Signature Amyloid β Profiles Are Produced by Different γ-Secretase Complexes*

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Hermien Acx,1,2 Lúcia Chávez-Gutiérrez,2,3 Lutgarde Serneels,1,5 Sam Lismont,1,5 Manasi Benurwar,1,5 Nadav Elad,1,5 and Bart De Strooper1,5,6

From the 5Center for the Biology of Disease, Flemish Institute for Biology (VIB), 3000 Leuven, Belgium, the 6Center for Human Genetics, Leuven Institute for Neurodegenerative Disorders and University Hospitals Leuven, University of Leuven, 3000 Leuven, Belgium, and the 7University College London, Institute of Neurology, Queen Square, London WC1N 3BG, United Kingdom

Background: γ-Secretase complexes generate amyloid-β (Aβ) in Alzheimer disease.

Results: Aβ profiles of the four γ-secretase complexes expressed in humans show that PSEN regulates total peptide levels and the Aβ38 pathway, whereas APH1 affects mainly the efficiency of the carboxypeptidase-like activity.

Conclusion: γ-Secretase subunit composition regulates Aβ generation.

Significance: These intrinsic differences could be used to advance AD therapeutic development.

γ-Secretase complexes are involved in the generation of amyloid-β (Aβ) in the brain. Therefore, γ-secretase has been proposed as a potential therapeutic target in Alzheimer disease (AD). Targeting γ-secretase activity in AD requires the pharmacological dissociation of the processing of physiological relevant substrates and the generation of “toxic” Aβ. Previous reports suggest the differential targeting of γ-secretase complexes, based on their subunit composition, as a valid strategy. However, little is known about the biochemical properties of the different complexes, and key questions regarding their Aβ product profiles should be first addressed. Here, we expressed, purified, and analyzed, under the same conditions, the endopeptidase and carboxypeptidase-like activities of the four γ-secretase complexes present in humans. We find that the nature of the catalytic subunit in the complex affects both activities. Interestingly, PSEN2 complexes discriminate between the Aβ40 and Aβ38 production lines, indicating that Aβ generation in one or the other pathway can be dissociated. In contrast, the APH1 subunit mainly affects the carboxypeptidase-like activity, with APH1B complexes favoring the generation of longer Aβ peptides. In addition, we determined that expression of a single human γ-secretase complex in cell lines retains the intrinsic attributes of the protease while present in the membrane, providing validation for the in vitro studies. In conclusion, our data show that each γ-secretase complex produces a characteristic Aβ signature. The qualitative and quantitative differences between different γ-secretase complexes could be used to advance drug development in AD and other disorders.

The γ-secretase complexes are membrane-associated aspartyl proteases involved in the pathogenesis of Alzheimer disease (AD). γ-Secretase complexes consist of four essential subunits: nicastrin (NCT), PSEN (presenilin), APH1 (anterior pharynx defective 1), and PEN-2 (presenilin enhancer 2) (1–3). Two different PSEN subunits (PSEN1 and PSEN2) and two different APH1 subunits (APH1A and APH1B) are encoded by separate genes in humans, which results in four different protease complexes (see Fig. IA). Furthermore, the mRNA for the PSEN and APH1 subunits can be alternatively spliced (4), contributing to the structural diversity of the γ-secretase population.

Cleavage of the amyloid precursor protein (APP) by β-secretase results in the release of the APP ectodomain into the extracellular environment and the generation of a 99-amino acid-long APP C-terminal fragment (APP-C99) within the membrane (5). APP-C99 is then sequentially processed by the γ-secretase complexes to release C-terminal heterogeneous amyloid-β (Aβ) peptides. Aβ40 is the main product of the γ-secretase (6), but the more aggregating-prone and neurotoxic Aβ42 and Aβ43 are also generated in the brain. Aβ42 and Aβ43 enhance (seed) the formation of neurotoxic oligomers in the brain leading to neuronal dysfunction in AD patients (7, 8).

According to the current model for the function of the γ-secretase complex, APP-C99 is sequentially processed along two production lines. The first endoproteolytic (ɛ-) cleavage occurs either between residues 50 and 51 or between 49 and 50 of APP-C99 and results in the release of an APP intracellular domain fragment called AICD50–99 and the corresponding

The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β; APP, amyloid precursor protein; APP-C99, 99-amino acid-long APP C-terminal fragment; AICD, APP intracellular domain; PEN-2, presenilin enhancer 2; PSEN, presenilin; APH1, anterior pharynx defective 1; CI, confidence interval; MEF, mouse embryonic fibroblast; NCT, nicastrin.
N-terminal fragment \( A\beta_{49} \) or AICD49–99 and the \( A\beta_{48} \) pep-
tide, respectively. \( A\beta_{49}/A\beta_{48} \) are subsequently shortened by con-
secutive carboxypeptidase-like \( \gamma \)-cleavages, which progres-
sively decrease their hydrophobicity and increase the probabil-
ity of their release into the extracellular environment (see Fig. 2)
(9, 10). Whereas the efficiency of the endopeptidase cleavage of
the \( \gamma \)-secretase defines AICD and total \( A\beta \) levels, the carboxy-
peptidase-like functionality defines the type of \( A\beta \) peptides that
are generated (\( A\beta \) product profiles). The latter is strongly
affected by mutations causing AD (11, 12), indicating that
changes in the profiles of \( A\beta \) peptides affect seeding and toxic-
ity of amyloid oligomers (13–15).

