Lipids for Protease Activity in independent Trafficking Functions and a Critical Role of Apart from its catalytic function, the γ-secretase appears to be dependent on functional assembly. We have simultaneously overexpressed all of these polypeptides, and we demonstrate functional assembly of the enzyme complex, a substantial increase in enzyme activity, and binding of all components to a transition state analogue γ-secretase inhibitor. Co-localization of all components can be observed in the Golgi compartment, and further trafficking of the individual constituents seems to be dependent on functional assembly. Apart from its catalytic function, γ-secretase appears to play a role in the trafficking of the β-amloid precursor protein, which was changed upon reconstitution of the enzyme but unaffected by pharmacological inhibition. Because the relative molecular mass and stoichiometry of the active enzyme complex remain elusive, we performed size exclusion chromatography of solubilized γ-secretase, which yielded evidence of a tetrameric form of the complex, yet almost completely abolished enzyme activity. γ-Secretase activity was reconstituted upon addition of an independent size exclusion chromatography fraction of lower molecular mass and nonproteinaceous nature, which could be replaced by a brain lipid extract. The same treatment was able to restore enzyme activity after immunoaffinity purification of the γ-secretase complex, demonstrating that lipids play a key role in preserving the catalytic activity of this protease. Furthermore, these data show that it is important to discriminate between intact, inactive γ-secretase complexes and the active form of the enzyme, indicating the care that must be taken in the study of γ-secretase.

γ-Secretase has been characterized as an unconventional aspartyl protease that processes type I membrane proteins after removal of their ectodomains by cleavage within their transmembrane domains. This enzyme has created great interest because it performs the final proteolytic cleavage step in the processing cascade of the β-amloid precursor protein (βAPP). γ-Secretase cleavage leads to the production of amyloid-β (Aβ) peptides, which are generally believed to be the causative agents for Alzheimer’s disease (AD). Because of its contribution to Aβ peptide production in the first instance and therefore to toxic events subsequently initiated by Aβ, inhibition of γ-secretase is also seen as a logical approach for the development of a disease-modifying treatment for this neurodegenerative condition. Extracellular clipping of βAPP by the aspartyl protease β-secretase (BACE1) (1–5) is a prerequisite for Aβ peptide generation. It generates the β-C-terminal fragment (β-CTF, C99) that serves as a direct γ-secretase substrate. Alternatively, βAPP can be metabolized by cleavage within the Aβ domain, which is mediated by members of the disintegrin and metalloprotease family TACE (6) and ADAM-10 (7) and represents the predominant pathway under normal physiological conditions. This precludes Aβ peptide production, and the remaining membrane-bound fragment (α-CTF, C83) is cleaved by γ-secretase to release a small peptide termed p3.

