The Large Hydrophilic Loop of Presenilin 1 Is Important for Regulating γ-Secretase Complex Assembly and Dictating the Amyloid β Peptide (Aβ) Profile without Affecting Notch Processing

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γ-Secretase is an enzyme complex that mediates both Notch signaling and β-amyloid precursor protein (APP) processing, resulting in the generation of Notch intracellular domain, APP intracellular domain, and the amyloid β peptide (Aβ), the latter playing a central role in Alzheimer disease (AD). By a hitherto undefined mechanism, the activity of γ-secretase gives rise to Aβ peptides of different lengths, where Aβ42 is considered to play a particular role in AD. In this study we have examined the role of the large hydrophilic loop (amino acids 320–374, encoded by exon 10) of presenilin 1 (PS1), the catalytic subunit of γ-secretase, for γ-secretase complex formation and activity on Notch and APP processing. Deletion of exon 10 resulted in impaired PS1 endoproteolysis, γ-secretase complex formation, and had a differential effect on Aβ-peptide production. Although the production of Aβ38, Aβ39, and Aβ40 was severely impaired, the effect on Aβ42 was affected to a lesser extent, implying that the production of the AD-related Aβ42 peptide is separate from the production of the Aβ38, Aβ39, and Aβ40 peptides. Interestingly, formation of the intracellular domains of both APP and Notch was intact, implying a differential cleavage activity between the ε/S3 and γ sites. The most C-terminal amino acids of the hydrophilic loop were important for regulating APP processing. In summary, the large hydrophilic loop of PS1 appears to differentially regulate the relative production of different Aβ peptides without affecting Notch processing, two parameters of significance when considering γ-secretase as a target for pharmaceutical intervention in AD.

Alzheimer disease (AD) is the most common form of dementia in elderly and is a multifactorial disease caused by a progressive neurodegeneration, leading to dementia and eventually to death. Neuropathological hallmarks of AD include intracellular neurofibrillary tangles composed of the hyperphosphorylated Tau protein and extracellular senile plaques, which are mainly deposits of the amyloid β peptide (Aβ) (1, 2). Aβ is generated from the amyloid precursor protein (APP) by a sequential cleavage of β-secretase and γ-secretase (3). Interfering with either β- or γ-secretase activities holds great promise as disease-modifying strategies for AD.

γ-Secretase is a member of a subset of proteases that cleaves its substrate within the membrane, a process that has been named regulated intramembrane proteolysis (RIP) (4). In contrast to other enzymes catalyzing RIP, γ-secretase is a multisubunit complex composed of four members; presenilin (PS), nicastrin, Pen-2, and Aph-1 (5–8). PS1 plays a central role in the complex as it provides the catalytic core of the complex via nicastrin, Pen-2, and Aph-1 (5–8). PS1 plays a central role in the complex as it provides the catalytic core of the complex via Asp-257 and Asp-385, located in transmembrane domains (TMD) 6 and 7, respectively (7). Apart from APP, γ-secretase is also involved in the processing of many other types of type I transmembrane proteins, most importantly the Notch receptor. Notch is an important signaling molecule in cell differentiation during development, but recent studies in adult mice using non-selective γ-secretase inhibitors have revealed its importance during adulthood as well. Perturbations of Notch signaling in adult mice affect cell homeostasis and tissue differentiation in lymphocytes, gastrointestinal tracts, pancreas, and the skin (9–12).