In addition to APP, \( \gamma \)-secretase complexes cleave many other
type I transmembrane proteins, such as Notch1–4, N-cad-
herin, ErbB4, and neuregulin (16). These substrates are
involved in various physiological processes, implying that the
pathological and clinical significance of \( \gamma \)-secretase goes
beyond AD (17, 18). In fact, the broad substrate specificity of
the \( \gamma \)-secretase complexes likely explains the serious side
effects (mostly related to Notch toxicity) observed in clinical
trials that targeted \( A\beta \) production by general inhibition of the
\( \gamma \)-secretase complexes (19, 20). For instance, in the phase III
clinical trial with the broad spectrum inhibitor semagacestat
(20), \( A\beta \) levels were not altered in the cerebrospinal fluid, but
toxic effects related to the inhibition of the processing of Notch
were observed. This negative outcome of the clinical trial is
actually not surprising as semagacestat has a stronger IC50 for
Notch processing than for APP processing (12). Importantly
and unfortunately, this trial did not address whether lowering
\( A\beta \) production would be beneficial in AD but actually con-
ﬁrmed that general inhibition of the \( \gamma \)-secretase complexes is
not a therapeutic option in AD.

The associated toxicity with \( \gamma \)-secretase inhibition could be
circumvented if the \( \gamma \)-secretase activity required for the pro-
cessing of physiological relevant substrates and the generation
of toxic \( A\beta \) can be pharmacologically dissociated in the brain.
In this scenario, the selective targeting of the \( \gamma \)-secretase com-
plex(es) involved in the production of toxic \( A\beta \) in the brain
could be applied in the clinic.

Gene targeting studies have indeed indicated that the struc-
tural heterogeneity of \( \gamma \)-secretase is important in physiology
and pathology. Although \( Psen1 \) knock-out and \( Aph1A \) knock-
out mice die during embryogenesis due to impaired Notch sig-
naling (21–24), \( Psen2 \) null mice do not exhibit embryonic lethal
phenotypes (21, 25–27). However, \( Psen1 \) type \( \gamma \)-secretase complexes seem to produce most of the \( A\beta \) in the CNS (28),
whereas \( Psen2 \) complexes have a minor contribution (29). But,
interestingly, \( Psen2 \) containing \( \gamma \)-secretase complexes appear
to carry out an important part of the physiological processing of
Notch in peripheral organs. The differential activity profiles of
\( Psen1/2 \) complexes were actually exploited in a study con-
ducted by Borgegård et al. (30) in which \textit{in vivo} inhibition of
APP processing by a \( Psen2 \)-sparing \( \gamma \)-secretase inhibitor
(MRK-560) circumvented to a large extent Notch-related side
effects. These results demonstrate that \( Psen1 \) and \( Psen2 \)
\( \gamma \)-secretase complexes can be targeted specifically and provide
the first preclinical proof of concept that differential targeting
of \( \gamma \)-secretase complexes is a worthwhile strategy in therapy
development for AD.

Also, the heterogeneity at the \( Aph1B \) subunit of the \( \gamma \)-secre-
tase complex might provide alternative ways to develop drugs
acting specifically on some types of complexes and therefore
inhibiting or modulating their physiological substrates. Genetic
ablation of the \( Aph1B \) subunit in mice causes no major pheno-
types during development and adulthood. In addition and
importantly, it reduced significantly amyloid pathology and
restored memory in a murine AD model, while leaving unaf-
ected the processing of the Notch receptor (31). Although
more in-depth analysis revealed that the \( Aph1B \) containing
\( \gamma \)-secretase complexes are involved in the processing of neu-
regulin in the brain (32). Thus, this work evidenced that \( Aph1B \)
containing \( \gamma \)-secretase complexes play a major role in the pro-
duction of toxic \( A\beta \) (amyloid pathology) without contributing
significantly to the biology of other substrates and pointed at
the selective inhibition of \( Aph1B \) containing \( \gamma \)-secretase com-
plexes as a potential approach to tackle amyloid pathology in
the AD brain. The underlying reason for the biological or path-
ological activity of the different protease complexes is not fully
understood. Although differential expression and co-localiza-
tion of substrates and enzyme complexes at the tissue, cellular,
or subcellular level needs certainly to be considered, distinctive
intrinsic enzymatic properties may account for differential
processing of substrates by the different \( \gamma \)-secretase complexes
(33, 34). In fact, the two possibilities are non-mutually
exclusive.

