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Authors

Kulic, Luka
Walter, Jochen
Multhaup, Gerd
[et al.](#)

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Separation of presenilin function in amyloid β -peptide generation and endoproteolysis of Notch

Luka Kulic*, Jochen Walter*, Gerd Multhaup[†], David B. Teplow[‡], Ralf Baumeister[§], Helmut Romig[¶], Anja Capell*, Harald Steiner*, and Christian Haass*^{||}

*Adolf-Butenandt-Institute, Department of Biochemistry, Laboratory for Alzheimer's Disease Research, Ludwig Maximilians University, 80336 Munich, Germany; [†]Center for Molecular Biology–Zentrum für Molekulare Biologie der Universität Heidelberg, University of Heidelberg, 69120 Heidelberg, Germany; [‡]Harvard Medical School, Center for Neurologic Diseases, Boston, MA 02115; [§]Gene Center, Ludwig Maximilians University, 81377 Munich, Germany; and [¶]Boehringer Ingelheim K.G., CNS Research, 55216 Ingelheim, Germany

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Most of the genetically inherited Alzheimer's disease cases are caused by mutations in the presenilin genes, PS1 and PS2. PS mutations result in the enhanced production of the highly amyloidogenic 42/43 amino acid variant of amyloid β -peptide ($A\beta$). We have introduced arbitrary mutations at position 286 of PS1, where a naturally occurring PS1 mutation has been described (L286V). Introduction of charged amino acids (L286E or L286R) resulted in an increase of $A\beta$ 42/43 production, which reached almost twice the level of the naturally occurring PS1 mutation. Although pathological $A\beta$ production was increased, endoproteolysis of Notch and nuclear transport of its cytoplasmic domain was significantly inhibited. These results demonstrate that the biological function of PS proteins in the endoproteolysis of β -amyloid precursor protein and Notch can be separated.

Alzheimer's disease | γ -secretase

Alzheimer's disease (AD) is the most common form of senile dementia. Pathologically, AD is closely associated with the invariant accumulation of senile plaques (1), which are predominantly composed of amyloid β -peptide ($A\beta$). $A\beta$ is generated from the β -amyloid precursor protein (β APP) through the consecutive activity of two proteases called β - and γ -secretase (2). Two predominant species of $A\beta$ are generated, terminating at either amino acid 40 ($A\beta$ 40) or amino acids 42/43 ($A\beta$ 42/43) (1). The less common $A\beta$ 42/43 species is specifically involved in AD pathogenesis, because it preferentially accumulates in senile plaques and its production is significantly enhanced by familial AD (FAD)-associated mutations in the β APP gene as well as to the two homologous presenilin genes, PS1 and PS2 (1).

PS proteins not only are involved in FAD but also play a pivotal role in the physiological metabolism of β APP, since ablation of PS1 results in a significant reduction of $A\beta$ 40 and $A\beta$ 42/43 generation (3). Mutagenesis of two critical aspartates (see Fig. 1A) within transmembrane domains (TM) 6 and/or 7 of PS1 (4, 5), or in TM7 of PS2 (6), also inhibits formation of $A\beta$ 40 and $A\beta$ 42/43 (2). Furthermore, reduced $A\beta$ generation appeared to correlate with the concomitant failure of the mutant PS1 variant to undergo endoproteolysis (4). Together with the finding that γ -secretase activity can be reduced by aspartyl protease inhibitors (7), this observation led Wolfe *et al.* (8) to the intriguing hypothesis that PS proteins may be unusual aspartyl proteases having a catalytically active center located within two adjacent TM domains. According to this hypothesis, PS proteins are activated by autoprolysis. The resulting N- and C-terminal fragments (9) bind to each other, forming a biologically active PS complex (10–13), which subsequently catalyzes the intramembranous cleavage of β APP (4, 8). However, recent evidence demonstrates that endoproteolysis of PS1 and PS2 may not be an absolute prerequisite for the function of presenilins in β APP endoproteolysis (14, 15).