There are two homologues of PS in humans, PS1 and PS2, that have been characterized as 9 TMD proteins (13–15) and share an average homology of 63% and up to 95% within the TMDs (for review, see Ref. 16). The PS molecules undergo endoproteolysis upon assembly of all γ-secretase members in the ER and/or early Golgi compartment, generating the active N-terminal and C-terminal fragment (NTF and CTF) (17). The Aβ-peptide produced from γ-secretase-mediated cleavage of APP can be of various lengths, 37–43 amino acids, where the
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most abundant forms are Aβ40 and Aβ42 (18–20). The Aβ42 peptide is more prone to aggregate and believed to be the toxic form that causes synaptic and neuronal damage in the brain (21). Many studies have recently pointed out the ratio between these C-terminal variants to be of importance for the formation of plaques. For instance many familial Alzheimer disease (FAD) mutations in PS1 and 2 cause an increase in Aβ42/Aβ40 ratio either by decreasing the production of Aβ40 or by increasing the Aβ42 generation (22, 23). This increased ratio is often considered as gain of function of the γ-secretase complex. However, most of the FAD PS mutants have reduced proteolytic activity, implying that the mutations are instead loss of function mutations (22–25). Although PS1 and PS2 share a high degree of homology and can form γ-secretase complexes that are both active on APP and Notch processing, there are some domains that differ extensively between these two proteins, especially the large cytoplasmic loop between TMD6 and -7 (26). These loops are 110 amino acids in length in PS1 and 84 amino acids in length in PS2 and share only 16% homology when performing protein blast alignment (www.blast.ncbi.nlm.nih.gov). It has been shown that the PS1 loop binds to β-catenin and N-cadherin, but it has been demonstrated that the β-catenin binding domain is not essential for γ-secretase activity as a PS1 molecule lacking the loop can rescue a PS1 -/- lethal phenotype (27). Moreover, the absence of the large cytoplasmic loop does not influence the increased Aβ42/Aβ40 ratio caused by PS1 and PS2 FAD mutations (28). Recently, however, Deng et al. (29) showed that PS1Δ exon 10 knock-in mice, which lacks a big portion of the large cytoplasmic loop, had increased amyloid pathology and impaired γ-secretase activity (assessed by accumulation of APP-CTF and reduced Aβ40 formation), indicating a more pronounced functional role for this large domain. To further probe the functional role of the large cytoplasmic loop in PS1, we have investigated this region systematically in cells devoid of both PS1 and PS2.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Full-length PS1wt were cloned into the pcDNA5FRT/TO vector (Invitrogen) on BamH1/Not1 sites. The PS1Δ exon 10 construct lacks the amino acids 320–374 (PS1 numbering) and was first created by using PCR with the BGH primer and the Δ exon 10 forward primer and the T7 and Δ exon 10 reverse primers (supplemental Table S1), respectively. After a second PCR, the two fragments were linked together using the T7 and BGH primers, and the PS1Δ exon 10 molecule was cloned into the pcDNA5FRT/TO vector on BamH1/Not1 sites. PS1 NTFwt and CTFwt have been described elsewhere (30). The PS1 CTF N-terminal truncated constructs were created by in vitro mutagenesis according to the QuikChange mutagenesis protocol (Stratagene) using the following primers: CTFcasp (start 345), CTF start 355, CTF start 365, and CTF start 375 (see supplemental Table S1). The mutagenesis was performed on CTFwt mentioned above. For CTF start 375 D385A, the same primers were used as for CTF start 375, but the template was CTF D385A, which has also been described elsewhere (30). The CTF molecules were cloned into BamH1/Not1 sites in the pcDNA5FRT/TO vector (Invitrogen). Introduction of the glycosylation acceptor sites on PS1wt and PS1Δ exon 10, both in pcDNA5FRT/TO, were performed using the OptC primers (supplemental Table S1) according to the QuikChange mutagenesis protocol (Stratagene). The APPwt in pcDNA3, used for generating a stable cell line, was cloned into the previously described pENTR2B vector (31) on NotI/EcoRV sites and then transferred to the pCAG-IRES-Puro vector using Gateway cloning technology (Invitrogen). The DNA sequence of all constructs was verified using the BigDye® Terminator Version 3.1 Cycle Sequencing kit (Applied Biosystems). The reporter constructs MH100, CMV-β-gal, C99-GVP, and NotchΔE-GVP used in the luciferase-based reporter gene assay have been described previously (32, 33).

Cell Culture and Transfection—Blastocyst-derived embryonic stem cells deficient for PS1 and PS2 BD8 cells (34) were cultivated in ES medium; Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, and nonessential amino acids (Invitrogen). Flp-In BD8 cells, established elsewhere (26), stably expressing APPwt in pcDNA3.1 (BD8:APP) were generated by transfection after maintenance in media supplemented with puromycin (1 μg/ml) for 2 weeks. Clonal picking and characterization of APP expression was preformed to avoid clonal variation. A clone with high APP expression was chosen for further use in these experiments. The same clone were also used for creating cell lines stably expressing the PS1wt- or PS1Δ exon 10- pcDNA5-FRT/TO vectors, generated according to the Flp-In protocol (Invitrogen). Briefly, by cotransfecting a vector containing the gene of interest and an Flp recombination target site together with a vector encoding the FLP recombinase, this system targets the gene to a specific genomic site. After transfection, cells were selected by supplementing the medium with hygromycin (750 μg/ml) for 2 weeks. The surviving cells were expanded and further analyzed for their PS1 expression.