In this study, we focus on the processing of APP by the dif-
ferent \( \gamma \)-secretase complexes and investigate how the structural
heterogeneity of the protease affects its endopeptidase and car-
oxypeptidase-like activities. We show that the \( Psen \) subunit
regulates mainly the endopeptidase activity levels whereas the
\( Aph1B \) subunit regulates the functionality of the carboxypepti-
dase-like activity, leading to important changes in the \( A\beta \) prod-
uct profiles. Most importantly, our data demonstrate that each
protease complex has different intrinsic biochemical properties
that result in distinctive \( A\beta \) product profiles.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Rabbit polyclonal antibodies against \( Aph1A \)
(B80.3), \( Aph1B \) (B78.2), \( PEN-2 \) (B126.2), and APP C terminus
(B63.3) and the mouse monoclonal antibody 9C3 against
nicasinr have been described (47, 48). Rabbit monoclonal neo-
epitope AICD was a gift from Eli Lilly and Co. Commercially
available antibodies were as follows: anti-\( Psen1 \) loop
(MAB5232) from Chemicon, anti-\( Psen2-CTF \) (D30G3) from
Cell Signaling, anti-\( \text{FLAG} \) M2 from Sigma, goat anti-mouse
IRDye800 from Rockland, goat anti-rabbit Alexa Fluor 680
from Invitrogen, 82E1 from Demeditec Diagnostics, biotinyl-
ated anti-mouse IgG from Vector Laboratories, and streptavi-
din-HRP from GE Healthcare. ELISA-capturing antibodies
were as follows: JRF AB038 for \( A\beta_{40} \), JRF/cAb40/28 for \( A\beta_{40} \),
and JRF/cAb42/26 for \( A\beta_{42} \) from Janssen Pharmaceutica
(Beere, Belgium). 9C4 for \( A\beta_{13} \) from Signet Labs, Inc. Detection
antibody huAB25–HRPO was obtained from Janssen Phar-
maceutica (Zhou et al. (49)). The anti-GFP nanobody used for
γ-Secretase Subunit Composition Defines Its Aβ Profile

the purification of the γ-secretase complexes was obtained from the VIB Nanobody Service Facility (35).

Purification of γ-Secretase Complexes—The human coding sequences of PSEN1 or 2, NCT, APH1A1, or B, and PEN-2 were cloned into a pAcAB4 transfer vector (BD Biosciences). Cotransfection of a transfer vector (containing the heterologous genes) and BaculoGoldTm DNA into Sf9 cells allowed homologous recombination and the production of recombinant viruses (36). The GFP was cloned at the C-terminal site of NCT. γ-Secretase complexes were purified using agarose beads (NHS-activated beads, GE Healthcare) coupled with anti-GFP nanobodies. A PreScission cleavage site was included between NCT and GFP and used to elute untagged γ-secretase complexes (see Fig. 1B). Partial removal of the PreScission protease was carried out by concentrating the sample through a 100 kDa cut-off Amicon Ultra centrifugal filter (Millipore).

Cell Culture and Generation of Stable Cell Lines—Conditional Psen1/2 double knock-out mice were crossed with conditional Aph1ABC triple knock-out mice. At embryonic day 7.5, embryos were dissected and dissociated, and cells were plated in the presence of DMEM/f12 + 50% FCS (Invitrogen). Primary mouse embryonic fibroblasts (MEFs) were immortalized by transduction with the LargeT antigen. Psen1/2 double knock-out/Aph1ABC triple knock-out MEFs were generated by transduction with a Cre-GFP expressing adenoviral vector and GFP-positive MEFs were sorted by FACS analysis. Psen1/2 Aph1ABC-deficient MEFs were maintained in DMEM/f12 + 10% FCS. To rescue γ-secretase expression, Psen1/2 double knock-out/Aph1ABC triple knock-out MEFs were transduced using pMSCV viral vectors (Clontech) containing the human coding sequences of the different PSEN and APH1 homologues and the zeocin selection marker. An IRES sequence was cloned between the coding sequences for PSEN and APH1 to ensure co-expression of both proteins. Stable transfected cell lines were selected using 500 μg/ml zeocin (Invitrogen). Four different combinations were made: PSEN1 and APH1A1, PSEN1 and APH1B, PSEN2 and APH1A1, and PSEN2 and APH1B. These cell lines were transduced with pMSCV viral vectors (Clontech) expressing APP-C99-GFP-puromycin. After puromycin selection (5 μg/ml), GFP-positive cells were selected through FACS sorting.

