A Loss of Function Mutation of Presenilin-2 Interferes with Amyloid β-Peptide Production and Notch Signaling*

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Presenilin-1 (PS1) facilitates γ-secretase cleavage of the β-amyloid precursor protein and the intramembrane cleavage of Notch1. Although Alzheimer's disease-associated mutations in the homologous presenilin (PS2) gene elevate amyloid β-peptide (Aβ42) production like PS1 mutations, here we demonstrate that a gene ablation of PS2 (unlike that of PS1) in mice does not result in a severe phenotype resembling that of Notch-ablated animals. To investigate the amyloidogenic function of PS2 more directly, we mutagenized a conserved aspartate at position 366 to alanine, because the corresponding residue of PS1 is known to be required for its amyloidogenic function. Cells expressing the PS2 D366A mutation exhibit significant deficits in proteolytic processing of β-amyloid precursor protein indicating a defect in γ-secretase activity. The reduced γ-secretase activity results in the almost complete inhibition of Aβ and p3 production in cells stably expressing PS2 D366A, whereas cells overexpressing the wild-type PS2 cDNA produce robust levels of Aβ and p3. Using highly sensitive in vivo assays, we demonstrate that the PS2 D366A mutation not only blocks γ-secretase activity but also inactivates PS2 activity in Notch signaling by inhibiting the proteolytic release of the cytoplasmic Notch1 domain. These data suggest that PS2 is functionally involved in Aβ production and Notch signaling by facilitating similar proteolytic cleavages.

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The abbreviations used are: PS1, presenilin 1; PS2, presenilin 2; Aβ, amyloid β-peptide; APP, amyloid precursor protein; βAPP, β-APP; NICD, Notch intracellular domain; CTF, C-terminal fragment; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; wt, wild-type.

PS1* is required for Aβ generation (1) as well as Notch signaling (2). Recent evidence demonstrated a deficiency in γ-secretase activity in mice lacking the PS1 gene (1). Neurons isolated from these animals secreted reduced levels of Aβ and accumulated the C-terminal fragments of βAPP, which are normally proteolyzed by γ-secretase (1). Moreover, mice lacking PS1 exhibit a phenotype, which resembles that caused by the deletion of the Notch1 gene (3, 4). A direct involvement of PS1 in Notch signaling has now been demonstrated by the finding that cells lacking PS1 show reduced levels of the proteolytically generated cytoplasmic domain of Notch1 (Notch intracellular domain (NICD)) (5) as well as by genetic evidence derived from multiple model systems including Caenorhabditis elegans, Drosophila melanogaster, and mice (2, 6–9). Work by Wolfe et al. (10) recently suggested that PS1 might be an unusual aspartyl protease, which exhibits the γ-secretase activity required for the proteolytic release of Aβ. Mutagenizing either one of the two critical Asp residues in transmembrane domain 6 or transmembrane domain 7 of PS1 inhibits Aβ production and results in the accumulation of C-terminal fragments of βAPP (10). The biochemical phenotype caused by this mutation therefore closely resembles the effects of the PS1 ablation on Aβ production (1).

Although detailed knowledge on the function of PS1 is accumulating, we do not know the biological function of the homologous PS2 protein. Interestingly, PS2 mutations elevate Aβ42 production like PS1 mutations (11). However, Alzheimer's disease-associated mutations in PS2 are very rare, whereas numerous mutations have been reported in the PS1 gene. Moreover, PS2 mutations appear to be less aggressive and in contrast to PS1 the age of onset caused by PS2 mutations is apparently modified by the apoE phenotype (12). To understand the biological function of PS2, we analyzed the phenotype of PS2 deleted mice. We also generated a loss function mutation and investigated its influence on Aβ generation, NICD production, and Notch signaling in cultured cells and in a sensitive functional rescuing assay in C. elegans. From our experiments we conclude that loss of function mutations of PS2 interfere with Aβ production as well as Notch signaling, suggesting that PS2 is functionally involved in the proteolytic release of Aβ and NICD.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Lines—Human embryonic kidney 293 cells (293) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 200 μg/ml G418 (to select for βAPP expression), and 200 μg/ml zeocin (to select for presenilin expression). 293 cells stably expressing PS2 D366A were generated by transfection of 293 cells stably expressing βAPP containing the Swedish mutation (13). 293 cells stably co-expressing Swedish βAPP695 and wt PS2 were described previously (14).