BD8 cells stably expressing PS1 NTFwt (BD8:NTF) have been previously generated in our laboratory (35). The cDNA constructs were transiently transfected into the BD8, BD8:APP, and BD8:NTF using the Lipofectamine2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Antibodies for Immunoblotting—Antibodies were PS1-NTF (NT-1, a gift from Dr. Paul M. Mathews, Nathan Kline Institute) recognizing the C-terminal loop region of PS1, NCT (N1660, Sigma) raised against C-terminal residues 693–709 of nicastrin, Pen-2 (3981 ProSci, Inc.) raised against residues 1–13 of Pen-2, and Aph-1al (O2C2, Covance) recognizing the residues 245–265 of Aph-1al and β-actin (Abcam).

SDS-PAGE and Western Blot—Cells were lysed 24–48 h post-transfection in cell lysis buffer (10 mM Tris, pH 8.1, 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40) supplemented with Protease Inhibitor Mixture (Roche Applied Science). Protein levels were determined by the BCA™ protein assay kit (Pierce), and equal amounts of protein in Laemmli sample buffer (Sigma) were separated using NuPAGE Bis-Tris pre-cast gradient 4–12% gel and MES buffer (Invitrogen). Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and blocked with 5% dry milk followed by incubation of primary antibody overnight at 4 °C. After applying the secondary horseradish peroxidase-linked antibodies (1:2000, GE Healthcare) for 1 h in room temperature, the blots
were developed with ImmobilonTM Western chemiluminescent horseradish peroxidase substrate (Millipore) using Amersham Biosciences HyperfilmTM ECL (GE Healthcare) or the CCD camera LAS-3000 (FUJIFILM Life Science). In the case of quantification, the respective bands were measured using ImageJ software (National Institutes of Health).

**Co-immunoprecipitation—** Co-immunoprecipitation experiments were carried out 48 h post-transfection from BD8 cells grown in 10-cm plates. Cells were harvested in phosphate-buffered saline, pelleted, and lysed by sonication in 200 μl of co-immunoprecipitation buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% CHAPSO, and protease inhibitor mixture (Roche Applied Science). The samples were kept on ice, and all incubations were performed at 4 °C. The lysate were subjected to ultracentrifugation at 100,000 × g for 20 min followed by preclearing of the supernatant by 50 μl/ml lystate with a mixture of protein A and protein G-Sepharose (Amersham Biosciences). Samples were then incubated with primary antibody diluted 1:250 overnight with end-over-end rotation. The nicastrin antibody N1660 and the Aph-1αL antibody O2C2 were used as primary antibodies or purified rabbit IgG as a negative control. The following day a mixture of protein A and protein G-Sepharose was added to the samples and incubated for an additional 1 h. Next, the precipitates were washed 3 times with co-immunoprecipitation buffer containing 0.5% CHAPSO and once in phosphate-buffered saline. Laemmli sample buffer (Sigma) was added and incubated at room temperature for 30 min before SDS-PAGE and Western blot. The membranes were then analyzed with the NT1 and 3981 antibodies.

**GCB Pulldown—** To analyze the presence of active γ-secretase complexes, BD8 cells stably expressing PS1wt or PS1Δexon 10 were subjected to GCB pulldown. GCB is composed of the γ-secretase inhibitor L685,458 and a biotin group separated by a long cleavable hydrophilic linker. The GCB pulldown, its structure, and characteristics have been described previously (36). Briefly, membrane preparations of the cells were dissolved in 0.5% CHAPSO, and protein levels were determined by the BCA™ protein assay kit (Pierce) before the GCB pulldown. The specificity of the pulldown was confirmed by the addition of excess amounts of the pulldown was confirmed by the addition of excess amounts of competing non-biotinylated L685,458. The captured molecule was analyzed from seven membranes by quantifying the intensity of the full-length (FL) and the NTF band of PS1wt and PS1Δexon 10 in the CCD camera. Next, the ratio of NTF/FL was calculated and compared with the ratio for the PS1wt.

**Luciferase Reporter Gene Assay—** BD8 cells were transfected using a total amount of 1500 ng of DNA consisting of 300 ng of PS1wt/PS1Δexon 10 or 300 ng of each NTFwt, and truncated CTF, empty pcDNA5 vector was also added to adjust the difference in DNA amounts. Furthermore, 300 ng of MH100, 200 ng of CMV-β-gal, 100 ng of green fluorescent protein, and 300 ng of C99-GVP/NotchΔE-GVP were added. Cells were lysed 36 h post-transfection and then analyzed as previously described (33). To adjust for differences in transfection efficiency, the β-galactosidase activity of the cell lysate was determined. The luciferase activity was also normalized for protein expression using a PS1-NTF antibody (NT-1). Experiments were performed in triplicate and repeated 4–5 times.