In Vitro Activity Assays Using Purified γ-Secretase—The γ-secretase in vitro assay was performed as described previously (37) with minor modifications. Briefly, in vitro reactions with 30 ng/μl purified γ-secretase and 1.75 μM APP-C99–3×FLAG were performed in 50 mM citric acid, pH 6.7, 0.25 mM sucrose, 1 mM EGTA, 1× EDTA-free complete proteinase inhibitors (Roche Applied Science), 2.5% dimethyl sulfoxide, and 0.1% phosphatidylcholine. Twenty μl reactions were incubated for 3 h at 37 °C. Lipids and substrates were extracted by adding 1 volume chloroform/methanol (2:1, v/v). Then, the aqueous fraction (ICD Products) was taken and subjected to SDS-PAGE and quantitative Western immunoblot. Known amounts of APP–C99–3×FLAG (0.1–0.5 pmol), were included as standards for AICD–3×FLAG quantifications. AICD–3×FLAG and standards were determined with the anti-FLAG M2 (Sigma) and goat anti-mouse IR800 (Pierce) antibodies, whereas the AICD50–99 product was determined with a neoepitope mAb and a goat anti-rabbit Alexa Fluor 680 secondary antibody (Rockland). Infrared signals were detected using the Odyssey Infrared Imaging System. Specific endopeptidase activities (pm/min) in reactions containing equal enzyme levels were calculated by dividing AICD product levels by incubation time.

Quantification of Soluble Aβ Peptides Using Sandwich ELISA—Ninety-six-well plates (Nunc) were coated with 1.5 μg/ml anti-Aβ capture antibodies, except for the anti-Aβ43 antibody that was coated at 7.5 μg/ml, all in a final volume of 50 μl of 10 mM Tris-HCl, 10 mM NaCl, 10 mM NaN3, pH 8.5. After overnight incubation at 4 °C, the plates were rinsed with PBS + 0.05% Tween 20 and blocked with 100 μl per well of casein buffer (1× phosphate-buffered saline with 1% casein, pH 7.4) for 4 h at room temperature. Standards and samples were spotted (synthetic human Aβ1–38, Aβ1–40, Aβ1–42, or Aβ1–43 peptides) were diluted in casein buffer. After overnight incubation at 4 °C, plates were rinsed and developed using 50 μl per well of 100 mM NaOAc, pH 4.9, 0.83 mM 3,3′,5,5′-tetramethylbenzidine (Sigma), 0.03% H2O2 (v/v). Reactions were stopped with 50 μl per well of 2 N H2SO4 and read on a Perkin-Elmer Life Science Envision 2103 multilabel reader at 450 nm. Aβ43 peptides in the cell-based assays were quantified by the Aβ1–43 ELISA kit from IBL, according to manufacturer’s instructions.

Urea Gels—Aβ peptides were analyzed by a modified version of the urea-based SDS-PAGE (11% T/5% C instead of 12% T/5% C polyacrylamide and 0.075 M instead of 0.1 M H2SO4 in the separation gel) (38). Western immunoblot was performed using 82E1 antibody, biotinylated anti-mouse IgG, and streptavidin-HRP. Signals were detected using ECL chemiluminescence with a Fujifilm LAS-3000 Imager.

Statistics—In vitro experiments were repeated 12 times. Cell-based experiments were repeated four times. Statistical significance of the data was tested with one-way analysis of variance and Dunnett’s post test. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

RESULTS

Reconstitution and Purification of γ-Secretase Complexes—The baculovirus expression system was used to produce independently the following γ-secretase complexes: NCT-PSEN1-APH1A1-PEN-2, NCT-PSEN1-APH1B-PEN-2, NCT-PSEN2-APH1A1-PEN-2, and NCT-PSEN2-APH1B-PEN-2. To express the different complexes, we generated four recombinant baculoviruses, each of them expressing the four essential components of the γ-secretase complexes mentioned above. Thus, infection of insect cells with any of these recombinant baculoviruses ensures co-expression of the four essential components of the respective γ-secretase complex in every infected cell, facilitating the reconstitution of a functional enzyme. For purification purposes, the NCT subunit was expressed as a fusion protein with the GFP separated by a PreScission protease cleavage site. Hi5 insect cells infected with each of the recombinant baculoviruses were collected at 72 h post infection and crude cellular extracts were prepared in 1% CHAPSO. After removal of insoluble material by ultracentrifugation, immunoaffinity purification of the respective γ-secretase complexes was performed using a high affinity anti-GFP nanobody coupled to agarose beads. GFP was then cleaved from NCT by specific proteolysis to elute untagged γ-secretase (Fig. 1B) (39). All of the
γ-secretase subunits (NCT, APH1, PSEN-CTF, PSEN-NTF, and PEN-2) were present in the purified samples, and purity of the different γ-secretase complexes was high as evaluated by SDS-PAGE followed by Coomassie staining (Fig. 1C). Because NCT and PEN-2 are the only common subunits among the different complexes and we purified through a GFP tag linked to the NCT subunit, we used the intensity of the PEN-2 immunostaining (Fig. 1D) instead of total protein to normalize for γ-secretase complex levels among different samples and purifications. We made all our estimations relative to the NCT-PSEN1·APH1A·PEN-2 complex, which was considered as the reference γ-secretase complex in this study.

