The Biological and Pathological Function of the Presenilin-1 ΔExon 9 Mutation Is Independent of Its Defect to Undergo Proteolytic Processing*

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Harald Steiner, Helmut Romig, Melissa G. Grim, Uwe Philipps, Brigitte Pesold, Martin Citron, Ralf Baumeister, and Christian Haass

From the Central Institute of Mental Health, Department of Molecular Biology, I4, 68159 Mannheim, Germany, &Boehringer Ingelheim KG, CNS Research, 55216 Ingelheim, Germany, *Genzentrum, Fodor-Lynen-Str. 25, 81377 Munich, Germany, and †Amgen, Inc., Thousand Oaks, California 91320-1789

The two homologous presenilins are key factors for the generation of amyloid β-peptide (Aβ), since Alzheimer’s disease (AD)-associated mutations enhance the production of the pathologically relevant 42-amino acid Aβ (Aβ42), and a gene knockout of presenilin-1 (PS1) significantly inhibits total Aβ production. Presenilins undergo proteolytic processing within the domain encoded by exon 9, a process that may be closely related to their biological and pathological activity. An AD-associated mutation within the PS1 gene deletes exon 9 (PS1Δexon9) due to a splicing error and results in the accumulation of the uncleaved full-length protein. We now demonstrate the unexpected finding that the pathological activity of PS1Δexon9 is independent of its lack to undergo proteolytic processing, but is rather due to a point mutation (S290C) that affects conserved amino acids (4, 5). However, due to a splicing error, the PS1Δexon9 mutation results in the deletion of the domain encoded by exon 9 (8). This domain contains the cleavage site for proteolytic processing (9, 10), and therefore PS1Δexon9 accumulates as an uncleaved protein (9). Since proteolytic processing is highly regulated (9, 11, 12) and appears to be altered by PS mutations (Refs. 13–15; summarized in Ref. 16), the lack of proteolytic processing caused by the exon 9 deletion was expected to be responsible for its pathological activity. We now demonstrate the unexpected finding that the pathological function of PS1Δexon9 as well as its reduced biological activity is independent of its lack to undergo proteolytic processing, but is rather due to a point mutation (S290C) that is the result of the aberrant exon 8/10 splice junction.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Lines—K293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1% 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). K293 cells stably expressing PS1Δexon9 C290S were generated by transfection of K293 cells stably expressing βAPP containing the Swedish mutation (17). K293 cells stably transfected with Swedish βAPP95, wt PS1, and PS1Δexon9 were described previously (18).

Construction of the cDNA Encoding PS1Δexon9 C290S—The cDNA encoding PS1Δexon9 C290S was constructed by mutagenizing the cysteine at codon 290 of the PS1Δexon9 cDNA (8) to serine in a two-step PCR procedure. The following primers were designed: first PCR, PS1-187-F (5′-CCGATT-TCGAGAAGCCACACTCA-3′) and ΔE9-C290S-R (5′-GTGACTCCCTTCTTCTGGGAGGTAATTAGGACG-3′), second PCR, ΔE9-C290S-F (5′-GGCTCTCACTTACTCCCTCCACAGAAAAAGGATGCAC-3′) and PS1-STOP-R (5′-CGCCCTGGACCAAATATGACTTAGATATA-3′).

After gel purification the PCR products were mixed and subjected to a final PCR with primers PS1-187-F and PS1-STOP-R. The resulting PCR product was digested with EcoRI/XhoI and cloned into the pCDNA3.1/Zeo+ expression vector (Invitrogen). The cDNA was sequenced to verify successful mutagenesis.

Antibodies—The polyclonal antibodies against amino acids 263–407 (BI.3D7) or 297–356 (BI.HF5C) were described previously (19, 20). The monoclonal antibodies to the PS1 and PS2 loop of PS1 (3027) and amino acids 297–356 of PS2 (3711) were described (21). The polyclonal antibodies against amino acids 263–407 (BI.3D7) or 297–356 (BI.HF5C) were described previously (19, 20). The monoclonal antibodies to the PS1 and PS2 loop were raised against fusion proteins containing amino acids 263–407 (BI.3D7) or 297–356 (BI.HF5C).

