p53-dependent control of transactivation of the *Pen2* promoter by presenilins

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Summary

The senile plaques found in the brains of patients with Alzheimer's disease are mainly due to the accumulation of amyloid β-peptides (Aβ) that are liberated by γ-secretase, a high molecular weight complex including presenilins, PEN-2, APH-1 and nicastrin. The depletion of each of these proteins disrupts the complex assembly into a functional protease. Here, we describe another level of regulation of this multimeric protease. The depletion of both presenilins drastically reduces *Pen2* mRNA levels and its promoter transactivation. Furthermore, overexpression of presenilin-1 lowers *Pen2* promoter transactivation, a phenotype abolished by a double mutation known to prevent presenilin-dependent γ-secretase activity. PEN-2 expression is decreased by depletion of β-amyloid precursor protein (APP) and increased by the APP intracellular domain (AICD). We show that AICD and APP complement for *Pen2* mRNA levels in APP/APLP1-2 knockout fibroblasts. Interestingly, overexpression of presenilin-2 greatly increases *Pen2* promoter transactivation. The opposite effect triggered by both presenilins was reminiscent of our previous study, which showed that these two proteins elicit antagonistic effects on p53. Therefore, we examined the contribution of p53 on *Pen2* transcription. *Pen2* promoter transactivation, and *Pen2* mRNA and protein levels were drastically reduced in p53$^{-/-}$ fibroblasts. Furthermore, PEN-2 expression could be rescued by p53 complementation in p53- and APP-deficient cells. Interestingly, PEN-2 expression was also reduced in p53-deficient mouse brain. Overall, our study describes a p53-dependent regulation of PEN-2 expression by other members of the γ-secretase complex, namely presenilins.

Key words: Alzheimer disease, Presenilins, PEN-2, AICD, p53, Transcription

Introduction

Alzheimer disease (AD) is characterized by the extracellular cortical deposition of senile plaques, the main component of which is a set of poorly soluble peptides named amyloid β-peptides (Aβ). Mutations in the β-amyloid precursor protein (APP) and presenilin (PS) 1 and PS2, are responsible for early onset and aggressive forms of AD (Tanzi, 1999) and both perturb the processing of APP, yielding modified levels of Aβ-like peptides (Checler, 1995). These considerations explain efforts aimed at identifying the enzymes responsible for Aβ genesis, because theoretically, any pharmacological compound that could interfere with Aβ production could arrest AD pathology or slow down its progression.

Aβ derives from the subsequent proteolytic attack of its transmembrane precursor APP by β- and γ-secretases (Checler, 1995). γ-secretase refers to both presenilin-dependent (De Strooper et al., 1998) and presenilin-independent activities (Armogida et al., 2001; Beglopoulos et al., 2004; Lai et al., 2006; Wilson et al., 2003; Wilson et al., 2002; Yagishita et al., 2008). The former exists in a high molecular weight complex composed of at least PS1 or PS2, PEN-2 (PSENEN), APH-1 and nicastrin (NCSTN) (Edbauer et al., 2003; Francis et al., 2002; Goutte et al., 2000; Herreman et al., 2000; Takasugi et al., 2003; Yu et al., 2000; Zhang et al., 2000), where each of these proteins behaves as a limiting factor for the build-up of a biologically active γ-secretase complex.

The corollary of such a stringent contribution of each of these proteins to the γ-secretase complex is that an important effort should be made to gain a better understanding of the regulation of their expression. Studies on the post-transcriptional regulation of the levels of the various members of the complex have investigated their catabolic fate. Clearly, proteins are stabilized when they are included in the complex, whereas the lack of one member apparently drastically accelerates the catabolism of the others. Thus, lowering APH1 expression using a siRNA approach clearly reduces presenilin expression (Lee et al., 2002), and expression of PEN-2 is drastically lowered in presenilin- and nicastrin-deficient fibroblasts (Dunys et al., 2006; Zhang et al., 2005).

Very little is known concerning the transcriptional regulation of the members of the γ-secretase complex. Here, we establish that presenilins regulate p53-dependent activation of the *Pen2* promoter via the production of the APP intracellular domain (AICD).