**PSEN2 Containing γ-Secretase Complexes Display Lower Endopeptidase Activities Than the Corresponding PSEN1 Complexes**—To investigate the functional relevance of the subunit composition of the γ-secretase complex, we characterized the intrinsic kinetic properties of the four different γ-secretase complexes. First, we evaluated their endopeptidase activity by measuring the de novo generation of AICD in an in vitro γ-secretase activity assay (12, 37), in which equivalent amounts of purified enzymes were tested against purified APP-C99-FLAG substrate at saturating concentrations. AICD product was quantified by SDS-PAGE and Western blotting against the FLAG tag. Addition of 10 μM γ-secretase inhibitor X

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**FIGURE 1.** Purification of γ-secretase complexes. A, schematic representation of the subunit composition and structural heterogeneity of the γ-secretase complex. A color code, indicated on top, is applied to the PSEN and APH1 subunits to show local homology between PSEN1 and PSEN2 or APH1A and APH1B subunits, respectively (based on ClustalW using Blosum matrix). PSEN1 and PSEN2 show 65% homology, whereas APH1A and APH1B show 56% homology at the amino acid level. Stars on presenilins TMD6 and TMD7 denote catalytic aspartate residues Asp-257 and Asp-385, respectively (46). Stars on the ectodomain of NCT indicate complex glycosylation. B, schematic representation of the purification methodology. Baculovirus expressed γ-secretase complex is purified using the GFP tag linked to the cytoplasmic domain of NCT. The complex is eluted with Prescission protease. C, Coomassie staining of purified γ-secretase complexes. Next to the γ-secretase subunits, the Prescission protease remains present in all purified samples. An unidentified band is also visible in all purifications (asterisk). D, Western blot of purified γ-secretase complexes. PEN-2 subunit immunoreactivity was used to estimate and normalize γ-secretase complex levels used in further in vitro activity assays.
γ-Secretase Subunit Composition Defines Its Aβ Profile

(L-685,458; InhX, Merck) blocked the production of AICD demonstrating the specificity of the proteolytic assay (see Fig. 3A). APH1A containing γ-secretase complexes produce similar AICD levels compared with the corresponding APH1B containing complexes. In contrast, the endopeptidase activity was decreased by ~35% in PSEN2 containing γ-secretase complexes (see Fig. 3B). Furthermore, we asked whether the subunit composition of the γ-secretase has an effect on the position of the (e-) endopeptidase cleavage and therefore on the product line preference of the enzyme (Fig. 2). To address this question, we used an antibody that recognizes specifically the AICD50 –99/total AICD ratio showed that all γ-secretase complexes cleave the APP-C99 substrate in a similar way with regard to the position of the endoproteolytic cleavage (Fig. 3C).

Subunit Composition of the γ-Secretase Complex Affects the Functionality of the Carboxypeptidase-like Activity—To evaluate the carboxypeptidase-like activity of the different γ-secretase complexes, we quantified Aβ39, Aβ40, Aβ42, and Aβ43 by ELISA. These Aβ peptides represent substrates (Aβ43 and Aβ42) and products (Aβ40 and Aβ38) of the fourth enzymatic turnover in each of the product lines of the APP-C99 processing by the γ-secretase (Fig. 2). Because the endopeptidase activities of the γ-secretase complexes differ, Aβ peptides were normalized relative to total AICD, which reflects total Aβ substrate (Aβ49 + Aβ48) available for the carboxypeptidase-like activity. Clearly, the four γ-secretase complexes appear to generate distinct Aβ product profiles (Fig. 4, A and C). For instance, the production of the short Aβ38 and Aβ40 peptides are decreased in the APH1B-containing complexes, compared with the corresponding APH1A complexes (Fig. 4A and Table 1 for statistical significance).

We took the sum of Aβ39, Aβ40, Aβ42, and Aβ43 peptides (third + fourth Aβ products) as a measure for the overall functionality of the carboxypeptidase-like activity of the different complexes (i.e. how efficient the enzymes are in hydrolyzing the long Aβ18 and Aβ49 peptides initially generated upon endoproteolysis). The significant reductions observed in these Aβ products for the PSEN1-APH1B and the two PSEN2 containing γ-secretase complexes, when compared with PSEN1-APH1A (Fig. 4B), implies the concomitant accumulation of longer Aβ precursors (Aβ43). This was confirmed by analyzing total Aβ products in urea based gels (Fig. 4C). Clearly, the PSEN1-APH1B complex (Fig. 4C, lane 3) or PSEN2 containing γ-secretase complexes (Fig. 4C, lanes 4 and 5) maintain proportionally longer Aβ peptide species than the reference complex (PSEN1-APH1A) (Fig. 4C, lane 2).