Overwhelming evidence for a key role of the two highly homologous presenilins (PS) 1 and 2 in γ-secretase enzyme function has evolved from their original identification as the main gene loci for early-onset, familial AD (8–11). PS are polytopic membrane proteins that undergo endoproteolysis within their cytosolic loop region. The endoproteolytic processing yields N-terminal and C-terminal polypeptides (PS-NTF and PS-CTF) (12), which are thought to consist of six and two transmembrane domains, respectively (15). Although not proven entirely to date, it is generally believed that PS endoproteolysis represents an activation step (14, 15) similar to the conversion of a proenzyme into an active enzyme. Apart from the finding that not only PS expression (16) but also the presence of two putative catalytic aspartates in transmembrane domains 6 and 7 (D257 and D385) (12), which are thought to consist of six and two transmembrane domains, respectively (15). Although not proven entirely to date, it is generally believed that PS endoproteolysis represents an activation step (14, 15) similar to the conversion of a proenzyme into an active enzyme. Apart from the finding that not only PS expression (16) but also the presence of two putative catalytic aspartates in transmembrane domains 6 and 7 (D257 and D385) (12), which are thought to consist of six and two transmembrane domains, respectively (15). Although not proven entirely to date, it is generally believed that PS endoproteolysis represents an activation step (14, 15) similar to the conversion of a proenzyme into an active enzyme. Apart from the finding that not only PS expression (16) but also the presence of two putative catalytic aspartates in transmembrane domains 6 and 7 (D257 and D385) (12), which are thought to consist of six and two transmembrane domains, respectively (15). Although not proven entirely to date, it is generally believed that PS endoproteolysis represents an activation step (14, 15) similar to the conversion of a proenzyme into an active enzyme. Apart from the finding that not only PS expression (16) but also the presence of two putative catalytic aspartates in transmembrane domains 6 and 7 (D257 and D385) (12), which are thought to consist of six and two transmembrane domains, respectively (15). Although not proven entirely to date, it is generally believed that PS endoproteolysis represents an activation step (14, 15) similar to the conversion of a proenzyme into an active enzyme. Apart from the finding that not only PS expression (16) but also the presence of two putative catalytic aspartates in transmembrane domains 6 and 7 (D257 and D385) (12), which are thought to consist of six and two transmembrane domains, respectively (15). Although not proven entirely to date, it is generally believed that PS endoproteolysis represents an activation step (14, 15) similar to the conversion of a proenzyme into an active enzyme. Apart from the finding that not only PS expression (16) but also the presence of two putative catalytic aspartates in transmembrane domains 6 and 7 (D257 and D385) (12), which are thought to consist of six and two transmembrane domains, respectively (15). Although not proven entirely to date, it is generally believed that PS endoproteolysis represents an activation step (14, 15) similar to the conversion of a proenzyme into an active enzyme. Apart from the finding that not only PS expression (16) but also the presence of two putative catalytic aspartates in transmembrane domains 6 and 7 (D257 and D385) (12), which are thought to consist of six and two transmembrane domains, respectively (15). Although not proven entirely to date, it is generally believed that PS endoproteolysis represents an activation step (14, 15) similar to the conversion of a proenzyme into an active enzyme.

APP can be metabolized by cleavage within the Aβ domain, which is mediated by members of the disintegrin and metalloprotease family TACE (6) and ADAM-10 (7) and represents the predominant pathway under normal physiological conditions. This precludes Aβ peptide production, and the remaining membrane-bound fragment (α-CTF, C83) is cleaved by γ-secretase to release a small peptide termed p3.
Co-expression of these polypeptides can reconstitute γ-secretase in yeast (23) (which does not express endogenous enzyme) or eukaryotic cell lines (24, 25) as measured by the increase of cell-free enzyme activity. The requirement for the assembly of a complex consisting of at least four membrane proteins demonstrates the intricacy of the enzyme. It is not implausible, however, that other co-factors may be constituents of γ-secretase and modulate its catalytic function.

The exact stoichiometry of the active complex is also elusive because not only have PS complexes of various molecular weights been described (24–28) but also γ-secretase enzyme activity fractionates at varying sizes depending on the source of enzyme (29, 30). More recent evidence suggests a PS dimer at the core of the enzyme complex, with substrates being cleaved at the interface between the two PS molecules (31).

During the formation of the enzyme complex, its known components must associate and undergo a maturation process to yield the functionally active complex. Several groups have detected the formation of subcomplexes of Aph-1 and nicastrin during complex formation (32, 33). Nicastrin appears to undergo a conformational change (34), and it has also been demonstrated that Pen-2 interacts with the PS1 holoprotein in the absence of Aph-1 and nicastrin (76) and is able to stabilize the PS1-NTF/CTF heterodimer (77).

Apart from these findings, down-regulation or genetic inactivation of one of the components of this complex directly affects the maturation and/or stability of the other interacting factors (22, 35–37). It therefore appears that the association and maturation of these cofactors, along with the activation of the mature complex, possibly through PS endoproteolysis is an ordered and highly regulated process.