Results

Presenilins regulate the transactivation of the *Pen2* promoter

As was consistently reported in previous studies (Bergman et al., 2004; Crystal et al., 2004; Dunys et al., 2006), PEN-2 expression

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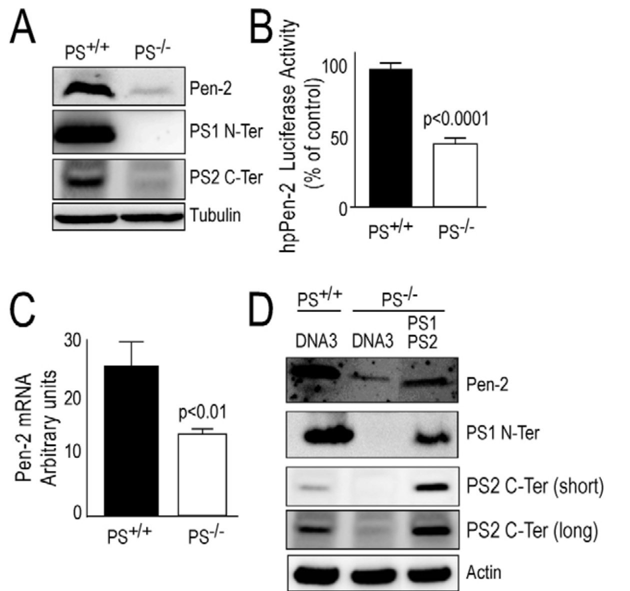

Fig. 1. The depletion of both PS1 and PS2 lowers PEN-2 expression, decreases the transactivation of its promoter and reduces mRNA levels. (A) Endogenous PEN-2 and β-tubulin (loading control) immunoreactivity were analyzed in wild-type ($PS^{+/+}$) and presenilin-deficient fibroblasts ($PS^{-/-}$) by western blot, using antibodies against PEN-2, the N-terminus of PS1 (PS1 N-ter) and the C-terminus of PS2 (PS2 C-ter). (B) Human *Pen2* promoter (hpPen-2) transactivation was analyzed in the indicated cell lines. Bars are the means ± s.e.m. of 15 independent determinations and are expressed as a percentage of control $PS^{+/+}$ fibroblasts. *P*-value compares luciferase activity with that obtained in $PS^{+/+}$ cells. (C) *Pen2* mRNA levels were monitored in the indicated cell lines. Bars represent the means ± s.e.m. of six independent experiments. (D) $PS^{-/-}$ fibroblasts were transfected with both PS1 and PS2 cDNA. Endogenous PEN-2 expression was assessed as above with PEN-2 antibody, and transfection efficiencies were established using anti-PS1-Nter and anti-PS2-Cter antibodies, respectively.

is drastically reduced by the depletion of both PS1 and PS2 in fibroblasts (Fig. 1A). We examined whether part of this phenotype could be accounted for by reduced *Pen2* gene transcription. Indeed, we established that presenilin-depleted fibroblasts displayed a significant reduction of *Pen2* promoter transactivation (Fig. 1B) and mRNA levels (Fig. 1C), indicating that reduced transcription of the *Pen2* promoter could also potentially contribute to the lowered levels of PEN-2 in these cells. Interestingly, PEN-2 protein levels in presenilin$^{-/-}$ fibroblasts could be enhanced by complementation with both PS1 and PS2 (Fig. 1D).

We examined whether PS1 and PS2 similarly influenced *Pen2* promoter transactivation. Interestingly, PS1 and PS2 elicited opposing effects. Thus, as was previously described (Alves da Costa et al., 2002; Alves da Costa et al., 2006; Kang et al., 2005), PS1 overexpression reduced PS2 expression (Fig. 2A), which was accompanied by a reduction of *Pen2* promoter transactivation (Fig. 2B). Conversely, PS2 overexpression reduced PS1 levels (Fig. 2A) and drastically enhanced *Pen2* promoter transactivation (Fig. 2B). Conversely, PS2 depletion decreased *Pen2* mRNA levels (Fig. 2C) whereas depletion of PS1 did not significantly affect them.