APH1B Containing γ-Secretase Complexes Are Less Functional Carboxypeptidases Than Their APH1A Counterparts—The Aβ39/Aβ42 and Aβ40/Aβ43 product/substrate ratios provide information on the efficiency of the fourth enzymatic turnover. These ratios reveal that in the PSEN1-APH1B complex the fourth turnover in both product lines is decreased by ~70%, relative to the reference complex (Fig. 5). Similarly, the PSEN2-APH1B complex lowers the Aβ40/Aβ43 ratio by ~50% relative to the PSEN2-APH1A complex (Fig. 5, gray bars). These decrements indicate that the APH1 subunit is involved in the mechanism(s) that regulate(s) the efficiency of the fourth catalytic turnover of the γ-secretase complex and likely the functionality of the carboxypeptidase.
PSEN2 Subunit Affects Differentially the Aβ/H925240 and Aβ/H925238 Production Pathways—Aβ/H925240/Aβ/H925243 ratios indicate that PSEN1/APH1A versus PSEN2/APH1A and PSEN1/APH1B versus PSEN2/APH1B complexes are equally efficient at the processing of Aβ/H925243 to Aβ/H925240 peptide (Fig. 5, gray bars). However, the Aβ/H925238/Aβ/H925242 ratios suggest that the processing of Aβ/H925242 by the PSEN2-catalytic subunit occurs in a less efficient manner than in the PSEN1 active site (Fig. 5, white bars). In addition, lower Aβ/H925242 levels are observed in the PSEN2 reactions, relative to the corresponding PSEN1 assays (Fig. 4A and Table 1). Because Aβ peptides are normalized toward total AICD (Aβ/H925249/Aβ/H11001Aβ/H925248) produced in each reaction, lower Aβ/H925242 levels likely result from lower turnover of the Aβ precursors (Aβ/H925245 and/or Aβ/H925248) in the PSEN2 (relative to PSEN1) reactions. In agreement, Aβ profiles in urea-based gels show higher levels of the longer Aβ peptides in the reactions for PSEN2 complexes than in those for PSEN1, when the APH1 subunit is constant (i.e. PSEN1-APH1A versus PSEN2-APH1A and PSEN1-APH1B versus PSEN2-APH1B)

TABLE 1

p values for pairwise comparisons of the Aβ product profiles in Fig. 4A

|          | Aβ/H925238 | Aβ/H925240 | Aβ/H925242 | Aβ/H925243 |
|----------|------------|------------|------------|------------|
| 1A vs. 1B | <0.0001    | <0.0001    | <0.0001    | 0.3296     |
| 1A vs. 2A | <0.0001    | 0.0534     | <0.0001    | 0.6509     |
| 1B vs. 2B | ND         | <0.0001    | 0.0019     | 0.1292     |
| 2A vs. 2B | ND         | <0.0001    | 0.9801     | 0.3087     |

FIGURE 4. γ-Secretase complexes generate distinctive Aβ product profiles. A, Aβ products were measured using ELISA techniques and normalized to initial AICD generation. Results are plotted as % of the Aβ products generated by the PSEN1/APH1A γ-secretase complex (mean ± 95% CI, n = 12). Aβ/H925240 peptides were undetectable in reactions with the PSEN2/APH1B complex (ND). B, the sum of Aβ/H925238, Aβ/H925240, and Aβ/H925242 was plotted as % of the PSEN1/APH1A complex (mean ± 95% CI, n = 12). Lower total Aβ levels suggest the accumulation of longer Aβ (>43) peptides. C, Aβ profiles analyzed in urea-based gels corroborate the accumulation of longer Aβ peptides by these complexes. Aβ/H925242 and Aβ/H925243 are running as one band, the asterisk denotes an aspecific band (between Aβ/H925240 and Aβ/H925238).

FIGURE 5. The APH1 subunit affects the fourth cleavage in vitro. Aβ product/substrate ratios for the fourth turnover were plotted as % of the ratio generated by the PSEN1/APH1A complex (mean ± 95% CI, n = 12). Aβ/H925238/Aβ/H925242 ratios could not be calculated for the PSEN2/APH1B complex because the levels of Aβ/H925238 were undetectable (ND). APH1B containing γ-secretase complexes lower the efficiency of the fourth cycle of γ-secretase activity, when compared with the corresponding APH1A containing complexes.