To study this enzyme further, we have generated a novel cell line referred to as γ(NRC-F8)-secretase cell line, by sequentially co-expressing the substrate precursor βAPP751 and the complex components PS1, nicastrin, Aph-1αL, and Pen-2. This cell line displays the characteristics of a γ-secretase overexpressing in vitro system, manifested by changes in nicastrin maturation and PS1 endoproteolytic processing, an ~14-fold increase in both cell-free γ(40)- and γ(42)-enzyme activities, and isolation of all components with the active site-directed (38) biotinylated transition state inhibitor Merck C (39). By demonstrating a successful and functional overexpression of the enzyme, we utilized a direct γ-secretase substrate to investigate the potential cause of the discrepancies that have been observed with respect to cell-free and cellular β peptide production in similar studies (24, 25, 40). Confocal immunofluorescence microscopy provided insights into the regulation of enzyme trafficking to the cell surface and implied a protease-independent role of the functional γ-secretase complex in the regulation of the maturation and trafficking of βAPP. Size exclusion chromatography (SEC) and immunoaffinity isolation have been employed to provide evidence for a tetrameric enzyme species, highlight several key considerations in the purification of γ-secretase, and most significantly to demonstrate that lipids are critical for preservation of the active conformation of the enzyme complex.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal and polyclonal antibodies were obtained from the following sources and diluted for Western blot analyses as indicated: anti-β-catenin (monoclonal mouse, BD Biosciences, 1:1000), anti-FLAG (monoclonal mouse, Sigma, 1:5000), anti-V5 (monoclonal mouse, Invitrogen, 1:1000), anti-nicastrin (rabbit polyclonal, Sigma, 1:10,000), anti-α-tubulin (monoclonal mouse, Sigma, 1:5000), and horseradish peroxidase-conjugated polyclonal goat anti-mouse and anti-rabbit (F(ab′)2) fragments (Amersham Biosciences, 1:5000). PS1-FL and its fragments were detected by using rabbit polyclonal antiserum 002/2 raised against the loop peptide 301–317 (41) (1:2000) and polyclonal antiserum 98/1 raised against residues 1–20 of PS1 (1:2500) (42). βAPP was detected by using polyclonal rabbit antiserum R7354 raised against residues 659–694 of βAPP (28). Antibodies for immunocytochemistry were diluted as follows: anti-FLAG (Sigma, 1:2000); anti-V5 (Invitrogen, 1:200); anti-nicastrin (Sigma, 1:1000); anti-Golgi 58K protein clone 58K-9 (Sigma, 1:500); anti-Pan-cadherin (CH-19, Abcam, 1:200); and anti-PS1 (98/1, 1:500), fluorescein isothiocyanate-α-, Cy3-, and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch, 1:100); βAPP was detected using antibodies either directed against the ectodomain, monoclonal antibody 6E10 (residues 61–81, Chemicon, 1:1000) and a polyclonal antibody against residues 1–100 (mAbP2–1, Affinity Bioreagents, 1:500), or polyclonal antibodies against the C terminus, residues 751–770 of βAPP (Calbiochem, 1:1000) and residues around Y668 βAPP (Cell Signaling, 1:500).

Complementary DNA Constructs—Expression vectors encoding nicastrin and Aph-1αL with a C-terminal V5 and 500 μM hexahistidine epitope tag were generated by assembling IMAGE clones (43) encoding the human sequences. Subcloning in the respective pcDNA vectors (Invitrogen) yielded the nicastrin-pcDNA3.1/Hygro (+) and Aph-1αL-pcDNA3.1/V5-His/Neo (+) constructs that were confirmed by DNA sequencing. The generation of Pen-2-pcDNA3.1/FLAG/Neo (+) vector has been described (44).

Generation of Stable Cell Lines—HEK cell lines stably overexpressing βAPP751 alone or in combination with human PS1 have been described previously (38). For sequential introduction of additional γ-secretase complex components, the βAPP751/PS1 cell line (clone B9) was transfected with nicastrin-pcDNA3.1/Hygro (+) using standard calcium phosphate methods. Transfectants were cultured in the presence of 1 μg/ml puromycin (Sigma) and 100 μg/ml Zeocin (Invitrogen) to monitor βAPP and PS1 expression, respectively, and penicillin G (Roche Applied Science) by trituration. After incubation for 30 min at 4 °C. Insoluble debris was removed by centrifugation for 10 min at 4 °C. Cell lysis was performed by Western blotting. Clone PS1, referred to as the γ(NRC-F8)-secretase cell line, was chosen for expansion because it showed the strongest expression of all exogenous polypeptides.