An endoproteolytic function of presenilins may further be supported by the finding that a PS1 knock-out (16, 17), as well as the aspartate mutations of PS1 or PS2 (6, 18), block the endoproteolytic cleavage of Notch. This cleavage appears to be very similar to the intramembranous cleavage of β APP by γ -secretase because it occurs close to or within the membrane (17, 19). Endoproteolysis of Notch can also be blocked by aspartyl protease inhibitors similar to the γ -secretase cleavage of β APP (16). Moreover, mutations in the *Drosophila* PS homolog also lead to a Notch-like phenotype and alter its endoproteolysis (20, 21). Endoproteolytic cleavage of Notch is required to liberate the Notch intracellular cytoplasmic domain (NICD), which translocates to the nucleus where it acts as a regulator of selected transcription factors (19). Nuclear translocation of NICD appears to be required for Notch signaling (19, 22), although recent findings suggest that alternative pathways, which are independent of endoproteolysis of Notch, may exist as well (23). A functional role of presenilins in Notch signaling is also strongly supported by the findings that a PS1 knock-out results in a phenotype that resembles a Notch knock-out to some extent (24, 25). Moreover, a double knock-out of both PS1 and PS2 results in a phenotype that is almost identical to the phenotype caused by the knock-out of Notch (26, 27). In *Caenorhabditis elegans*, artificial mutations of its PS homologue, sel-12 (28), also cause a Notch phenotype, and this phenotype can be functionally rescued by human PS1 or PS2 (29, 30). Moreover, a functional deficit of both sel-12 and its homologue hop-1 further enhance the Notch phenotype of mutant sel-12 alleles in the worm (31). Although all FAD-associated PS mutations analyzed so far failed to fully rescue the sel-12 mutant phenotype in *C. elegans*, FAD mutations of PS1 appear to rescue the PS1^{-/-} phenotype in mice (32, 33). Based on the latter results, it appears that PS mutations cause a gain of malfunction and are not due to a loss of function (32, 33).

We now wanted to investigate whether point mutations in PS1 equally affect endoproteolysis of β APP and Notch. To do so, we mutagenized a previously identified FAD mutation of PS1 and monitored the functional consequences on $A\beta$ production and NICD formation. We identified point mutations that inhibited

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Abbreviations: $A\beta$, amyloid β -peptide; AD, Alzheimer's disease; β APP, β -amyloid precursor protein; PS, presenilin; CTF, C-terminal fragment; NICD, Notch intracellular domain; TM, transmembrane domain; wt, wild type; FAD, familial AD.

^{||}To whom reprint requests should be addressed. E-mail: chaass@pbm.med.uni-muenchen.de.

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NICD formation but caused increased production of the pathologically relevant A β 42/43. This indicates that PS mutations could simultaneously cause a loss of function in Notch signaling but a gain of pathological dysfunction in A β production.

Materials and Methods

cDNA Constructs. The cDNAs encoding PS1 L286I, PS1 L286F, PS1 L286R, PS1 L286E, PS1 L286W, PS1 L286Q, PS1 L286P, and PS1 L286S were constructed by oligonucleotide-directed mutagenesis using PCR. The respective PCR products were cloned into *EcoRI/BamHI* restriction sites of the expression vector pcDNA3.1 containing a zeocin resistance gene (Invitrogen) and sequenced to verify successful mutagenesis. The expression plasmid containing the Notch Δ E cDNA was described before (6).

Cell Culture and Cell Lines. HEK 293 cells stably expressing wild-type (wt) β APP or Swedish mutant β APP (34) were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 200 μ g·ml⁻¹ G418. These cells were transfected with the respective cDNAs encoding wt or mutant PS1 using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; Roche Molecular Biochemicals) according to the manufacturer's instructions and selected in 200 μ g·ml⁻¹ zeocin. To analyze proteolytic processing of Notch, cell clones stably expressing the indicated PS1 derivatives were transfected with the cDNA encoding Notch Δ E and selected in the above described medium containing 50 μ g·ml⁻¹ hygromycin.

Amino Acid Sequencing. Radiosequencing was performed as described (35).

Mass Spectrometry. Immunoprecipitated A β was extracted twice with 500 μ l of 50% acetic acid from the Sepharose beads and dried under vacuum using a Speed-Vac centrifuge. The protein was redissolved in 20 μ l of 50% formic acid and diluted 1:10 in 10% formic acid. Approximately 0.5 μ l of the antibody/A β -peptide solution was mixed with 0.5 μ l of a saturated solution of sinapinic acid in a 1:1 acetonitrile/water solvent containing 0.5% trifluoroacetic acid on a target sample plate. Alternatively, α -cyano-4-hydroxycinnamic acid was used as the matrix. As external mass calibrants insulin, CLIP (corticotropin-like intermediate lobe peptide, ACTH-18–39) and CIP (corticotropin-inhibiting peptide, the 7–38 fragment of human ACTH-1–39) were used. After drying in air for several minutes, the target was placed in a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE STR, Perkin-Elmer) and then analyzed.