Construction of the cDNA Encoding PS2 D366A—The cDNA encod-
ing PS2 D366A was constructed by polymerase chain reaction-mediated mutagenesis of codon 366 of the PS2 cDNA (15, 16) using appropriate primers as described previously (17). The mutant cDNA was cloned into expression vector pcDNA3.1 containing a zeocin resistance gene (Invitrogen) and sequenced to verify successful mutagenesis.

**Antibodies**—The polyclonal and monoclonal antibodies against amino acids 263–407 of PS1 (3027, BL3D7) and against amino acids 297–356 of PS2 (3711, BL.HF5c) were described previously (18–20). Antibodies 3926 to synthetic Aβ (21), C7 (to the last 20 C-terminal amino acids of βAPP) (22) were described before. Antibody 5313 is raised to the ectodomain of βAPP (amino acids 444–592) and antibody 5618 is raised to the cytosolic domain of βAPP (amino acids 652–695).

**Generation of PS2 KO Mice**—Mice carrying an ablated PS2 gene were generated by targeting exon 5 of the mouse gene in ES cells. A small deletion in exon 5 and insertion of the neomycin resistance gene was sufficient to disrupt translation. Northern blot analysis was carried out according to standard procedures. For Western blot analysis, brain extracts were prepared according to standard procedures. Brain extracts from PS2+/−, PS2−/−, and PS2−/− mice were analyzed for PS1, PS2, and βAPP expression using a combined immunoprecipitation/immunoblotting protocol (see below).

**Metabolic Labeling and Immunoprecipitation of PS2**—To analyze expression of PS2, 293 cells were starved for 1 h in methionine- and serum-free minimum Eagle’s medium and subsequently metabolically labeled with 700 μCi [35S]methionine (Promix, Amersham Pharmacia Biotech) in methionine- and serum-free minimum Eagle’s medium for 30 min. The PS2 holoprotein was immunoprecipitated from cell extracts as described (17, 20).

**Combined Immunoprecipitation/Western Blotting**—Extracts from brains or stably transfected 293 cell lines were prepared and subjected to immunoprecipitation using the polyclonal antibody 3027 to PS1 (18), 3711 to PS2 (19), or C7 to βAPP (22). Following gel electrophoresis, immunoprecipitated PS proteins were identified by immunoblotting using the monoclonal antibody BL3D7 (PS1; Ref. 20) or BL.HF5C (PS2; Ref. 20). βAPP-CTFs were identified using the polyclonal antibody 5618. Bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Analysis of βAPP Metabolites**—Stably transfected 293 cell lines were grown to confluence. For the analysis of Aβ in conditioned media, cells were metabolically labeled with 450 μCi [35S]methionine (Promix, Amersham Pharmacia Biotech) for 2 h, and chased for 2 h in medium containing excess amounts of unlabeled methionine. Aβ and p3 were immunoprecipitated from conditioned media with antibody 3926 (21) and separated on 10–20% Tris–glycine gels (Novex), and analyzed by fluorography. To analyze βAPP-CTFs, cell lysates were subjected to immunoprecipitation with antibody C7 (22), separated on 10–20% Tris–glycine gels (Novex), and analyzed by fluorography. Quantitation of βAPP-CTFs, Aβ, and p3 was done by phosphomager analysis. APP was immunoprecipitated from conditioned media using antibody 5313.

**Analysis of Aβ40 and Aβ42**—Conditioned media (2 ml) were collected from confluent 293 cells grown in 6-well dishes for 24 h. The media were assayed for Aβ40 and Aβ42 using a previously described enzyme-linked immunosorbent assay (17, 20).