Presenilin-dependent γ-secretase controls *Pen2* promoter transactivation via AICD

To investigate the role of presenilin-dependent γ-secretase activity in the regulation of *Pen2* promoter transcription, we analyzed the

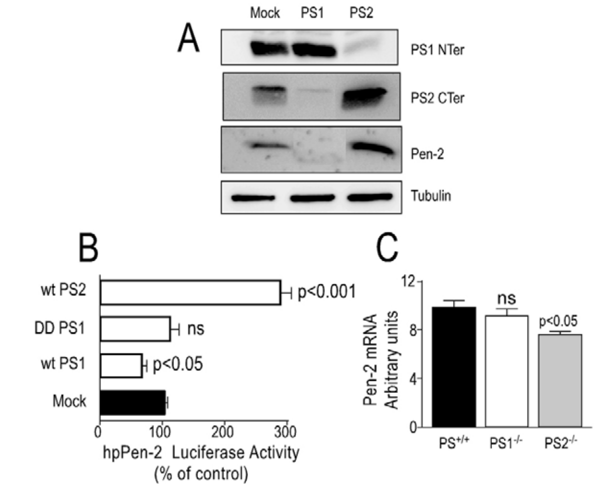

Fig. 2. Overexpression of PS1 and PS2 triggers opposite effects on PEN-2 expression and *Pen2* promoter transactivation. (A) Stably transfected HEK293 cells overexpressing empty cDNA (Mock), PS1 or PS2 were analyzed for PS1 and PS2 expression, as well as for endogenous PEN-2 immunoreactivity by western blot using anti-PS1-Nter, anti-PS2-Cter and PNT2 antibodies, respectively. (B) Stably transfected HEK293 cells overexpressing empty cDNA (Mock), wild-type PS1 (wtPS1), Asp257→Ala/Asp385→Ala-PS1 (DD PS1) or wild-type PS2 were cotransfected with both *Pen2* promoter luciferase (hpPen-2-luciferase) and β-galactosidase (to normalize the transfection efficiencies) reporter gene constructs then *Pen2* promoter transactivation was measured. Bars are the means ± s.e.m. of eight independent determinations. *P*-values compare luciferase activity with that obtained in mock-transfected cells. (C) *Pen2* mRNA levels were monitored in the indicated fibroblast knockout cell lines. Bars represent the means ± s.e.m. of six independent experiments. ns, not significant.

effect of the substitution of aspartate residues 257 and 385 of PS1 by alanines. This double mutation (DD-PS1) has been reported to abolish PS1-associated γ-secretase activity (Wolfe et al., 1999). Fig. 2B shows that this double mutation prevented the PS1-induced inhibition of *Pen2* promoter transactivation, suggesting a role of a γ-secretase-derived product in the control of *Pen2* transcriptional regulation. Two distinct lines of evidence suggest that this product could be AICD, the intracellular domain of APP that is released upon cleavage of APP by γ-secretase (Passer et al., 2000). First, the overexpression of AICD59 (C59 in Fig. 3A) increases *Pen2* promoter transactivation (Fig. 3A, lower panel). Second, fibroblasts devoid of APP display reduced PEN-2 expression (Fig. 3B), and lowered *Pen2* promoter activation (Fig. 3C) and mRNA levels (Fig. 3D). Importantly, we established that the transfection of APP, AICD50 (C50) or AICD59 cDNA in APP/APLP2-deficient fibroblasts rescued the levels of *Pen2* mRNA (Fig. 3E). Overall, this suggests that the lack of endogenous AICD probably accounts for the reduced *Pen2* promoter activation, mRNA levels and expression in APP-deficient fibroblasts.

p53 regulates *Pen2* promoter transactivation

We envisioned the possibility that presenilin-dependent and AICD-induced regulation of *Pen2* promoter transactivation could be mediated by p53 for two main reasons. First, the opposite effects of PS1 and PS2 on the transactivation of the *Pen2* promoter were strikingly similar to those triggered by these proteins on p53 expression, activity, promoter transactivation and mRNA levels (Alves da Costa et al., 2006). Second, we previously demonstrated