Antibodies. The polyclonal and monoclonal antibodies against PS1 [3027 (36), BI.3D7 (37)] and PS2 [BI.HF5c (37)], as well as the anti-A β antibody 3926 (38), were described previously. The monoclonal anti-myc antibody 9E10 developed by J. Michael Bishop (University of California, San Francisco) was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Science (Iowa City, IA).

Metabolic Labeling and Immunoprecipitations. To analyze proteolytic processing of β APP, HEK 293 cells were starved for 1 h in methionine- and serum-free MEM and subsequently metabolically labeled for 2 h with 700 μ Ci (1 μ Ci = 37 kBq) of [³⁵S]methionine (Promix; Amersham Pharmacia) in methionine- and serum-free MEM. Cells were chased in the presence of excess amounts of unlabeled methionine for 2 h. To monitor NICD formation, cells were pulse labeled for 15 min and chased for 60 min.

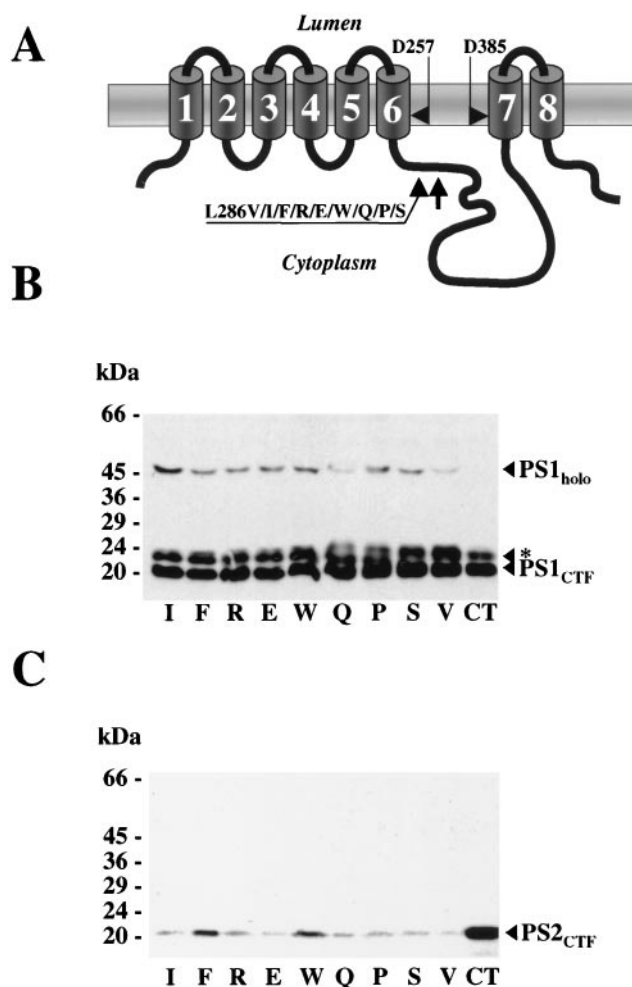


Fig. 1. (A) Schematic representation of PS1. The critical aspartates in TM 6 and 7 as well as the endoproteolytic cleavage site(s) (arrow) and the mutations introduced at codon 286 are indicated. (B) Expression and endoproteolytic processing of the mutant PS1 derivatives (CT, untransfected control expressing endogenous PS). Cell lysates were immunoprecipitated with antibody 3027, and precipitated PS1 derivatives (CTFs and holoprotein) were visualized by immunoblotting using antibody BI.3D7. *, Phosphorylated PS1 CTF (13, 36). Note that endoproteolysis of PS1 occurs in all cell lines investigated. (C) Overexpressed mutant PS1 derivatives displace endogenous PS2 CTFs. Cell lysates were immunoblotted with the monoclonal antibody BI.HF5c.