Metabolic Labeling and Immunoprecipitation of PS—Stably transfected K293 cells were grown to confluence in 10-cm dishes. After starvation for 1 h in 4 ml of methionine- and serum-free MEM (MEM supplemented with 1% l-glutamine and 1% penicillin/streptomycin) cells were metabolically labeled with 700 μCi of [35S]Smethionine (Promix, Amersham Pharmacia Biotech) in 4 ml of methionine- and serum-free MEM for 1 h. Cell extracts were prepared and subjected to immunoprecipitation of PS as described (12). PS immunoprecipitates were solubilized in sample buffer containing 4 M urea for 10 min at 65 °C,

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** To whom correspondence should be addressed: Central Institute of Mental Health, Dept. of Molecular Biology, J5, 68159 Mannheim, Germany, †Amgen Inc., Thousand Oaks, California 91320-1789.
The Biological and Pathological Function of the PS1ΔExon9 Mutation

FIG. 1. Generation and expression of PS1Δexon9 and PS1Δexon9 C290S. A, schematic representation of the genomic structure (exon 8 to exon 10) of wt PS1, PS1Δexon9 and the amino acid sequence at the splice junctions of exons 8, 9, and 10. The nucleotide exchange (G to T) in the PS1 gene identified in the DNA of patients with the PS1Δexon9-linked mutation results in an amino acid exchange at the exon 8/10 splice junction, which changes codon 290 from a serine to a cysteine (bold letters). B, K293 cells expressing endogenous presenilins (control) as well as K293 cells stably overexpressing PS1Δexon9, or PS1Δexon9 C290S were pulse labeled with [35S]methionine for 1 h, and cell lysates were immunoprecipitated with antibody 3027 (19) to the large loop of PS1. Full-length PS (β-PS) proteins accumulate in the cell lines expressing PS1Δexon9 and PS1Δexon9 C290S but not in cells expressing endogenous presenilins. The asterisk indicates dimeric forms of PS1 (19). C and D, stable expression of PS1Δexon9 and PS1Δexon9 C290S inhibits formation of endogenous PS1 (C) and PS2 C-terminal fragments (CTF) (D). Cell lysates from K293 cells and cell lines overexpressing PS1Δexon9 and PS1Δexon9 C290S were immunoprecipitated with the previously described antibodies (see “Experimental Procedures”) specific to the large loop of PS1 (3027; Ref. 19) and PS2 (3711; Ref. 20) and detected by immunoblotting using the monoclonal antibodies BI.3D7 and BI.HF5C.

separated on SDS-urea gels, and analyzed by fluorography (19).

Combined Immunoprecipitation/Western Blotting—Stably transfected K293 cell lines were grown to confluence. Cell extracts were prepared and subjected to immunoprecipitation as described (12). Following gel electrophoresis, immunoprecipitated proteins were identified by immunoblotting (12). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Analysis of Aβ40 and Aβ42—Culture medium (2 ml) was collected from confluent K293 cells grown in six-well dishes for 24 h. The medium was assayed for Aβ40 and Aβ42 using a previously described ELISA assay (12). Aβ peptides were immunoprecipitated using antibody 6E10 (Senetek) according to established protocols (21).

Transgenic Lines of Caenorhabditis elegans and Rescue Assays—Expression constructs were generated as described previously (22). Transgenic lines were established by microinjection of plasmid DNA mixtures into the C. elegans germ line to create extrachromosomal arrays as described previously (22). All plasmids used in this study were injected at a concentration of 20 ng/µl into sel-12(ar171) or sel-12(ar171) unc-1(e653B) hermaphrodites along with ttx-3:GFP as a cotransformation marker. Successful transformation was monitored by the expression of GFP in the AIY interneurons of F1 and F2 generation animals. Four independent lines from the progeny of F2 generation animals were established.

As the sel-12(ar171) animals never lay eggs (23), rescue of the sel-12 defect can be quantified by scoring egg laying behavior in transgenic animals (22, 24). Consequently, for each transgenic line, we examined 50 transgenic animals for their ability to lay eggs and determined their brood size. The number of eggs laid by individual transgenic animals was counted and placed into four categories: “Egl+++,“ robust egg laying, more than 30 eggs laid (wt phenotype); “Egl++,” 15–30 eggs laid; “Egl+,” 5–15 eggs laid; “Egl−,” no eggs laid.

RESULTS

The PS1Δexon9 mutation changes a G to a T at the splice site for exon 9. It therefore destroys the minimal required consensus sequence for the splice acceptor site (8), which then results in an aberrant deletion of the domain encoded by exon 9 (Ref. 8; Fig. 1A). However, the mutation also changes codon 290 at the exon 8/10 splice junction from a serine to a cysteine (Ref. 8; Fig. 1A). We therefore have investigated if the single amino acid exchange or the deletion of the domain required for pro-}