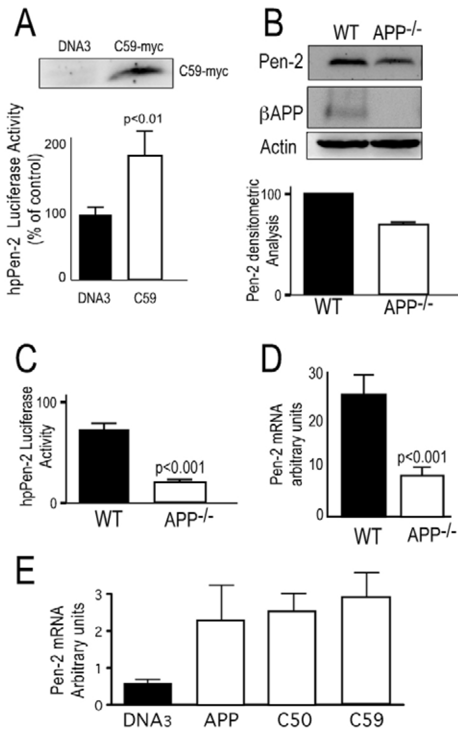

Fig. 3. Influence of AICD and APP on PEN-2 expression, promoter transactivation and mRNA levels. (A) Mock-transfected HEK293 cells were transiently transfected with empty pcDNA3 vector or AICD (C59) then C59 expression (upper panel) and *Pen2* promoter transactivation (lower panel) were measured. Bars are the means ± s.e.m. of six independent determinations and are expressed as control luciferase activity (taken as 100) obtained in mock-transfected cells. *P*-value compares luciferase activity with that obtained in mock-transfected cells. (B) Endogenous PEN-2 and β-actin (loading control) immunoreactivities were analyzed by western blot in wild-type (WT) and APP-deficient ($APP^{-/-}$) fibroblasts. Bars represent densitometric analyses of endogenous PEN-2 immunoreactivity in five independent experiments and are expressed as a percentage of PEN-2 expression recovered in WT fibroblasts. (C) *Pen2* promoter transactivation was analyzed in the indicated cell lines. Bars are the means ± s.e.m. of three independent determinations. *P*-value compares luciferase activity with that obtained in WT fibroblasts. (D) *Pen2* mRNA levels were monitored in the indicated cell lines. Bars represent the means ± s.e.m. of six independent experiments. (E) APP/APLP2-deficient fibroblasts were transiently transfected with empty vector (DNA3) or the indicated cDNAs then *Pen2* mRNA levels were quantified. Bars represent the means ± s.e.m. of three independent experiments.

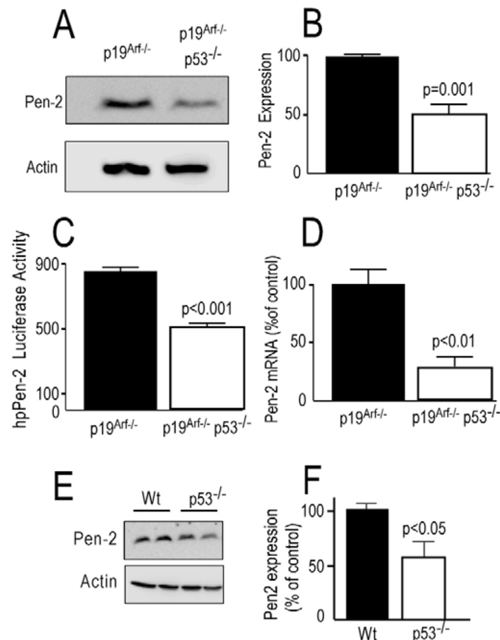

Fig. 4. PEN-2 expression, promoter transactivation and mRNA levels are decreased by p53 deficiency, in vitro and in vivo. Fibroblasts deficient for $p19^{Arf}$ ($p19^{Arf-/-}$) or for both $p19^{Arf}$ and p53 ($p19^{Arf-/-}$ $p53^{-/-}$) were analyzed for their endogenous PEN-2 content (A,B). Panel B represents the densitometric analysis of PEN-2 immunoreactivity expressed as percentage of control expression observed in $p19^{Arf-/-}$ fibroblasts and are the means ± s.e.m. of four independent experiments. (C,D) *Pen2* promoter activation (C) and *Pen2* mRNA levels (D) were monitored using the human *Pen2* promoter-luciferase reporter gene construct and by real-time quantitative PCR. Bars are the means ± s.e.m. of nine (C) and 3-4 (D) independent determinations. *P*-values compare $p19^{Arf-/-}$ $p53^{-/-}$ and $p19^{Arf-/-}$ fibroblasts. (E,F) Analysis (E) and quantification (F) of PEN-2 expression in wild-type (Wt) and p53-deficient mouse brains. Bars are the means ± s.e.m. of four determinations.