Combined Immunoprecipitation/Western Blotting. Extracts from HEK 293 cells were prepared and subjected to immunoprecipitation using the polyclonal antibody 3027 to PS1 (36). After gel electrophoresis, immunoprecipitated PS1 proteins were identified by immunoblotting using the monoclonal antibody BI.3D7 (37). PS2 C-terminal fragments (CTFs) were identified by immunoblotting using antibody BI.HF5c (6). Notch Δ E was immunoprecipitated as described (6). NICD generation was monitored by phosphorimaging.

Immunocytochemistry. Immunocytochemistry was carried out as described (39).

Transgenic Lines of *C. elegans* and Rescue Assays. These experiments were carried out as described (6, 30, 37).

Results

Point mutations were inserted at codon 286 of PS1 (Fig. 1A). This codon was chosen because a FAD-associated mutation has

been mapped previously to this position (40). Moreover, codon 286 is located within a critical domain of presenilins, to which many other FAD-associated mutations have been mapped (41). This domain is highly conserved in all PS species identified so far (5). Finally, the site(s) of endoproteolysis of PS1 and PS2 have also been located close to this mutation (9, 14, 15, 42).

Mutagenesis at Amino Acid 286 of PS1 Does Not Interfere with Endoproteolysis. The FAD-associated leucine to valine exchange appears to be an apparent conservative mutation. Additional mutants were made to substitute leucine with conservative and more nonconservative mutations, i.e., amino acids with nonpolar side chains (I), polar side chains (S, Q), large bulky aromatic side groups (F, W), acidic (E) and basic side chains (R), and the imino acid proline to create a fixed kink in the polypeptide chain (Fig. 1A). These mutations, as well as wt PS1 and the FAD-associated PS1 L286V mutation, were stably transfected into human kidney 293 (HEK 293) cells expressing Swedish mutant β APP (34). HEK 293 cells coexpressing PS derivatives, together with Swedish mutant β APP, are suitable for the analysis of PS function as demonstrated previously (6, 11, 12, 14, 37, 42–46). Pooled cell clones were first analyzed for PS1 expression and endoproteolysis. Cell lysates were immunoprecipitated with antibody 3027 (36). Immunoprecipitated PS1 derivatives were then visualized by immunoblotting using the monoclonal antibody PS1 BI.3D7 (37). Consistent with previous results (9), no PS1 holoprotein could be identified in cells expressing endogenous PS1 (Fig. 1B). However, in all cell lines overexpressing exogenous PS1, the holoprotein accumulated (Fig. 1B). In these cell lines, formation of the PS1 CTF was observed, which demonstrates that endoproteolysis was not inhibited by any of the mutations introduced (Fig. 1B). As observed previously (9), no increased expression of the PS1 CTFs was obtained on overexpression, which is due to the fact that PS expression is tightly controlled and that overexpressed PS fragments displace endogenous fragments (47). To demonstrate that the overexpressed PS1 derivatives shown in Fig. 1B replace their endogenous counterparts, we analyzed the same extracts for the expression of the PS2 CTF. PS2 CTFs were identified by immunoblotting using the monoclonal antibody BI.HF5c (37). As reported previously, HEK 293 cells endogenously express PS2 CTFs (6) (Fig. 1C). Expression of the PS2 CTFs was reduced in all cell lines overexpressing PS1 derivatives (Fig. 1C). Displacement of endogenous presenilins is a prerequisite for stable expression of exogenous PS because PS derivatives not displacing endogenously expressed fragments are unstable and are rapidly degraded (11, 43, 47). The results in Fig. 1B and C therefore demonstrate that the overexpressed mutant PS1 derivatives undergo endoproteolysis and displace endogenous PS.