**Quantification of Secreted Aβ—** A sandwich immunoassay using the Meso Scale Discovery Sector Imager 6000 was used to quantify secreted Aβ peptides in conditioned medium. All reagents were from Meso Scale Discovery if not stated otherwise. BD8:APP was transfected with 800 ng of PS1wt/PS1Δexon 10 or 800 ng of each NTFwt and truncated CTF, empty pcDNA5 vector was added to adjust for differences in DNA amounts, and 200 ng of CMV-β-gal and 100 ng of green fluorescent protein was added. Next, cells were incubated in 240 μl of fresh E5 medium containing either 1 μM L685,458 or vehicle (DMSO) for 36 h before analysis of the conditioned medium. Multi-Array plates precoated with either Triplex C-terminal-specific anti-Aβ40/38/42 antibodies or 6E10 were blocked and washed according to manufacturer’s instructions. Media samples and Aβ peptide standards (Meso Scale Discovery and AnaSpec) as well as a ruthenylated 6E10 or C-terminal-specific anti-Aβ42 (SULFO-TAG™) detection antibodies or primary anti-Aβ40 and anti-Aβ39 antibodies (both from Astrazeneca) were added to the samples before incubation at 4 °C overnight. The next day the 6E10 precoated plates with primary anti-Aβ40 or anti-Aβ39 antibodies were subjected to a secondary ruthenylated anti-rabbit (SULFO-TAG™) antibody. For total Aβ detection, samples were incubated overnight and then subjected to ruthenylated 4G8 (SULFO-TAG™) at room temperature for 2 h before all plates were washed. All antibodies were diluted in 1% blocking buffer. For detection, Meso Scale Discovery Read buffer was added, and the light emission at 620 nm was measured after electrochemical stimulation. The corresponding concentrations of Aβ peptides in the samples were calculated using the Aβ peptide standard curves. Next, the β-galactosidase activity of the cell lysate was determined to adjust for differences in transfection efficiency. The secreted Aβ40 peptide was also normalized for protein expression using a PS1-NTF antibody (NT-1). Experiments were performed in triplicate or duplicate and repeated 4–6 times.

**Cycloheximide Treatment of Cells—** BD8 and BD8:NTF cells were transfected in 1 well of a 6-well tissue culture plate with 500 ng of CMV-β-gal and 200 ng of green fluorescent protein as well as 2000 ng of PS1wt or PS1Δexon 10 for BD8 cells or 2000 ng of each of CTFwt or CTF start 375 for BD8:NTF. 6 h post-transfection, the cells were divided to 8 wells and incubated for 36 h. Transfected cells were exposed to 50 μg/ml cycloheximide for 0, 0.5, 1, 2, 4, 8, 12, and 16 h and then lysed in 200 μl of whole cell extraction buffer (20 mM HEPES, pH 7.8, 0.42 mM NaCl, 0.5% Nonidet P-40, 25% glycerol, 0.2 mM EDTA, 1.5 mM MgCl₂, 1 mM dithiothreitol) supplemented with protease inhibitor mixture (Roche Applied Science) for 30 min at 4 °C. Protein levels

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were determined by the BCA™ protein assay kit (Pierce), and equal amounts of protein from the cell extracts from the \( t = 0 \) time point was analyzed for full-length PS1 and/or PS1 NTF expression by SDS-PAGE and immunoblotting using the NT1 antibody. \( \beta \)-Galactosidase measurements were done as described above to ensure equal transfection efficiency. After
under conditions preserving complex integrity. By using two different capture antibodies, we showed that the PS1Δexon 10 molecule interacted with nicastrin, Aph-1, and Pen-2 (Fig. 1C). In addition, by using GCB pulldown, we confirmed that an active site-directed transition state analogue inhibitor of γ-secretase binds to the PS1-NTF of the PS1Δexon 10 molecule (Fig. 1D). This demonstrates that PS1Δexon 10 can assemble into a bona fide authentic and active γ-secretase complex. However, we observed a tendency of decreased binding of PS1Δexon10 compared with PS1wt (15–70% reduction, three independent experiments) to the inhibitor (the ratio pulldown NTF/input NTF). This suggests a conformational change of the active site in the PS1Δexon 10 molecule.

Next, we wanted to study the possibility that the removal of the loop had altered the topology of the PS1 molecule. Our group has previously shown that PS1 has nine TMDs (13) where the N terminus is facing the cytosol and the C terminus is facing the lumen. To determine the topology for the PS1Δexon 10 molecule, we introduced a glycosylation acceptor site (Asn-Ser-Thr) in the C-terminal end. Glycosylation patterns for the transiently expressed constructs in BD8 cells were assessed by immunoblotting. The introduction of the glycosylation acceptor site resulted in a shift in migration for both the full-length PS1wt and PS1Δexon 10 (Fig. 1E, fourth and eighth lanes). Endoglycosidase H treatment reduced the size of the protein (Fig. 1F, fifth and ninth lanes), confirming that the shift was caused by glycosylation and indicating that the C terminus in the PS1Δexon 10 molecule had a luminal/extracellular orientation. Thus, the exon 10 deletion did not appear to induce an aberrant topology of the molecule.