**Analysis of Notch1 Cleavage**—DNAs encoding Myc-tagged Notch1 derivatives (23) were cloned into the pcDNA3.1/Hygro expression vector (Invitrogen) and stably transfected into 293 cells. To analyze cleavage of Notch1, cells were starved for 1 h in methionine- and serum-free minimum Eagle’s medium, subsequently metabolically labeled with 300 μCi [35S]methionine (Promix, Amersham Pharmacia Biotech) for 20 min, and chased for 1 h in medium containing excess amounts of unlabeled methionine. Cell extracts were prepared and subjected to immunoprecipitation of Notch1 derivatives using the anti-Myc antibody 9E10 as described previously (23).

**Transgenic Lines of C. elegans and Rescue Assays**—Constructs for transgenic expression of PS2 and PS2 D366A in C. elegans were generated as described previously (7). Transgenic lines were established by microinjection of plasmid DNA mixtures into the C. elegans germ line to create extrachromosomal arrays (7). Four independent lines from the progeny of F2 generation animals were established. As the sel-12(or171) animals never lay eggs (7), rescue of the sel-12 defect can be quantified by scoring egg-laying behavior in transgenic animals (7). 50 transgenic animals of each line were analyzed for their ability to lay eggs. The numbers of egg laid by individual transgenic animals were counted and plotted into four categories: Egl−/−, robust egg laying, more than 30 eggs laid; Egl+/−, 15–30 eggs laid; Egl+, 5–15 eggs laid; Egl−, no eggs laid.

**RESULTS**

To understand the function of PS2 we generated mice in which the PS2 gene is ablated. Northern blot analysis confirmed the lack of PS2 expression (Fig. 1, A and B). In contrast, no significant changes were observed in the levels of PS1 and PS2. C57Bl/6 mice from a cross between targeted line (129sv/ev) and C57Bl/6. Neonates (wt, left) and null mice (right) grossly differ in gross brain architecture and throughout adulthood (D–G). Gross brain architecture in 8-month-old animals is essentially similar between null and wt mice as shown by cresyl violet Nissl staining. Wt mouse (D) and null mutant (E) show normal layering in the hippocampus. Wt mouse (F) and null mutant (G) show the overall architecture of the hippocampal fields.

**A**. Northern blot analysis of PS2 mRNA in brains from PS2+/+ (left) and PS2−/− (right) and PS2−/− mice. Note the absence of PS2 mRNA in PS2−/− mice. B, PS2−/− mice fail to express the PS2 protein and do not accumulate APP CTFs. Brain lysates derived from PS2+/+ (left) and PS2−/− were analyzed for the presence of the PS2 CTF (top panel), the PS1 CTF (middle panel), and APP CTFs (bottom panel) by immunoprecipitation/immunoblotting. The PS2 CTF is observed in wt mice, reduced in PS2−/− and absent in mice lacking the PS2 gene. There is no obvious difference in the amount of the PS1 CTF and the APP CTFs. Note difference in the expression levels of PS1 and PS2. C, F1 mice derived from a cross between the targeted line (129sv/ev) and C57Bl/6. Neonates (wt, left) and null mice (right) grossly differ in birth and throughout adulthood (D–G). Gross brain architecture in 8-month-old animals is essentially similar between null and wt mice as shown by cresyl violet Nissl staining. Wt mouse (D) and null mutant (E) show normal layering in the hippocampus. Wt mouse (F) and null mutant (G) show the overall architecture of the hippocampal fields.

**Fig. 1.** A. A gene ablation of PS2 in mice does not result in an obvious phenotype. A, Northern blot analysis of PS2 mRNA in brains from PS2+/+ (left) and PS2−/− (right) and PS2−/− mice. Note the absence of PS2 mRNA in PS2−/− mice. B, PS2−/− mice fail to express the PS2 protein and do not accumulate APP CTFs. Brain lysates derived from PS2+/+ (left) and PS2−/− were analyzed for the presence of the PS2 CTF (top panel), the PS1 CTF (middle panel), and APP CTFs (bottom panel) by immunoprecipitation/immunoblotting. The PS2 CTF is observed in wt mice, reduced in PS2−/− and absent in mice lacking the PS2 gene. There is no obvious difference in the amount of the PS1 CTF and the APP CTFs. Note difference in the expression levels of PS1 and PS2. C, F1 mice derived from a cross between the targeted line (129sv/ev) and C57Bl/6. Neonates (wt, left) and null mice (right) grossly differ in birth and throughout adulthood (D–G). Gross brain architecture in 8-month-old animals is essentially similar between null and wt mice as shown by cresyl violet Nissl staining. Wt mouse (D) and null mutant (E) show normal layering in the hippocampus. Wt mouse (F) and null mutant (G) show the overall architecture of the hippocampal fields.