Effects of Mutations at Codon 286 on A β 42/43 Production. To analyze a potential pathological function of the introduced PS mutations, we investigated the production of A β 40 and A β 42/43. Cells were metabolically labeled with [35 S]methionine for 2 h and subsequently chased for an additional 2 h in the presence of excess amounts of unlabeled methionine. Conditioned media were immunoprecipitated with antibody 3926 (38) to isolate all species of A β . Immunoprecipitated A β species were separated on a previously described gel system, which allows the specific identification and quantification of A β 40 and A β 42/43 (37, 48). As expected (45, 49, 50), cells overexpressing the L286V mutation increased the relative secretion of A β 42/43 as compared with cells overexpressing wt PS1 or the endogenous PS genes (Fig. 2A). The introduced mutations showed individual and characteristic effects on A β production. PS1 L286F and PS1 L286I did not enhance A β 42/43 production to pathological levels (Fig. 2A and B). All other mutations significantly increased A β 42/43 production to levels similar (L286S, L286P,

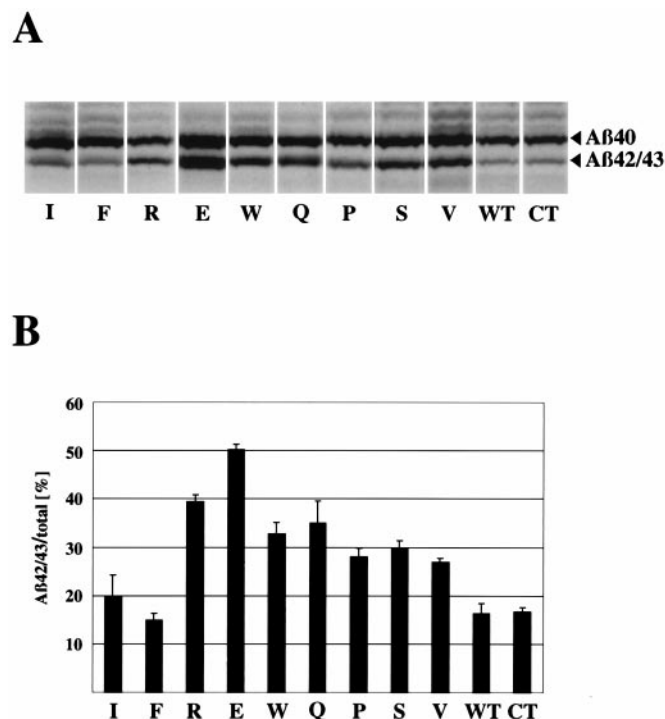


Fig. 2. Effects of the mutant PS1 derivatives on A β production. (A) A β species were immunoprecipitated from conditioned media of metabolically labeled cells with antibody 3926. Isolated A β species were separated on a previously described gel system, which allows the specific identification of A β 40 and A β 42/43 (37, 48). The identity of the observed A β species produced from cells expressing the charged amino acids, as well as wt PS1, was confirmed by amino acid sequencing (data not shown). Mass spectrometry revealed A β 40 and A β 42 but no A β 43 in cells expressing wt PS1. In cells expressing the PS1 mutants L286E and L286R, a significantly increased production of A β 43 was observed (data not shown) (WT, control transfected with wt PS1; CT, untransfected control). (B) Quantification of the (A β 42/43)/(A β total) ratio by phosphorimaging. Bars represent the mean \pm SE of three independent experiments.

L286Q, L286W) or even above (L286E, L286R) the A β 42 concentrations observed in cells expressing the originally described FAD-associated L286V mutation (Fig. 2A and B). Interestingly, on expression of the L286E mutation, the A β 42/43 level reached about 50% of total A β produced (Fig. 2A and B), whereas the A β (42/43):A β total ratio in cells expressing the natural-occurring L286V mutation was below 30% (Fig. 2A and B). High levels of A β 42/43 production were also obtained by the expression of L286R (Fig. 2B). As reported for several FAD-associated PS mutations (44, 45), effects were independent of the expression levels of PS1 (compare the amount of the PS1 holoprotein identified in Fig. 1B to the effects on A β 42/43 production).

PS1 L286E and PS1 L286R Selectively Inhibit Endoproteolysis of Notch.