The Exon 10 Coding Region Is Vital for γ-Secretase Complex Assembly, and the Complexes Formed Remain Stable—To address if the apparent lower levels of NTFwt produced from PS1Δexon 10 are due to impaired endoproteolysis or if it is a consequence of less stable γ-secretase complexes, we generated

quantifying the intensity with the CCD camera, protein levels for the different constructs were correlated to the construct showing the lowest expression, i.e., PS1wt versus PS1Δexon 10 and CTFwt versus the CTF start 375. Proteins from various time points for each construct were separated by SDS-PAGE on 4–12% Bis-Tris gels and analyzed as above. The same blots were also stained with anti-β-actin antibody to ensure for equal amounts of loaded protein. The experiment was repeated three times.

Statistics—All experiments in this paper were performed at least three times in duplicate or triplicate. The non-parametric Mann-Whitney U test by the STATISTICA 8 software from StatSoft was used for statistical analysis.

RESULTS

Metabolism and Topology of PS1 Lacking Exon 10—Although the large hydrophilic loop in PS1 has been suggested to be dispensable for γ-secretase activity (27, 28), we wanted to further explore the altered Aβ generation observed in a knock-in mouse model lacking a big portion of this loop (29). To address this we have used cells deficient for PS1 and PS2 (BD8) to nullify the influence from endogenous PS, thereby simplifying interpretation of results. We constructed a cDNA that encodes the human PS1 protein without the loop region (∆320–374), termed PS1Δexon 10 (Fig. 1A). Because the endoproteolytic site is still present in the construct (Met-292) (38, 39), we did not expect to disrupt this cleavage event. Indeed, a 30-kDa PS1Δexon 10-derived NTF, which co-migrated with NTF derived from FL PS1wt was observed when these constructs were transfected into BD8 cells (Fig. 1B). As expected, the “full-length” PS1Δexon 10 also migrated faster on the gel compared with full-length PS1wt due to the deletion of 55 amino acids in the loop (Fig. 1B).

To investigate if PS1Δexon 10 could form γ-secretase complexes, we performed co-immunoprecipitation studies

Interestingly, we noticed that lower levels of NTF were formed from PS1Δexon 10 compared with PS1wt (Fig. 1B). We, therefore, compared the endoproteolytic pattern of PS1Δexon 10 to PS1wt. By quantifying the FL and the NTF band of PS1wt and PS1Δexon 10 and calculating the ratio of NTF/FL, we demonstrated that endoproteolysis of the PS1Δexon 10 molecule was 10-fold decreased compared with PS1wt (Fig. 1E).

To address if this endoproteolytic pattern was due to a transiently overexpressed system, we also generated stable BD8 cell lines expressing PS1wt and PS1Δexon 10, respectively, and observed the same endoproteolytic pattern (data not shown). Thus, the large cytoplasmic loop seems to affect the rate of endoproteolysis of the PS1 molecule.

FIGURE 2. The exon 10 region is important for efficient γ-secretase complex formation but does not affect the stability of formed complexes. A, a schematic presentation shows the CTF construct lacking exon 10, CTF start 375, B, expression analysis is shown of lysed BD8 or BD8:NTF cells transfected with PS1wt, PS1Δexon 10, CTFwt, CTF start 375, and empty vector using a PS1-NTF antibody (NT-1). A β-actin antibody was used to ensure equal amounts of loaded protein. C and D, BD8:NTF cells transfected with CTF wt, CTF start 375, and empty vector and BD8 cells expressing PS1wt and PS1Δexon 10 were treated with cycloheximide (50 μg/ml) for seven different time points between 0 and 16 h. Lysates were analyzed for NTF expression with a PS1-NTF antibody (NT-1). A β-actin antibody was used to ensure equal amounts of loaded protein.
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A lysates from BD8 cells expressing PS1wt and PS1Δexon 10 were monitored with a luciferase reporter gene assay for AICD and Notch intracellular domain (NICD) production using transiently transfected C99-GVP/Notch3E-GVP, MH100, and CMV-β-gal constructs. Luciferase activity was normalized for protein expression (right panel) and transfection efficiency. PS1wt was set to 100% AICD formation. The right panel is a representative Western blot from four independent experiments using a PS1-NTF antibody (NT-1). (NT-1).