(Fig. 4C) (band refer as > Aβ/H925246, may contain peptides longer than 46 amino acids). Thus, although all the different complexes do not change the product line preference (position of
We calculated the product/substrate ratios ($\text{A}_\beta_{38}/\text{A}_\beta_{43}$ and $\text{A}_\beta_{38}/\text{A}_\beta_{42}$) for the fourth enzymatic turnover of the $\gamma$-secretase complex. With regard to the APH1 subunit, the $\text{A}_\beta_{40}/\text{A}_\beta_{43}$ and $\text{A}_\beta_{40}/\text{A}_\beta_{42}$ ratios indicate that PSEN1-APH1B complexes are less functional than their PSEN1-APH1A counterparts in the cell (Fig. 6C), which supports the observations in vitro. Additionally, the $\text{A}_\beta_{40}/\text{A}_\beta_{43}$ ratio for the PSEN2-APH1B complex evidences a less functional turnover of $\text{A}_\beta_{43}$ with respect to the PSEN2-APH1A complex, but this difference does not reach statistical significance on the other product line ($\text{A}_\beta_{38}/\text{A}_\beta_{42}$ ratio). However, PSEN2 complexes show an increased turnover of $\text{A}_\beta_{43}$ but decrease the conversion of $\text{A}_\beta_{42}$ into $\text{A}_\beta_{38}$ with respect to their PSEN1 counterparts. Thus, as expected from our in vitro observations, the effect of the PSEN2 subunit on the processing of APP depends on the product line (Fig. 6C).

**DISCUSSION**

We demonstrate that the structural heterogeneity of the $\gamma$-secretase has an effect on the intrinsic enzymatic properties of the complex by analyzing the processing of APP-C99 towards $\text{A}_\beta$ peptides. Using purified $\gamma$-secretase complexes expressed in insect cells (Fig. 1, B–D), we found that the endopeptidase activity levels are regulated by the nature of the catalytic subunit of the complex. The PSEN2 type complexes appear to be the less active endopeptidases (Fig. 3B). These results expand previous observations that show lower activity levels in samples containing PSEN2 type $\gamma$-secretase complexes, relative to those with PSEN1 as catalytic subunit (solu-
bilized microsomal membranes from MEFs, blastocyst derived cell cultures or mouse brain lyastes) (12, 33, 34, 40). Our results differ from data published by Yonemura et al. in 2011 (42), who used a yeast reconstitution system to express the different γ-secretase complexes (41). They found that solubilized microsomes prepared from cells expressing the PSEN1-APH1A γ-secretase complex produced ~24-fold more Aβ than those containing PSEN2-APH1A complexes (42), but γ-secretase levels were ~28-fold higher in microsomes containing PSEN1 than in those with PSEN2. Thus, apparent specific activities for the PSEN1-APH1A and PSEN2-APH1A γ-secretase complexes in their experiments seemed similar. However, the use of microsomes complicates considerably the interpretation of the data. Questions such as to what extent substrate and enzyme interact or to what extent the interaction occurs under similar conditions in the different preparations are critical issues that cannot be controlled nor addressed under these conditions. Our approach using purified enzymes and purified substrate allows control of critical variables such as substrate and enzyme concentrations or lipids present in the assay. Our work further differentiates from previous publications because we evaluated the specificity of the PSEN1 and PSEN2 endopeptidase cleavage by quantifying the position of the initial (ε-) cleavage in the APP sequence. Our results show that PSEN2 and PSEN1 complexes differ at their endopeptidase activity levels but display similar endopeptidase cleavage specificities against the APP-C99 subsequence. Our results show that PSEN2 and PSEN1 complexes interact or to what extent the interaction occurs under similar conditions in the different preparations are critical issues that cannot be controlled nor addressed under these conditions. Our approach using purified enzymes and purified substrate allows control of critical variables such as substrate and enzyme concentrations or lipids present in the assay. Our work further differentiates from previous publications because we evaluated the specificity of the PSEN1 and PSEN2 endopeptidase cleavage by quantifying the position of the initial (ε-) cleavage in the APP sequence. Our results show that PSEN2 and PSEN1 complexes differ at their endopeptidase activity levels but display similar endopeptidase cleavage specificities against the APP-C99 substrate (Fig. 3C), which results in lower Aβ40 and Aβ42 product levels in the PSEN2 reactions, but similar Aβ40/Aβ42 ratios, compared with PSEN1 reactions.