To investigate whether the artificial PS1 mutants affect endoproteolysis of Notch, we transfected the above described cell lines with the Notch Δ E cDNA (19). Similar cell lines overexpressing multiple transgenes, including β APP, PS1/PS2, and Notch Δ E, have been used previously to analyze Notch endoproteolysis (6, 46). The N-terminal truncated version of Notch has been used previously to monitor NICD generation in transfected cells lines (6, 18, 19). NICD formation was then followed in pulse–chase experiments. Cells were metabolically labeled with [35 S]methionine for 15 min and chased for 60 min in the presence of excess amounts of unlabeled methionine. We specifically

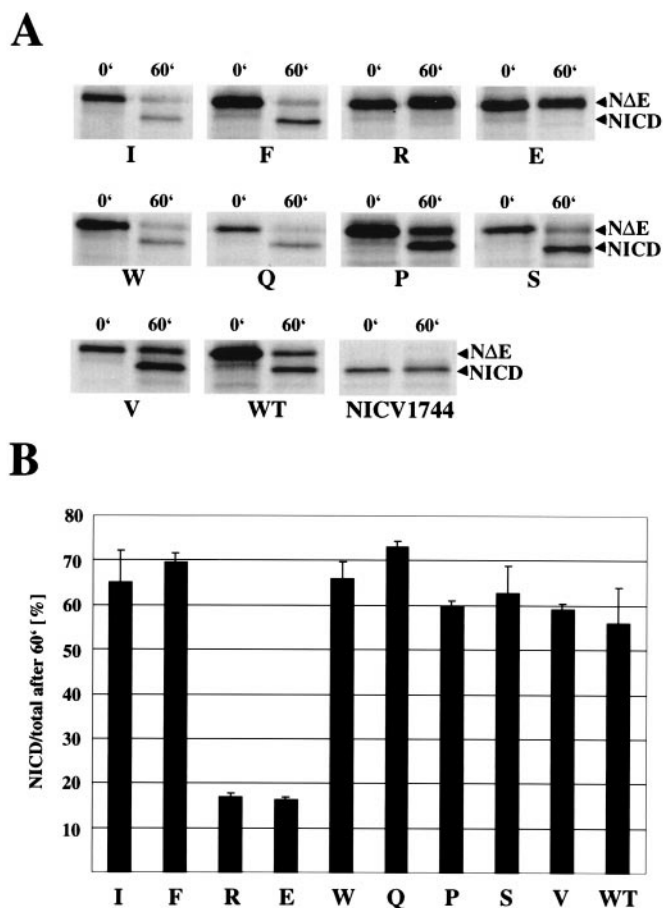


Fig. 3. Effects of the mutant PS1 derivatives on endoproteolysis of Notch. (A) Mutant cell lines were transfected with the Notch Δ E cDNA (19). NICD formation was analyzed in pulse–chase experiments as described (6). Note that PS1 L286E and PS1 L286R inhibit NICD formation. All other PS1 derivatives, including the naturally occurring PS1 L286V mutation, allow normal NICD formation. NICV1744 represents recombinant NICD, which was used as a marker to monitor NICD production (6, 19). (B) Quantification of the experiments shown in A by phosphorimaging. Bars represent the mean \pm SE of three independent experiments.

chose a 60-min chase time because we have demonstrated previously that maximum NICD formation occurred at this time point (6). Cell lysates were prepared, and Notch derivatives were immunoprecipitated with an antibody to the myc epitope fused to C terminus of Notch Δ E (6, 19). As reported before (6), robust levels of NICD were generated after 60 min in cells expressing wt PS1 (Fig. 3A). Similar results were obtained with cells expressing PS1 mutations (including the naturally occurring L286V), which contain uncharged amino acids at codon 286 (Fig. 3A). In contrast, PS1 L286R and PS1 L286E significantly inhibited NICD formation (Fig. 3A). Quantitation of independent experiments fully confirmed the inhibitory effects of the PS1 L286R and L286E mutations on Notch endoproteolysis (Fig. 3B). Moreover, transgenic expression of PS1 L286R in sel-12 mutant *C. elegans* completely failed to rescue the egg-laying phenotype (data not shown). This demonstrates the functional inactivity in the facilitation of Notch signaling of a PS1 variant with a charged amino acid artificially inserted at codon 286.