teolytic processing is responsible for the pathological activity of the PS1Δexon9 mutation in regard to Aβ42 generation. For this purpose we mutagenized amino acid 290 of PS1Δexon9 to a serine (PS1Δexon9 C290S), thus correcting the point mutation (Fig. 1A). cDNAs encoding PS1Δexon9 and PS1Δexon9 C290S were stably transfected into kidney 293 cells expressing endogenous PS, as well as cell lines overexpressing PS1Δexon9 or PS1Δexon9 C290S. Conditioned medium from metabolically labeled kidney 293 cells expressing the indicated presenilin variants was immunoprecipitated with antibody 6E10. Aβ40 and Aβ42 were separated on a previously described gel system, which allows the specific resolution of both species (25). As reported before, Aβ42 migrates faster as Aβ40 (25). Note that cells expressing PS1Δexon9 produced increased amounts of Aβ42, whereas cells expressing PS1Δexon9 C290S do not show such an increase. Two individual immunoprecipitations of each cell lysate are shown. B, quantitation of the Aβ42 and Aβ40 concentrations in conditioned medium of kidney 293 cells expressing the indicated presenilin variants using a previously described highly specific ELISA (12). Expression of PS1Δexon9 but not the expression of PS1Δexon9 C290S results in a 3-fold increase of Aβ42 production. Identical results were observed with independent cell clones.

It has been shown before that overexpression of PS proteins results in the replacement of endogenous PS fragments and the accumulation of PS1Δexon9 prevents the formation of stable PS fragments (9, 11, 12). To prove that overexpression of PS1Δexon9 C290S still allows the reduction of endogenous PS fragments, cell lysates from unlabeled cells were immunoprecipitated with antibodies specific to the PS1 or PS2 loop domain (see “Experimental Procedures”), and PS fragments were identified with the PS1/PS2-specific monoclonal antibodies BI.3D7 and BI.HF5C (see “Experimental Procedures”). Whereas cell
lines expressing endogenous presenilins produced PS1 as well as PS2 C-terminal fragments (Fig. 1, C and D), overexpression of PS1\textsuperscript{Δexon9} and PS1\textsuperscript{Δexon9 C290S} inhibited the formation of endogenous PS1 and PS2 fragments (Fig. 1, C and D). This demonstrates that PS1\textsuperscript{Δexon9 C290S}, like PS1\textsuperscript{Δexon9}, fully replaced presenilins derived from the endogenous genes and also proves that PS1\textsuperscript{Δexon9 C290S} does not undergo proteolytic processing but rather accumulates as an uncleaved full-length protein.

In order to investigate the pathological activity of the mutant PS derivatives on Aβ production, control cells as well as cells overexpressing PS1\textsuperscript{Δexon9} and PS1\textsuperscript{Δexon9 C290S} were metabolically labeled with [\textsuperscript{35}S]methionine, and Aβ40 and Aβ42 peptides were immunoprecipitated from the conditioned medium using antibody 6E10. This antibody is raised to Aβ1–17 and therefore recognizes Aβ40 as well as Aβ42 (see “Experimental Procedures”). Isolated Aβ peptides were then separated on a previously described gel system, which allows the specific resolution of Aβ40 and Aβ42 (25). As shown in Fig. 2A, cells overexpressing PS1\textsuperscript{Δexon9} secreted elevated levels of Aβ42 as compared with control cells. In contrast, the cell line stably transfected with PS1\textsuperscript{Δexon9 C290S} produced significantly lower amounts of Aβ42 as cells expressing the Alzheimer’s disease-associated PS1\textsuperscript{Δexon9} mutation. This suggests that the pathological activity of the PS1\textsuperscript{Δexon9} mutation is due to the point mutation generated at the aberrant splice junction. In order to quantitate Aβ42 and Aβ40 production, we used a previously described specific ELISA (12a). In this assay, two highly specific monoclonal antibodies are used for the discrimination of both peptide species (12). As reported before (12a, 18, 26), the PS1\textsuperscript{Δexon9} mutation results in an approximately 3-fold increase of the Aβ42/Aβ\textsubscript{total} ratio (Fig. 2B). In contrast, the cell line stably overexpressing PS1\textsuperscript{Δexon9 C290S} showed no increase in Aβ42/Aβ\textsubscript{total} ratio (Fig. 2B). Therefore, using two different approaches we can show that the pathological activity of PS1\textsuperscript{Δexon9} is independent of its lack of proteolytic processing but is rather caused by the single amino acid change at the aberrant splice junction.

Having demonstrated that PS1\textsuperscript{Δexon9 C290S} is pathologically inactive, we also wanted to test if this protein is sufficient to rescue a lin-12-mediated signaling defect that is a result of a mutant PS homologue (sel-12) in Caenorhabditis elegans (23). As reported previously, transgenic expression of human presenilins rescues the phenotype caused by mutations of sel-12 (22–24). In contrast, transgenic expression of the PS1\textsuperscript{Δexon9} variant in C. elegans resulted in an incomplete rescue of the sel-12 mutant phenotype, as indicated by a significantly reduced brood size and reduced egg laying (Refs. 22 and 24; see also Table I). In order to assess the in vivo function of PS1\textsuperscript{Δexon9 C290S}, we tested its ability to rescue the putative sel-12 null allele ar171 (23). Four independent transgenic strains expressing PS1\textsuperscript{Δexon9 C290S} displayed robust egg laying (Table I).