**RESULTS**

To understand the function of PS2 we generated mice in which the PS2 gene is ablated. Northern and Western blot analysis confirmed the lack of PS2 expression (Fig. 1, A and B). In contrast, no significant changes were observed in the levels of PS1 (Fig. 1B). Surprisingly, in contrast to the very severe effects of the PS1 gene ablation on mouse embryonic development (3, 4) the lack of the PS2 gene in mice does not result in an obvious phenotype. Gross external examination of neonates (Fig. 1, A and B) and adults, heterozygote, and wt mice showed that the PS2-ablated mice develop normally up to the oldest age studied (1 year). Gross analysis of brain cytoarchitecture by Nissl stain (Fig. 1, D–G) showed that ablated and wt mice have similar neuroanatomy. In addition, adult-ablated mice do not show gross skeletal abnormalities reminiscent of those seen in PS1 null mutant embryos (3, 4) when examined by x-ray analysis (data not shown). Although it is possible that subtle cellular abnormalities exist in the PS2-ablated mice, it is clear that the PS2 null phenotype is grossly different from that seen in the PS1 null mouse (3, 4). This suggests that PS2 is not obligatorily required for normal embryonic development. Moreover, in contrast to the PS1 deletion (1, 24), analysis of βAPP processing revealed no accumulation of APP CTFs (Fig. 1B) and no change in Aβ production (data not shown) in the PS2−/− mice. However, the lack of a phenotype in the gene-ablated animals...
could be due to the compensation of PS2 activity by the significantly (5-fold) higher expression levels of PS1 during mouse development (25) and might therefore not exclude an essential function of PS2.

To allow a direct assessment of the functional role of PS2 in Aβ production and Notch signaling, we mutagenized a conserved aspartate residue (PS2 D366A) (15, 16), which was previously shown to be required for the γ-secretase promoting activity of PS1 (10), and generated stably transfected cell lines. As a control, cells were stably transfected with the wt PS2 cDNA. Overexpression of the mutation inhibits PS2 endoproteolysis, whereas the wt PS2 protein is proteolytically cleaved to generate the characteristic C-terminal fragments observed for presenilins (26–29) (Fig. 2A). The lack of endogenous PS2 fragments is due to the previously observed replacement upon ectopic overexpression (17, 20, 27, 28). Cells expressing the PS2 aspartate mutation accumulate C-terminal fragments of βAPP, which are known to be turned over by the γ-secretase activity to produce p3 and Aβ (10, 11) (Figs. 2B and 3A). Analysis of Aβ/p3 generation revealed that cells stably expressing PS2 D366A secrete significantly less Aβ and p3 than cells overexpressing wt PS2 (Fig. 2B). The reduced Aβ and p3 production is not due to a decrease in protein secretion, because no difference in the secretion of APP was found in the two cell lines (Fig. 2B). Quantitation demonstrates that Aβ/p3 production is inhibited by ~90% as compared with wt PS2 expressing cells (Fig. 3, B and C). Moreover, inhibition of Aβ production affects both Aβ species, Aβ40 (Fig. 3D) and Aβ42 (Fig. 3E). As we (Ref. 30; see also Figs. 2B and 3B) and others (14, 26) have shown earlier, Aβ/p3 production occurs in the presence of overexpressed PS2, which also displaces both endogenous presenilins (Refs. 28 and 30; and data not shown). We therefore conclude that the PS2 D366A mutation interferes with Aβ/p3 production.