that AICD could indeed act as a transcriptional regulator of p53 (Alves da Costa et al., 2006). Four lines of independent data support the view that p53 is responsible for the presenilin-associated regulation of *Pen2* promoter transactivation. First, PEN-2 expression (Fig. 4A,B), promoter transactivation (Fig. 4C) and quantitative real-time PCR measurements of *Pen2* mRNA levels (Fig. 4D) were all drastically reduced by depletion of endogenous p53. Second, PEN-2 expression was also reduced in p53-deficient mouse brain (Fig. 4E,F). Third, the transient transfection of p53 cDNA in $p19^{Arf-/-}$ $p53^{-/-}$ fibroblasts restores PEN-2 expression (Fig. 5A,B) and promoter activation (Fig. 5C). Fourth, p53 cDNA transfection also increases PEN-2 expression in APP-deficient fibroblasts (Fig. 5D).

To confirm the association between AICD-induced upregulation of *Pen2* promoter transactivation and p53, we examined the influence of the depletion of endogenous p53 on the AICD-associated phenotype. Clearly, AICD increased *Pen2* mRNA levels in $p19^{Arf-/-}$ cells but this effect was fully abolished by the lack of endogenous p53 (Fig. 5E), thereby confirming the fact that AICD-induced upregulation of *Pen2* promoter transactivation was fully mediated by p53 (Fig. 5E). Overall, our data demonstrate for the first time that p53 is a regulator of *Pen2* promoter transactivation and indicate that presenilins could modulate *Pen2* transcription via AICD-mediated control of this oncogene.

Discussion

Protein homeostasis results from a complex set of cellular regulations, implying the involvement of both genesis and catabolism. Most neurodegenerative diseases are associated with increased levels of proteins that are prone to aggregation, giving rise to intracellular or extracellular lesions thought to be involved in the degenerative processes (Bucciantini et al., 2002). This is true in Alzheimer disease, where both extracortical lesions, called senile plaques, and intracellular accumulation of abnormally phosphorylated tau protein are observed at a late stage of the disease (Selkoe, 1991).

The biology of the presenilin-dependent γ-secretase, the enzymatic machinery that generates Aβ peptides, is poorly understood, although several cell biology studies emphasize the crucial role of each of the proteins in the build-up of the complex and reveal a highly coordinated sequence of events. This implies

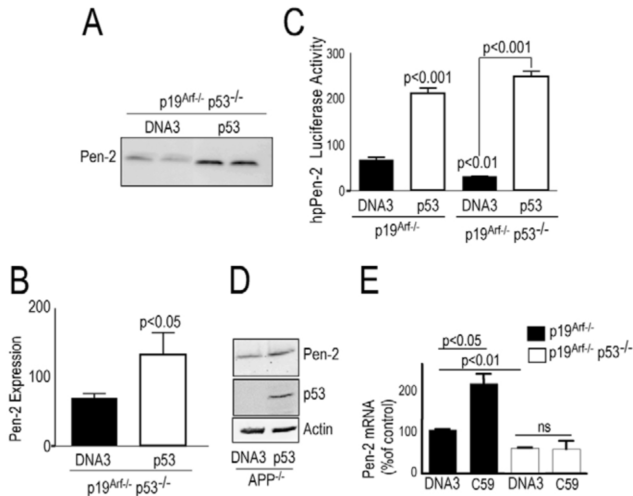

Fig. 5. p53 complementation increases PEN-2 expression and promoter transactivation in p19$^{Arf-/-}$ p53$^{-/-}$ and APP$^{-/-}$ fibroblasts. (A-C) p19$^{Arf-/-}$ p53$^{-/-}$ fibroblasts were transiently transfected with empty pcDNA3 vector (DNA3) or p53 cDNA, then PEN-2 expression (A,B) or promoter transactivation (C) were analyzed. Bars are the means ± s.e.m. of 3-5 (B) or four (C) independent determinations. *P*-values compare PEN-2 expression or luciferase activity with those observed in mock-transfected p19$^{Arf-/-}$ p53$^{-/-}$ fibroblasts. (D) APP-deficient fibroblasts were transfected with empty vector (DNA3) or p53 cDNA then PEN-2 expression was monitored. (E) The indicated fibroblastic cell line was transfected with empty pcDNA3 or C59 then *Pen2* mRNA levels were monitored. Bars are the means ± s.e.m. of four independent determinations. ns, not significant