Based on both numbers of laid progeny and numbers of eggs in utero, the phenotype of these strains is almost indistinguishable of that of wild type worms (Table I). These results demonstrate that PS1\textsuperscript{Δexon9 C290S} rescues the egg laying phenotype of the sel-12 (ar171) mutant animals significantly better than PS1\textsuperscript{Δexon9} (Table I). Therefore, the reduced biological activity of PS1\textsuperscript{Δexon9} is also due to a single point mutation and completely independent of the lack of proteolytic processing.

**DISCUSSION**

Our results demonstrate that the pathological effect of the PS1\textsuperscript{Δexon9} mutation is independent of its lack of proteolytic cleavage and the deletion of the exon 9-encoded domain. Reverting the cysteine residue generated by the exon 8/10 splice junction at codon 290 back to its wt residue (serine) still inhibits its proteolytic processing, causes an accumulation of the uncleaved protein, and prevents formation of endogenous PS fragments. Although the artificially generated PS1\textsuperscript{Δexon9 C290S} variant behaves like PS1\textsuperscript{Δexon9} in regard to the characteristic biochemical features described above, it does not allow pathological overproduction of Aβ42. Moreover, PS1\textsuperscript{Δexon9 C290S} regains full biological activity in a very sensitive in vivo assay system. This demonstrates that not only the pathological overproduction of Aβ42 but also the reduced biological function is due to the single amino acid exchange. Therefore, similar to all other known PS mutations, the pathological effect of the PS1\textsuperscript{Δexon9} mutation is due to a rather subtle amino acid exchange at a single highly conserved codon whereas the large deletion of the complete domain encoded by exon 9 does not affect the biological and pathological function. However, consistent with our previous results (27), we suggest that PS molecules lacking the domain encoded by exon 9 mimic a proteolytically processed and biologically active PS complex and therefore rescue the phenotype of the mutant nematode. Consequently, the rescuing activity of PS1\textsuperscript{Δexon9} is significantly enhanced when the pathologically relevant point mutation at codon 290 is reverted to the wt residue.

It remains to be shown if the mutation at codon 290 is also pathologically active within the full-length protein like all other AD-associated point mutations. It may however be possible that the mutation only exhibits a pathological activity if it is aberrantly flanked by the domains encoded by exons 8 and 10. Structural changes that may specifically occur in the PS1\textsuperscript{Δexon9} molecule (12, 19) might be responsible for the pathological activity of this very unusual mutation.

Based on our results it would be interesting to investigate if the point mutations in the PS genes require the endoproteolytic cleavage for their pathological activity. Therefore artificial PS molecules should be generated containing an AD associated mutation as well as the smallest possible alteration of the sequence at the cleavage site which would inhibit PS processing. However, such mutations might be very difficult to gener-

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**Table I**

Rescue of the sel-12 egg laying defect by human PS genes expressed from the sel-12 promoter

For 50 transgenic animals each, the numbers of progeny were counted and grouped in the following categories: +++, over 30 progeny laid by individual animal; +, 15–30 progeny laid; +, 5–15 progeny laid; −, no progeny laid. The sel-12 (ar171) strains carried an additional unc-1(e538) marker that did not affect egg laying or rescuing frequency. It was backcrossed several times from the strain originally published (23).

| Strain | Transgene | Genotype | Egg laying behavior |
|--------|-----------|----------|---------------------|
| N2     | Vector    | Wild type| +++                 |
| BR1029 | PS1\textsuperscript{Δexon9} | sel-12(ar171) | ++                  |
| BR1083 | PS1\textsuperscript{Δexon9} | sel-12(ar171) | +                   |
| BR1092 | PS1\textsuperscript{Δexon9 C290S} | sel-12(ar171) | +                   |
| BR1093 | PS1\textsuperscript{Δexon9 C290S} | sel-12(ar171) | −                   |
| BR1094 | PS1\textsuperscript{Δexon9 C290S} | sel-12(ar171) | −                   |
| BR1095 | PS1\textsuperscript{Δexon9 C290S} | sel-12(ar171) | −                   |
ate since PS can be cleaved at multiple sites (10, 12) and PS molecules containing larger deletions at the cleavage sites might mimic a proteolytically processed PS molecule (27).

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Note Added in Proof—While this manuscript was in press, we found that expression of PS1ΔExon9 C290S containing a FAD-associated mutation (M146L) results in elevated Aβ(42) production, in further support of the findings presented here.

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