Because PS2, like PS1, appears to be involved in Aβ production, we also investigated whether the PS2 aspartate mutation interferes with Notch signaling. To investigate if functional PS2 is required for cell fate decisions mediated by Notch signaling, we expressed PS2 D366A in a mutant strain of C. elegans, which lacks a functional PS homologue (sel-12) (Ref. 2). The sel-12 (ar171) allele is known to cause a phenotype, which is due to reduced Notch signaling (2). Consistent with previous results (6), transgenic expression of PS2 in the mutant worm fully rescued the egg-laying phenotype (Table I). However, expression of PS D366A failed to exhibit any rescuing activity (Table I). Therefore, the same mutation, which interferes with the amyloidogenic function blocks the activity of PS2 in Notch signaling in vivo.

To prove whether the PS2 D366A mutation blocks the potentially intramembranous cleavage of Notch1 (5, 23, 31), we

**FIG. 2.** Expression of PS2 D366A interferes with PS2 endoproteolysis and βAPP processing. A, the PS2 holoprotein accumulates in cells overexpressing wt PS2 or PS2 D366A. Untransfected 293 cells or 293 cells stably transfected with wt PS2 or with PS D366A were metabolically labeled with [35S]methionine for 30 min, and cell lysates were immunoprecipitated with the PS2-specific antibody 3711 (19). Overexpression of both PS2 derivatives results in the accumulation of the PS2 holoprotein. As observed previously (26, 27), no endogenous holoprotein could be observed. To detect the PS2 CTF, cell lysates from untransfected 293 cells, or 293 cells stably expressing wt PS2 or the PS2 D366A mutation were immunoprecipitated with antibody 3711 (19), and PS2 CTFs were detected with the monoclonal antibody 1.H.F5C (20). Robust levels of PS2 CTFs are detected in untransfected cells as well as cells stably expressing wt PS2, whereas generation of the PS2 CTF is inhibited in cells stably expressing PS2 D366A. As observed before, overexpression of PS does not result in an increased production of the proteolytic fragments (28). B, expression of PS2 D366A results in the accumulation of C-terminal proteolytic fragments of βAPP and a marked reduction of Aβ/p3 production. 293 cells stably expressing wt PS2 or D366A were metabolically labeled with [35S]methionine for 2 h followed by a cold chase for an additional 2 h. Cell lysates were immunoprecipitated with antibody C7 (22) to detect the C-terminal fragments of βAPP, and conditioned media were immunoprecipitated with antibody 5313 revealed no difference in the secretion of APP.

**FIG. 3.** Quantitation of the effect of the PS2 D366A mutation on Aβ/p3 production as well as the accumulation of βAPP C-terminal fragments. A, expression of PS2 D366A results in a marked accumulation of C-terminal fragments of βAPP generated by α- and β-secretase. B, expression of PS2 D366A inhibits total Aβ production. C, expression of PS2 D366A inhibits p3 production. D and E, the PS2 D366A mutation affects production of both Aβ40 and Aβ42. Bars represent the mean ± S.E. of three independent experiments.
analyzed the proteolytic release of the NICD. Control cells or cells expressing the PS2 D366A mutation were stably transfected with the previously described NotchΔE cDNA (NAE) construct (23). To monitor the generation of NICD from NAE, we also generated cell lines expressing the NotchICV cDNA, which only encodes the derivative corresponding to NICD (23). In control cells, proteolytic release of NICD from NAE was observed (Fig. 4). In agreement with previous results (23), the proteolytically released NICD co-migrates with the NotchICV-encoded protein (Fig. 4). In contrast, overexpression of the PS2 D366A mutation interferes with the proteolytic release of NICD (Fig. 4). Because this domain is required for Notch signaling (23, 31), the lack of processing may explain why this construct failed to rescue the sel-12 mutant phenotype (see Table I).

### DISCUSSION

In this work, we have demonstrated that mutagenesis of a highly conserved aspartate residue of PS2 results in a functionally inactive PS2 variant. This variant interferes with the proteolytic release of Aβ and NICD and is totally inactive in an in vivo assay monitoring Notch signaling.