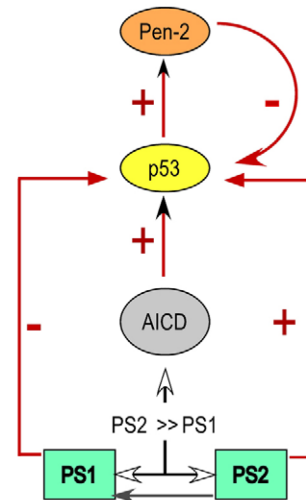

Fig. 6. Schematic representation of regulation between presenilins, p53 and PEN-2. Scheme of the pathway linking PS1, PS2, AICD, p53 and PEN-2. As we previously described (Alves da Costa et al., 2006), PS1 lowers the level of p53 whereas PS2 increases p53. Both proteins functionally interact, but PS2 is dominant for the p53-dependent pro-apoptotic phenotype (PS2>>PS1) (Alves da Costa et al., 2006). AICD positively modulates p53 (Alves da Costa et al., 2006) and p53 increases *Pen2* transcription (present study). Conversely, there exists a feedback loop by which PEN-2 downregulates p53 (Dunys et al., 2007).

that the level of each of the components can be seen as rate-limiting step for γ-secretase formation and therefore conditions the resulting phenotypes (Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003).

Several studies suggest that the catabolism of the members of the γ-secretase complex is drastically enhanced when proteins occur outside of the complex. Thus, reduction of APH-1 leads to drastic reduction of presenilin expression (Lee et al., 2002). A few works have suggested that the catabolism of the members of the complex can be regulated by proteasomal degradation (Bergman et al., 2004; Crystal et al., 2004), but a recent study indicated that these observations probably result from an artifactual effect of proteasome inhibitors that could nonspecifically upregulate CMV-driven promoter transcription (Dunys et al., 2006).

Relatively little data concern upstream regulation of these proteins and particularly the fact that they might be modulated at a transcriptional level. Previous studies have demonstrated the regulation of *PSEN1* promoter transcription by ETS proteins, particularly Elk-1 and ER81 (Pastorcic and Das, 2000; Pastorcic and Das, 2003), cAMP-response element-binding protein (CREB) (Mitsuda et al., 2001) or p53 (Pastorcic and Das, 2000; Roperch et al., 1998). The transcription of *PSEN2* involves Sp1 and Egr-1 (Renbaum et al., 2003). Recently, *APH1A* and *Pen2* promoter sequences have been described (Wang et al., 2006b). The *APH1A* promoter seems to be regulated by HIF-1α under hypoxic conditions (Wang et al., 2006b), whereas the *Pen2* promoter contains CREB-binding domains (Wang et al., 2006a). Until now, the sequence of the nicastrin gene promoter was unknown and its transcriptional regulation has not been documented.

Our study clearly establishes that PS1 and PS2 modulate *Pen2* promoter transactivation by a p53-dependent mechanism (Fig. 6). Thus, depletion of presenilin reduces *Pen2* gene expression, promoter activation and mRNA levels. This effect is related to presenilin-associated catalytic activity, because it is prevented by a double mutation known to abolish γ-secretase activity. This phenotype was apparently due to a γ-secretase-derived product of APP because depletion of endogenous APP mimics that triggered by the deficiency of both PS1 and PS2 on PEN-2 protein and mRNA levels as well as *Pen2* promoter transactivation. That this product corresponded to AICD was supported by our demonstration that AICD-induced upregulation of *Pen2* promoter transactivation was fully abolished by the depletion of endogenous p53. These results agree perfectly with our previous demonstration that AICD acts as a transcriptional regulator of p53 (Alves da Costa et al., 2006), as demonstrated for various other proteins (Baek et al., 2002; Kim et al., 2003; Pardossi-Piquard et al., 2005; von Rotz et al., 2004; Zhang et al., 2007). This is the first demonstration that p53 can upregulate *Pen2* transcription.