We investigated the effect of subunit composition on the functionality of the carboxypeptidase-like activity of the γ-secretase complex. Importantly, Aβ peptides were normalized relative to total AICD produced in the assay, i.e. for the initial endopeptidase activity of the complexes (Fig. 4A and Table 1). PSEN2 type carboxypeptidases appeared to be less functional than the PSEN1 counterparts, as indicated by the Aβ38, Aβ40, Aβ42, and Aβ43 product levels (Fig. 4B) and the accumulation of longer and more hydrophobic Aβ peptides in the reactions. Aβ45 is relatively increased in the PSEN2/APH1B profile compared with the PSEN1/APH1B (Fig. 4C, lane 5 versus 3); whereas the >Aβ46 band suggests a higher relative abundance of longer Aβ peptides in the PSEN2/APH1A profile versus PSEN1/APH1A (Fig. 4C, lane 4 versus 2). However, and interestingly, the Aβ product profiles show that although Aβ38 and Aβ42 levels are decreased in PSEN2 versus corresponding PSEN1 reactions, Aβ40 and Aβ43 products are produced to similar levels (Fig. 4A). This results in decreased Aβ38/Aβ42 but similar Aβ40/Aβ42 ratios in PSEN2 versus PSEN1 counterpart reactions (PSEN2-APH1A versus PSEN1-APH1A and PSEN2-APH1B versus PSEN1-APH1B) (Fig. 5). Our results thus indicate that PSEN2-containing complexes affect differentially the Aβ product lines, being less functional at the Aβ38 product line. Furthermore, the fact that PSEN2 type complexes produce lower Aβ42 than the corresponding PSEN1 complexes suggests that the product line is actually affected at the second and/or third enzymatic cycles of the Aβ38 product line (Aβ40 to Aβ45 and/or Aβ45 to Aβ43). Recently, Okochi et al. (43) reported that 40% of Aβ38 is actually derived from Aβ43 in HEK cells. We cannot truly evaluate the extent to which this observation influences our results, as it is unknown what type of γ-secretase complexes are present in HEK cells and the proportion in which they are expressed. In addition, it is unclear whether this observation applies to all the different complexes. In case this observation is valid for all, then the Aβ38/Aβ42 ratio actually underestimates the magnitude of the effects.

Taken together, the nature of the catalytic subunit in the γ-secretase complex is determinant for both endo- and carboxypeptidase-like activities, but surprisingly, it mainly affects the carboxypeptidase functionality in one production line. The mechanism(s) of this observation needs further functional and structural understanding of the γ-secretase complex, probably requiring a deep comparison of PSEN1 versus PSEN2 complexes once the structure of the γ-secretase complex is available at atomic resolution.

In contrast, structural heterogeneity at the APH1 subunit affects mainly the carboxypeptidase-like activity of the complex (Fig. 4A). Both APH1A and APH1B type γ-secretase complexes display similar endopeptidase activities (Fig. 3B). However, APH1B containing complexes are less efficient carboxypeptidases compared with the corresponding APH1A complexes (Figs. 4 and 5). This reinforces the idea that endo- and carboxypeptidase-like activities of γ-secretase are uncoupled (12, 44) and also confirms our previous work (31), demonstrating that APH1B complexes generate longer Aβ peptides. Interestingly, the impact of APH1B on the fourth enzymatic turnover of the γ-secretase results in relative more Aβ42 or Aβ43 (Figs. 4 and 5) than in the APH1A product profiles; an effect that has also been observed with γ-secretase complexes containing familial AD-linked PSEN mutants. We have previously suggested that this could reflect a “premature” release of intermediary Aβ products, i.e. a less efficient retaining of longer Aβ by the γ-secretase complex containing the APH1B subunit similar to what is seen with familial AD-linked PSEN mutants (12). These effects (APH1A versus APH1B complexes or WT versus familial AD-linked complexes) correlate with a change in the conformation of the γ-secretase complexes as indirectly measured by fluorescence lifetime imaging microscopy technology (31, 45). Taken together, this suggests that the APH1 subunit stabilizes PSEN in particular conformations, referred to as open/closed (31), and the conformational status of PSEN has an effect on the efficiency of the carboxypeptidase-like activity. Interestingly, these data indicate that APH1 may act as an allosteric subunit that activates/inhibits the production of short Aβ peptides but does not regulate the generation of APP intracellular domain (endopeptidase activity). Theoretically, this opens the possibility that molecules that bind to APH1 could affect Aβ generation.

Finally, the results obtained in the in vitro enzymatic characterization are well supported by the results obtained in cell lines expressing only one γ-secretase complex. Thus, APH1B complexes present a lower Aβ40/Aβ42 ratio than their APH1A counterparts and PSEN2 affects considerably the efficiency of the carboxypeptidase-like activity in the Aβ38 production line compared with PSEN1 (Fig. 6C). We conclude that the functionality and product line preference of the carboxypeptidase-like activity are intrinsic attributes of the enzyme complexes.
and that those properties are well maintained in solubilized conditions, something that was also observed when characterizing the familial AD linked PSEN mutations in vitro and in cell culture (12).

In conclusion, this is the first time that both endo- and carboxypeptidase-like activities of human γ-secretase complexes are compared under the same conditions. Our results show that the nature of the PSEN catalytic subunit present in the complex affects both γ-secretase activities. Interestingly, PSEN2 complexes discriminate between the Aβ40 and Aβ38 production lines, indicating that Aβ40 and Aβ38 generation can be dissociated at the level of the carboxypeptidase-like activity. In contrast, the APH1 subunit mainly affects the carboxypeptidase-like activity, with APH1B complexes favoring the generation of longer Aβ peptides. Clearly, each γ-secretase complex produces a characteristic Aβ signature. Such differences could be used to advance AD therapeutic development.

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