Accumulation of Notch on the Cell Surface. To further confirm these results, we followed the cellular transport of Notch in immunocytochemical experiments. Cells expressing wt PS1, the naturally occurring PS1 L286V mutation or the artificial PS1 L286E,

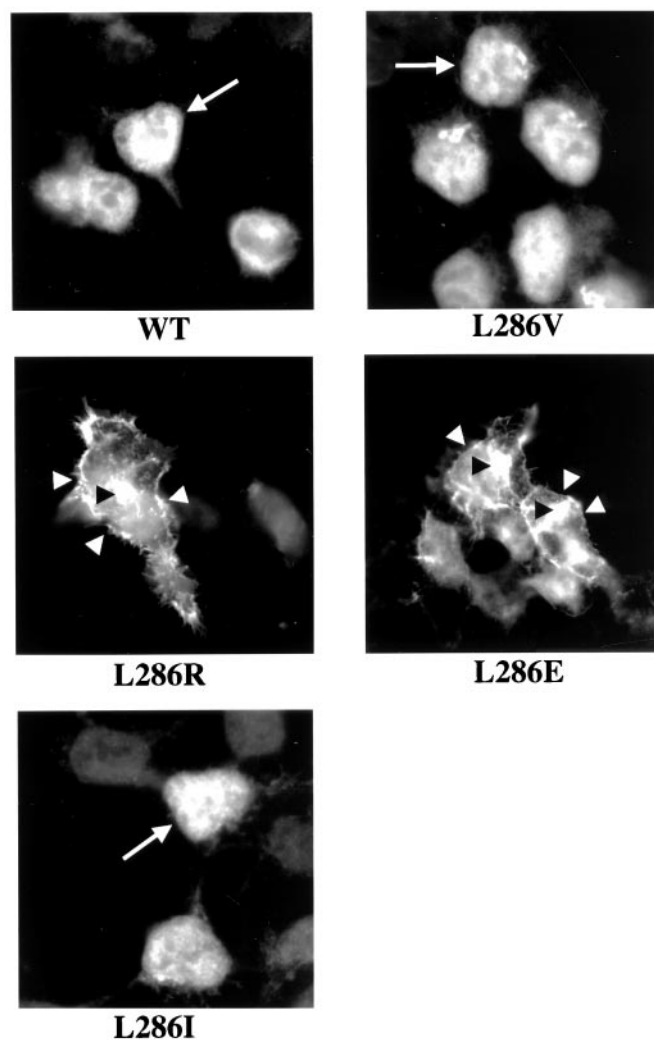


Fig. 4. Cellular distribution of Notch in cells expressing the indicated PS1 derivatives. After fixation in paraformaldehyde, cells were permeabilized and stained with antibody 9E10 to the cytoplasmic myc tail of Notch Δ E. As observed before (22), cells expressing wt PS1 show an accumulation of Notch epitopes (most likely NICD) within the nucleus (arrows). In contrast, cells expressing PS1 L286E or PS1 L286R accumulate Notch epitopes at the plasma membrane (white arrowheads) and within the Golgi (46) (black arrowheads).

L286R, and L286I mutations were permeabilized and stained with an antibody to the myc tail of the recombinant Notch. Cells expressing wt PS1, L286V, or L286I showed a prominent nuclear staining (Fig. 4). Together with the results in Fig. 3, this demonstrates efficient nuclear transfer of NICD. In contrast, cells expressing PS1 L286R or L286E showed very little, if any, nuclear staining of Notch epitopes (Fig. 4). In these cells Notch epitopes accumulated on the plasma membrane (Fig. 4, white arrowheads). Together with the above described inhibition of NICD formation (Fig. 3), these results demonstrate that the uncleaved Notch Δ E precursor accumulates at the cell surface, whereas NICD is not produced and consequently not transported to the nucleus.

Discussion

The above described experiments demonstrate that mutagenesis of a FAD-associated codon of PS1 can result in a further increase in the pathological activity of the original mutation. Of eight mutations introduced, only two had no pathological activity in

regard of A β 42/43 production. In contrast, the remaining six mutations substantially increased A β 42/43 production. The highest A β 42/43 level was reached when the leucine at amino acid 286 was exchanged to a glutamate. Interestingly, introduction of a positively charged amino acid (arginine) also significantly increased the pathological potential of the resulting PS variant. Insertion of charged amino acids (R and E) therefore appears to have the most prominent effects on the pathological function of PS1. In that regard, it is interesting to note that only those PS1 derivatives that contained charged amino acids at position 286 affected endoproteolysis of Notch. These mutations inhibited NICD formation and its nuclear translocation to a very similar extent as the previously described aspartate mutations within the potentially catalytic center of PS1 or PS2 (6, 18). Therefore, PS1 L286E and PS1 L286R behave like the aspartate mutations blocking almost completely the biological function of PS1 in the facilitation of Notch endoproteolysis. Furthermore, this indicates that a very severe effect of PS1 mutations on A β 42/43 production may be correlated with a loss of function in at least one of the biological activities of PS1. In contrast to the PS1 knock-out (3) or the active site mutations in TM6 or TM7 (4–6), the mutations in the large cytoplasmic loop result in a selective loss of function exclusively in endoproteolysis of Notch.