The question arose as to whether p53 directly modulates *Pen2* transcription. This appears unlikely because we did not delineate any in silico consensus sequences corresponding to putative sites targeted by p53. Another possibility could be that NFκB, which lowers p53 activity (Ozes et al., 1999), could be downregulated, but it has been reported that NFκB did not modulate *Pen2* promoter transactivation (Wang et al., 2006a). Finally, we envisioned the possibility that p53 could lead to a CREB-associated increase of *Pen2* transcription because CREB was reported to activate *Pen2* promoter transactivation (Wang et al., 2006a). However, our data indicate that endogenous p53 downregulates CREB transcriptional activity in opposition to the p53-induced increase of *Pen2* transcription (data not shown). Altogether, this indicates that p53 indirectly controls *Pen2* promoter transactivation via a molecular intermediate that remains to be identified.

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It is interesting to emphasize the fact that PS1 and PS2 trigger opposite effects on PEN-2 protein level and *Pen2* promoter transactivation. PS1 overexpression lowers PEN-2 protein expression whereas PS2 exacerbates its expression. In agreement with these data, PS2 depletion diminished *Pen2* mRNA levels. These observations fit perfectly with the opposite influence of PS1 and PS2 on p53 (Alves da Costa et al., 2006; Kang et al., 2005) and indirectly confirm that p53 accounts for the distinct and opposite effects of PS1 and PS2 on PEN-2 levels. Furthermore, this scheme suggests two distinct loops of regulation of PS1 (anti-apoptotic) and PS2 (pro-apoptotic) phenotypes. Previous studies have demonstrated that PS1 lowers p53 (Mitsuda et al., 2001; Roperch et al., 1998), whereas PS2 increases the expression of this oncogene (Alves da Costa et al., 2002; Alves da Costa et al., 2006; Janicki and Monteiro, 1997; Nguyen et al., 2005). The opposite phenotype triggered by PS1 and PS2 adds support to previous studies suggesting that these proteins could indeed display their own function and elicit their specific pharmacological spectrum, some of them could be linked to p53-dependent functions unrelated to the control of cell death (Chen et al., 2003; Gu et al., 2004; Lai et al., 2003).

The present study has several conceptual implications. First, this work identifies p53 as a common effector modulating the transcriptional regulation of various members of the γ-secretase complex. Second, this is the first indication that a member of the γ-secretase complex could participate in the transcriptional regulation of another member of this complex. Third, we demonstrate that PS1 and PS2 distinctly influence PEN-2 via an AICD- and p53-dependent mechanism, suggesting that the generic term of γ-secretase probably refers to various complexes with specific composition harboring various pharmacological functions. Overall, this study shows that proteins of the complex are not only associated with post-tranductional events but could also be intimately linked to upstream events, implying a transcriptional control of their expression by p53 via AICD-dependent mechanisms.

Materials and Methods

Cell culture and transfection

Stably transfected HEK293 cells expressing wild-type (wt) PS1, mutated D257A/D385A PS1 or wt PS2 were obtained after transfection of 3 μg cDNA using Lipofectamine reagent (Invitrogen) according to the manufacturer's recommendations and selection of positive transfectants by western blot as described below. Fibroblasts were transfected with Lipofectamine 2000 reagent (Invitrogen) or JetPEI reagent (Polyplus-transfections) according to the manufacturer's recommendations. In some experiments, fibroblasts were transfected by means of the mouse embryonic fibroblasts Nucleofector™ kit (Amaxa Biosystems, Cologne, Germany) as described (Alves da Costa et al., 2006). Mouse embryonic fibroblasts (MEFs) depleted of PS1 ($PS1^{-/-}$), PS2 ($PS2^{-/-}$), PS1 and PS2 ($PS^{-/-}$) or APP ($APP^{-/-}$) were cultured as previously described (Alves da Costa et al., 2006; Dunys et al., 2006). MEFs devoid of $p19^{Arf-/-}$ or of both $p19^{Arf-/-}$ and $p53^{-/-}$ were cultured as previously described (Dunys et al., 2007). These two cell lines allow monitoring the influence of p53 without interference with p53-associated $p19^{Arf}$-dependent control of cell cycle (Kamijo et al., 1998). Telencephalon-specific murine cells (TSM-1) overexpressing Myc-tagged PEN-2 were obtained and cultured as previously described (Dunys et al., 2006).