FIGURE 3. PS1 lacking exon 10 possesses a differential loss of function on AICD and Aβ formation. A, lysates from BD8 cells expressing PS1wt and PS1Δexon 10 were monitored with a luciferase reporter gene assay for AICD and Notch intracellular domain (NICD) production using transiently transfected C99-GVP/Notch3E-GVP, MH100, and CMV-β-gal constructs. Luciferase activity was normalized for protein expression (right panel) and transfection efficiency. PS1wt was set to 100% AICD formation. The right panel is a representative Western blot from four independent experiments, using a PS1-NTF antibody (NT-1). B, sandwich immunossay was used for detection of secreted total Aβ production using 4G8 and 6E10 antibodies. Total Aβ was set to 100% for PS1wt. C, detection is shown of secreted Aβ40/42 generation. AP40, Aβ42, and/or 6E10-specific antibodies for detection. Levels of Aβ38, Aβ39, Aβ40, and Aβ42 for PS1wt were set to 100%, respectively. # indicates that the peptide were below the detection limit regarding the standard curve but <10% of wt. D, when secreted Aβ40 was normalized for protein expression (right panel), the same production was observed as in C. The right panel is a representative Western blot from four independent experiments using a PS1-NTF antibody (NT-1). E, shown is the Aβ42/Aβ40 ratio of PS1wt and PS1Δexon 10. Error bars indicate the S.D., and statistical significance is calculated by the non-parametric Mann-Whitney U test. *, p < 0.05; **, p < 0.01; *** , p < 0.001.

and 16 h (Fig. 2C). The same was observed for the PS1Δexon 10 molecule, in which the half-time was comparable with the PS1wt (Fig. 2D). This indicates that the loop is important for complex formation, as we still observed lowered NTF levels even under conditions by-passing PS endoproteolysis. The complexes formed from PS1 molecules lacking the loop remain as stable as wild type complexes.

PS1Δexon 10 Affects Aβ Formation but Not AICD Generation—Next, we wanted to assess the functional impact of PS1Δexon 10 on AICD and Aβ production. The PS1Δexon 10 molecule was transiently expressed in BD8 cells, and γ-secretase activity was determined by monitoring AICD generation through the use of a luciferase reporter gene assay (32, 33). Expression of PS1Δexon 10 did not reduce AICD generation compared with PS1wt when normalizing for protein expression (Fig. 3A). This finding is not compatible with in vivo data from Deng et al. (29), which indirectly showed a decreased AICD formation by demonstrating an accumulation of APP-CTF in the PS1Δexon 10 knock-in mice. However, when we also monitored Notch intracellular domain production we also found an unperturbed processing with the PS1Δexon 10 molecule (Fig. 3A).

Furthermore, we investigated the influence of PS1Δexon 10 on Aβ production by transfecting PS1wt or PS1Δexon 10 cDNA into BD8 cells stably expressing APPwt (BD8-APP). Sandwich immunoassay quantification revealed that the total Aβ production was strikingly impaired in a PS1 CTF construct lacking exon 10, CTF start 375 (Fig. 2A). When co-expressed with PS1 NTFwt in BD8 cells, thus bypassing endoproteolysis, we observed lower levels of NTFwt in the presence of CTF start 375 than in the presence of CTFwt. We also observed a lower expression level of NTFwt when using BD8 cells stably expressing NTFwt, BD8:NTF (Fig. 2B). To analyze the stability of the complexes, we treated BD8:NTF cells expressing CTFwt and CTF start 375 molecules as well as BD8 cells expressing PS1Δexon 10 and PS1wt with 50 μg/ml cycloheximide for different time points. We found that NTFwt co-expressed with CTF start 375 had a comparable half-life with NTFwt co-expressed with CTFwt when monitoring between 1 cells transfected with PS1Δexon 10 compared with PS1wt (Fig. 3B). Importantly, the production of Aβ38, Aβ39, and Aβ40 was severely impaired, whereas the effect on Aβ42 generation was affected to a much lesser extent (Fig. 3C). Importantly, we observed the same production of Aβ40 when normalizing for NTF expression, indicating that the result is not due to lower levels of PS1 (Fig. 3D). Thus, the PS1Δexon 10 protein seems to affect Aβ40 generation more than Aβ42, similar to many PS1 FAD mutations (22, 23). This also resulted in a 4.4-fold increase in the Aβ42/Aβ40 ratio for PS1Δexon 10 compared with PS1wt (Fig. 3E). This suggests that the large hydrophilic loop of PS1 affects ε/S3-site and γ-site cleavage differentially.
APP Processing Is Affected When the Last 10 Amino Acids in the Loop Are Removed—To assay how γ-secretase activity is affected by the absence of the loop under conditions bypassing endoproteolysis, we examined the impact of the CTF start 375 molecules on APP processing. We have previously shown that cotransfection of PS1 CTFwt and NTFwt can interact and restore γ-secretase activity in PS null cells (30). Using a luciferase reporter gene assay, we observed that co-expression of PS1 NTFwt and CTF start 375 had the same effect on AICD formation as CTFwt and the PS1 lacking exon 10. Interestingly, the sandwich immunoassay also revealed a remarkably similar production pattern of different Aβ peptides as well as the total Aβ generation for the co-expressed NTFwt and CTF start 375 compared with the PS1Δexon 10 molecule (Fig. 4 cf. Fig. 3).

