellner, J. A., Aguzzi, A., Rülicke, T., Kretzschmar, H., von Koch, C., Sisodia, S. S., Tremml, P., Lipp, H.-P., Wolfer, D. P., and Müller, U. (2000) J. Neurosci. 20, 7951–7963

40. El-Deiry, W., Kern, S., Pietenpol, J., Kinzler, K., and Vogelstein, B. (1992) Nat. Genet. 1, 45–49

41. Ginsberg, D., Oren, M., Yaniv, M., and Piette, J. (1990) Oncogene 5, 1285–1290

42. Lee, S.-F., Shah, S., Li, H., Yu, C., and Yu, G. (2002) J. Biol. Chem. 277, 45013–45019

43. Steiner, H., Winkler, E., Edbauer, D., Prokop, S., Basset, G., Yamashita, A., Kostka, M., and Haass, C. (2002) J. Biol. Chem. 277, 39062–39065

44. Chun, J., and Jaenisch, R. (1996) Mol. Cell. Neurosci. 7, 304–321

45. Rosen, A., and Casciola-Rosen, L. (1997) J. Cell. Biochem. 64, 50–54

46. Decker, P., and Muller, S. (2002) Curr. Pharm. Biotechnol. 3, 275–283

47. Chen, F., Hasegawa, H., Schmitt-ulms, G., Kawarabayashi, T., Shoji, M., Checler, F., Iwaki, T., Makifuchi, T., Takeda, K., Kira, I.-i., and Tabira, T. (2005) FASEB J. 19, 255–257

48. Loo, D. T., Copani, A., Pike, C. J., Whitemore, E. R., Walenciwicz, A. J., and Cotman, C. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7951–7955

49. Ivins, K. J., Thornton, P. L., Rohn, T. T., and Cotman, C. W. (1999) Neurobiol. Dis. 6, 440–449

50. Guo, Q., Wu, S., Sopher, B. L., Miller, M. W., Ware, C. B., Martin, G. M., and Mattson, M. P. (1999) Nat. Med. 5, 101–106

51. Hong, C.-S., Caromille, L., Nomata, Y., Mori, H., Bredesen, D. E., and Koo, E. H. (1999) J. Neurosci. 19, 637–643

52. Araki, W., Yuasa, K., Takeda, S., Shiotani, K., Tkahashii, K., and Tabira, T. (2001) J. Neurochem. 79, 1161–1168

53. Roperch, J.-P., Alvaro, V., Priere, S., Tynder, M., Nenami, M., Lethrosne, F., Piuouffe, L., Gendron, M.-C., Israel, D., Dausset, J., Oren, M., Amson, R., and Telerman, A. (1998) Nat. Med. 4, 835–838

54. Pastorcic, M., and Das, H. K. (2000) J. Biol. Chem. 275, 34938–34945

55. Serneels, L., Dejaegere, T., Craessaerts, K., Horre, K., Jorissen, E., Tousseyn, T., Hebert, S., Coolen, M., Martens, G., Zwijsen, A., Annaert, W., Hartmann, D., and De Strooper, B. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1719–1724

56. Campbell, W. A., Yang, H., Zetterberg, H., Baulac, S., Sears, J. A., Liu, T., Wong, S. T. C., Zhong, T. P., and Xia, W. (2006) J. Neurochem. 96, 1423–1440