Site-directed mutagenesis.

PS1 construct in which both aspartates D285 and D357 were replaced by an alanine residue (DD-PS1) was obtained by oligonucleotide-directed mutagenesis from human wild-type *PS1* cDNA by means of a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis was performed according to manufacturer's conditions using the set of primers: 5′-GGC TGT GAT TTC AGT ATA TGC TTT AGT GGC TGT TTT GTG TCC G-3′ and 5′-CGG ACA CAA AAC AGC CAC TAA AGC ATA TAC TGA AAT CAC AGC C-3′ (Cybergene, Saint-Malo, France) containing the D257A mutation. Then, this mutant construct was used to produce the double mutant using the primers: 5′-CTT GGA TTG GGA GCT TTC ATT TTC TAC AGT GTT CTG G-3′ and 5′-CCA GAA CAC TGT AGA AAA TGA AAG CTC CCA ATC CAA G-3′ containing the D385A mutation. Final cDNA constructs were entirely sequenced to verify mutations.

Western blot analyses and antibodies

Cells were gently scraped with PBS-EDTA 5 mM, pelleted by centrifugation, and then lysed in 50-100 μl of 25 mM HEPES, pH 7.5 containing a cocktail of protease inhibitors (Roche Molecular Biochemicals). Protein analyses in brains from 3-month-old wild-type or p53-knockout mice were carried out after homogenization in 10 mM Tris-HCl (pH 7.5) or in Prüsiner buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate). Equal amounts of proteins were separated on SDS-PAGE gels containing 8-12% acrylamide (Euromedex) for analysis of APP, PS1 and PS2 or on 16.5% acrylamide Tris-Tricine gels for PEN-2. Proteins were then wet-transferred to Hybond C membranes (GE HealthCare). Membranes were blocked with non-fat milk and incubated overnight at 4°C with the following primary antibodies: anti-PEN-2 (PNT2, rabbit polyclonal, Calbiochem; 1:1000), anti-actin (mouse monoclonal, Sigma; 1:5000), anti-PS1-Nter (rabbit polyclonal, a gift from Gopal Thinakaran, University of Chicago, IL; 1:1000), anti-PS2-Cter 2192 (rabbit polyclonal, Cell Signaling; 1:1000) and anti-βAPP 22C11 (mouse monoclonal, Boehringer; 1:1000). Immunological complexes were revealed by enhanced electrochemiluminescence (Roche Molecular Biochemicals) with either anti-rabbit or anti-mouse IgG antibodies (1:5000) coupled to peroxidase (Jackson ImmunoResearch).

Real-time quantitative polymerase chain reactions

RNAs from fibroblastic cell lines were extracted by means of the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and treated with DNase I and 2 μg of total RNA were reverse transcribed as previously described (Alves da Costa et al., 2006). Real-time PCR was performed as described (Dunys et al., 2007) with gene-specific primers for mouse *Pen2* and mouse β-actin to normalize mRNA concentrations.

Promoter activity measurements

The human *Pen2* promoter (hpPen-2) in frame with luciferase reporter gene has been previously described (Mitsuda et al., 1997; Renbaum et al., 2003; Wang et al., 2006a). Cells were grown in 12-well plates until they reach 60-70% confluency and were then cotransfected with 1 μg *Pen2*-luciferase cDNA and 0.5 μg of a β-galactosidase transfection vector (to normalize transfection efficiency) with or without 1 μg of either pcDNA3, AICD59, AICD50 or p53 cDNA by means of the Lipofectamine 2000 reagent (Invitrogen) or the Amaxa Transfection System (Amaxa Biosystems) according to the manufacturer's protocol. Luciferase and β-galactosidase activities were then analyzed (Promega).

Statistical analysis

Statistical analyses were performed using Prism Software (Graphpad Software, San Diego, CA) by the mean of the Neuman-Keuls multiple comparison test.

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