To narrow down the region in exon 10 that is responsible for proper PS1 activity, we created an array of N-terminal-truncated PS1 CTF molecules (Fig. 5A). When γ-secretase activity was assayed for these different truncated CTFs (expressed together with NTFwt), an unchanged activity at the e-site (monitored by AICD formation) was observed for all truncations when normalizing for protein expression (Fig. 5B). However, a gradual decline in activity at the γ-site was seen paralleling the extent of truncation (Fig. 5C). The most dramatic shift in both activity and cleavage pattern could be seen between expressing CTF start 365 and CTF start 375 (Fig. 5D). Strikingly, the formation of Aβ42 was relatively unperturbed between the constructs, whereas expression of CTF start 375 resulted in a drastic decrease in formation of the shorter Aβ variants (Aβ40, Aβ39, Aβ38). Taken together, these results suggest that the loop domain controls the extent of Aβ formation without affecting AICD production and that the last C-terminal 10 amino acids in the PS1 loop seem to differentially affect production of shorter Aβ variants.

**DISCUSSION**

PS is the catalytic moiety of the γ-secretase complex, which mediates both essential cell biological processes such as Notch signaling as well as cleavage of APP, resulting in production of the AD-related Aβ-peptide. Despite its critical role in normal biology and pathobiology, the enzymatic mechanism underlying γ-secretase-mediated Notch and APP processing remains in large part elusive. To gain further insight into γ-secretase mediated catalysis, we have in this study focused on PS1. In particular, we concentrated our studies on the evolutionarily non-conserved hydrophilic loop, located between TMD6 and -7 in PS1, which has been suggested to be important for
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A

B

C

D

E

F

NTFwt

CTFwt

CTFcasp

CTF start 365

CTF start 375

CTF start 355

NTFwt

pCDNA5

NTFwt

CTF wt

CTF casp

CTF start 355

CTF start 365

CTF start 375

pCDNA5

NTFwt

Aβ generation (% CTFwt)

pCDNA5

NTFwt

Aβ generation (% CTFwt)

Aβ40

Aβ42

Aβ38

Aβ39

0

0,1

0,2

0,3

0,4

0,5

0

20

40

60

80

100

120

0

20

40

60

80

100

120

0

20

40

60

80

100

120

0

20

40

60

80

100

120

0

20

40

60

80

100

120

0

20

40

60

80

100

120
γ-secretase function. Our data show that this region plays a pivotal role for γ-secretase biology. First, the loop is required for efficient γ-secretase assembly and endoproteolysis. Second, it regulates γ-secretase activity differentially between the ε/S3-site and the γ-site. Third, the loop region is important for regulating Aβ production, where its absence causes a dramatic decrease in the formation of the shorter Aβ38, Aβ39, and Aβ40 isoforms and total Aβ released. Notably, the production of Aβ42 is only partially impaired under the same conditions. It is interesting to note that this effect on Aβ production resembles the Aβ phenotype of many FAD-linked PS1 mutants (22, 23). Similar to the PS1Δexon 10 mutant, a total decrease in Aβ production in combination with a less impaired Aβ42 generation results in an increased Aβ42/Aβ1-X ratio (where X = 38, 39, or 40) for the FAD associated PS mutants. This Aβ phenotype causes an enhanced amyloidosis in FAD as well as transgenic models harboring FAD-linked PS. The FAD-like Aβ phenotype of PS1Δexon 10 also translates to increased amyloidosis, as recently demonstrated by Deng et al. (29) in transgenic mice.