Ectopic expression of PS proteins is now well known to result in the efficient replacement of the endogenous presenilins (17, 20, 27, 28). This is also the case when we overexpress PS2 variants (Ref. 30, Fig. 2A, and data not shown). One might therefore argue that we simply followed the loss of PS1 function due to the displacement by the PS2 D366A mutation. However, consistent with previous data, overexpression of wt PS2 (Refs. 14, 26, and 30; Fig. 2B) and familial Alzheimer’s disease-associated PS2 mutations allows robust Aβ production (14, 26, 30). Therefore PS2 D366A is not only displacing PS1 but also appears to interfere directly with Aβ production and Notch cleavage. We can however not exclude that the effects observed by the overexpression of the PS2 D366A mutation are due to the inhibition of PS2 function (by the loss of function mutation) together with the inhibition of PS1 function (by replacement). To prove that PS2 is directly involved in Notch signaling we used an in vivo rescuing assay in C. elegans. In this system the PS homologue, the sel-12 gene, is nonfunctional, which leads to a prominent Notch phenotype. PS2 expression in the mutant worm leads to a full rescue (Table I) directly demonstrating PS2 activity in Notch signaling. In contrast, expressing PS2 D366A fails to exhibit any rescuing activity demonstrating that Asp-366 is critically required for PS2 function in Notch signaling. The very same mutation also blocks the proteolytic release of NICD and significantly reduces Aβ production. Based on these data, we conclude that PS2 promotes Aβ and NICD production by facilitating the endoproteolytic processing of their precursors. Therefore PS2 (data shown here) and PS1 functions (1, 2, 5, 6–10, 32) appear to be similar. This may also explain our finding that a PS2 ablation causes no obvious phenotype, because the highly expressed PS1 could take over PS2 function (25). Based on these findings one would predict that a double knock-out of PS1 and PS2 would enhance the pathological phenotype. Moreover, reverse genetics in C. elegans revealed a redundant function of sel-12 and hop-1 in Notch signaling (32). A redundant function of PS1 and PS2 is also supported by the findings that wt PS2 can functionally replace sel-12 in C. elegans like PS1 (Table I). However, further work is required to support these predictions. Based on the work reported here it may be interesting to prove if co-expression of nonfunctional PS1 and PS2 derivatives could increase the effects on proteolytic processing of βAPP. It may also be possible to rescue the reduced Aβ production in cells derived from PS1−/− mice by overexpression of PS2. We also want to note that additional functions of PS2, which may differ from PS1 cannot be excluded from our work. Indeed, PS2 may be functionally involved in apoptosis (34, 35). In that regard it is interesting to speculate that PS2 activity in apoptosis may also be required for intramembranous processing of receptor proteins involved in signal transduction during programmed cell death.

Whether the aspartate mutations inactivate an intrinsic aspartyl protease activity as suggested by Wolfe et al. (10) or indirectly affect cellular transport of certain target proteins (24) remains to be determined. However, based on our data not only PS1 but also PS2 may be a potential target for Aβ-lowering drugs. Because very minor amounts of the cytoplasmic Notch domain are required for signal transduction (23, 31), a partial inhibition of PS2/PS1 activity may be sufficient to reduce Aβ and amyloid plaque formation.

### Note Added in Proof

While this manuscript was in press, De Strooper and colleagues (36) found that a double knock-out of PS1 and PS2 leads to a full Notch phenotype.

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### FIG. 4.

The PS2 D366A mutation inhibits the release of the NICD. The indicated cell lines were pulse labeled with [35S]methionine for 20 min and chased for 1 h. Notch I derivatives were immunoprecipitated with the antibody 9E10. In control cells the release of NICD from NAE is observed consistent with previous results (23). The cleaved derivative co-migrates with the NotchICV-encoded protein as expected (23). In contrast, expression of PS2 D366A blocks the proteolytic generation of NICD.

### TABLE I

| Strain       | Transgene   | Genotype | Egg-laying behavior |
|--------------|-------------|----------|---------------------|
| N2           | Wild type   | + + + +  | 50                   |
| BR1129       | sel-12 (ar171) | 0 0 0 50 |                      |
| BR1336–38    | PS2 wild type | 119 10 2 |                      |
| BR1227       | PS2 D366A   | 0 0 0 50 |                      |
| BR1322       | PS2 D366A   | 0 0 0 50 |                      |
| BR1390       | PS2 D366A   | 0 0 0 50 |                      |
| BR1391       | PS2 D366A   | 0 0 0 50 |                      |

* Data of three independent lines were combined.
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