Interestingly, all FAD-associated human PS1 mutations analyzed so far failed to fully rescue the sel-12 mutant phenotype (29, 30, 37). The *ar131* allele of sel-12 is a point mutation (C60S) that occurs at a position that is highly conserved during evolution (28). Interestingly, this point mutation results in reduced Notch activity (28), and, at such conserved positions, FAD-associated mutations are usually observed (41). Moreover, Song *et al.* (22) demonstrated that some FAD-associated PS1 mutations also failed to rescue the lack of NICD production in cells derived from PS1 knock-out mice. However, FAD-associated PS1 mutations were able to rescue embryonic development of PS1 knock-out mice (32, 33). The latter is consistent with our finding that the naturally occurring L286V mutation did not affect Notch endoproteolysis. In contrast, insertion of the two charged amino acids results in a PS1 derivative, which does not functionally support Notch endoproteolysis. This may indicate that the mutations L286R and L286E cause a loss of function of PS1 in Notch endoproteolysis. On the other hand, γ -secretase cleavage of β APP is not inhibited but pathologically modified (by increasing A β 42/43), suggesting a gain of misfunction. Therefore, separation of the PS1 function in endoproteolysis of β APP and Notch has implications for the hypothesis that presenilins are identical with the γ -secretase (4, 8). If PS1 were identical with the protease mediating the γ -secretase cleavage of β APP as well as the endoproteolytic production of NICD, one would expect that a loss of function mutation affects both cleavages equally. However, the two mutations, which block NICD production and its nuclear translocation, do not inhibit A β generation like the

aspartate mutations in TM6 or TM7 of PS1 and PS2 (4–6) or the PS1 knock-out (3). In clear contrast to the aspartate mutations in TM6 and/or TM7, the mutations described here even caused an opposite phenotype by increasing the pathological production of A β 42/43 to an extent, which is way above that of the naturally occurring mutation. Although these data may in fact challenge Wolfe's hypothesis (8), we cannot exclude that the mutations described here may selectively affect cellular trafficking of Notch and β APP. Retention of β APP transport may promote A β 42/43 production within an early cellular compartment, such as the endoplasmic reticulum (ER) and the intermediate compartment, locations, which overlap with the expression of the majority of PS1 (51–53). However, only the production of intracellular A β 42/43 was associated with β APP processing within the ER or the intermediate compartment (38, 54). In contrast, generation of secreted A β 40 and A β 42/43 requires cell-surface transport and reinternalization of β APP (55). Therefore, a specific retention of β APP by mutant PS derivatives within the ER appears to be unlikely. A second possibility would be that the mutations inserted at amino acid 286 affect the sequence specificity of the proteolytic activity potentially exhibited by PS1. In that case, β APP is cleaved preferentially at the alternative position (after amino acid 42/43 of A β), whereas Notch could not be cleaved at the corresponding site. This also appears to be unlikely because substantial amounts of A β 40 are still produced (Fig. 2) and one would consequently expect significant NICD production. Finally, and most likely, the two mutations may affect the conformation of PS1 in a way which would specifically allow PS1 interaction with β APP CTFs but not with Notch. In that regard, one may argue that the two mutations interfere with the Notch/PS1 binding site, whereas the potentially distinct site of β APP/PS1 interaction is not affected. This may be indirectly supported by the previous finding that PS-mediated Notch endoproteolysis occurs closer to the cytoplasmic face of the membrane than does the cleavage of the β APP CTFs (19). Therefore, our data do not exclude the interesting possibility that PS proteins are identical with the γ -secretase (4, 8), but rather indicate that different domains of PS1 are required for distinct functions.

Similar to the previously identified dominant-negative mutations within TM6 and TM7 of PS1 (4, 5, 18) or PS2 (6), the mutations located in the cytoplasmic loop domain of PS1 provide tools to understand the complicated functions of presenilins in endoproteolysis of its target proteins.

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