The mechanism underlying FAD-linked production of different species (causing an increased Aβ42/Aβ1-X ratio) is not known. One hypothesis that has been raised is that FAD-linked mutations give rise to a partial loss of function of the γ-secretase, which in turn causes a more severe effect on shorter Aβ peptides compared with the longer Aβ42 peptide. Our results are to some extent congruent with this hypothesis, as removal of the cytoplasmic loop causes a partial reduction in Aβ42, whereas the amount of the shorter secreted Aβ38, Aβ39, and Aβ40 peptides were decreased with 80% or more. Accordingly, our data would fit a partial loss of function model, except that the PS1Δexon 10 molecule is fully active at the ε/S3-site, which is not the case for most of PS FAD mutants. This suggests that PS1Δexon 10 γ-secretase processing is initiated by unperturbed ε-cleavage but gradually results in shorter peptides, especially Aβ40 and shorter peptides. Several mechanisms could lead to a partial loss of γ-secretase processing. Our data show that the stability of the γ-secretase complex is not changed in the absence of the loop region, ruling out this mechanism as responsible for the impaired γ-secretase-mediated APP processing.

We have previously shown that it is possible to reconstitute γ-secretase activity in the absence of PS endoproteolysis by expressing PS NTF- and CTF-encoding constructs in PS null cells (30). This experimental paradigm enables a powerful system to study different domains and amino acids in PS for different aspects of PS biology. By utilizing this system we could observe that partial deletions of the loop region from the N-terminal end to only a few amino acids remaining of the C-terminal end of the loop region cause a progressive loss of both Aβ38, Aβ39, and Aβ40 as well as of Aβ42 production, which is in agreement with a gradual loss of function. Interestingly however, when removing the last C-terminal amino acids of the loop, Aβ42 production is not further impaired, whereas the production of Aβ38–40 is dramatically lowered. Thus, it appears that the C-terminal amino acids of exon 10 are more important for Aβ38–40 production than for Aβ42. These data also imply that the differential effect on Aβ42 versus shorter Aβ peptides could not solely be explained by a general loss of function, as we would then have expected a concomitant lowering in Aβ42 and AICD upon removing the most C-terminal amino acids.

We do not currently understand why this region has a differential effect on Aβ peptide production, but the primary structure may be important. This is supported by the observation that expression of full-length Δexon 10 results in the same phenotype as expression of NTFwt and CTF start 375; endoproteolysis of Δexon 10 results in a CTF with an extension of 20 N-terminal amino acids (residues 300–320; encoded by exon 9). Although the removal of Δexon 10 results in an Aβ phenotype similar to many FAD mutants, no FAD-causing mutation has so far been identified in this region. Irrespective of the relationship between Aβ42 and the shorter peptides, the present study and the data on FAD mutants suggest that Aβ42 production in particular seems to be less sensitive to genetic modifications of the presenilin gene. Indeed, in efforts identifying amino acids critical for inhibition by γ-secretase-directed small molecules, Basi and co-workers (40) made a similar observation, i.e. that many artificial mutants cause a more severe loss of shorter Aβ variants than Aβ42. Future studies are warranted to explain the differential sensitivity of PS mutations on Aβ42 production versus other shorter Aβ peptides.

Because many studies have pointed out the importance of sustaining the Notch signaling pathway in adulthood (9–12), the differences in Notch S3-site/app-ε-site and APP γ-site processing that we observe in the absence of the loop is of great interest. One reason for the different cleavage activity observed could be due to a structural change in the docking or the active site. The removal of the loop may induce an altered topology in these sites, leading to a weaker binding to the substrates. Indeed, Kornilova et al. (41) have shown that PS FAD mutations have reduced photolabeling by a transition state analogue. Moreover, Berezovska et al. (42, 43) have published two reports that show the influence of both genetic and pharmacological manipulations, which increase the Aβ42/Aβ40 ratio, to be associated with a uniform conformational change in the catalytic site of PS1. Our data using a biotin-labeled transition state analogue inhibitor of γ-secretase show a trend of less binding (15–70% reduction) of PS1Δexon 10-NTF compared with

![Figure 5](image-url)
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PS1wt-NTF when normalizing for their corresponding input NTFs. This implicates that the active site of PS1Δexon 10 has a more close conformation than the PS1 wt. It remains to be established whether the loop region is critical for the activity of γ-secretase modulators. Thus, the loop can be important for the positioning of the substrates, but the initial processing at the e/S3-site is not affected, whereas a gradual decrease occurs in the processing toward shorter peptides.

In conclusion, the data presented here suggest that the production of Aβ42 is distinct from Aβ38, Aβ39, and Aβ40 and that the integrity of the large hydrophilic loop between TMD6 and -7 of PS1 is important for proper γ-secretase complex assembly, PS1 endoproteolysis, and determination of Aβ peptide profiles. The large hydrophilic loop does not, however, affect the AICD or Notch intracellular domain production. This result is of importance, as avoiding Notch processing is a prerequisite when designing drugs that target the γ-secretase complex in the treatment of AD.

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