Transient Transfections and Solubilization of βAPP-CTFs—Cells were plated at ~2 × 10^6 cells/10-cm dish and transfected on the following day with 5 μg of SPA4C or SPA4D cDNA (45) or empty vector (mock control) using FuGene 6 Transfection reagent according to the manufacturer’s instructions (Novagen). The cells were harvested after 2 days and trituated in 1 ml of TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). Membranes were collected by centrifugation for 30 min at 65,000 rpm in a TLA-100.2 rotor (Beckman) at 4 °C. Membrane proteins were solubilized in TBS, 1% Triton X-100, 1:1 EDTA-free protease inhibitor mixture (Roche Applied Science) by trituration. After incubation for 30 min on ice, insoluble debris was removed by centrifugation, and the samples were processed for Western blotting as described below.

Extraction of Protein from Whole Cells for Western Blot Analysis—Cells were collected in phosphate-buffered saline (PBS) followed by centrifugation for 5 min at 6500 × g at 4 °C. Cell lysis was performed by incubation for 25 min under constant shaking in 50 ml Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5 mM Nonidet P-40, 0.2% SDS, 1 mM EDTA, 1:1 EDTA-free protease inhibitor mixture (Roche Applied Science) at 4 °C. Insoluble debris was removed by centrifugation for 10 min at 20,000 × g, and protein levels were determined by using the bichinonic acid assay in a 96-well plate format according to the manufacturer’s instructions (Pierce and Warriner). Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Probing of the membranes was subsequently (28) with various antibodies, as indicated in the figure legends, using the enhanced-chemiluminescence system (ECL, Amersham Biosciences).

Immunocytochemistry—~85 × 10^6 cells/ml were seeded on polylysine-coated glass coverslips the day before the experiment and washed with PBS, fixed with 4% paraformaldehyde, and permeabilized for 10 min at 4 °C with 0.5% Triton X-100. After blocking for 1 h at room temperature with 10% normal goat serum (Sigma), the cells were incubated for 1 h...
at room temperature with primary antisera followed by a fluoro-
phore-conjugated secondary antibody (Jackson ImmunoResearch). For
co-immunostaining, the cells were further incubated with a second
primary and secondary antibody conjugated to an alternative fluoro-
phore. The cells were washed with PBS and nuclei stained with TOTO-3
iodine for 30 s (Molecular Probes). The coverslips were mounted onto
glass slides using Vectashield mounting medium (Vector Laboratories),
and images were collected on a Leica confocal microscope using a Leica
TCS NT “Image” program version 1.6.587.

Quantification of Aβ Peptides in Conditioned Cell Culture Media—
Cells were plated at 6 × 10^6 cells/10-cm dish, and the media were
exchanged on the following day. Aβ peptide secretion into the fresh
media after overnight incubation was quantified by an Origen™
electrochemiluminescence assay (Orogen M-Series™ analyzer, Igen) as
described previously (28).

Membrane Preparation for Affinity Precipitations and Cell-free γ-Sec-
tase—Ras assays were performed using the stable cell lines that were
preserved essentially as described previously (28). Briefly, after collection
in phosphate-buffered saline (PBS), 2 mM EDTA cells were hypotonically
shocked by incubation for 8 min in 20 mM HEPES-HCl, pH 7.3, 10 mM
KCl and sedimented by centrifugation for 10 min at 1000 × g. Cells
were homogenized in 20 mM HEPES-HCl, pH 7.3, 90 mM KCl, and
nuclei and cellular debris were removed by centrifugation for 10 min at 1000
× g. Cell membranes were collected by centrifugation for 1 h
at 45,000 rpm (50.2 Ti rotor, Beckman), resuspended in PBS, 5% gly-
cerol, and stored at −80 °C prior to further use.

CHAPSO Solubilization of Active γ-Sec-
tase—Cell membranes (stored in PBS, 5% glycerol) were collected by centrifugation for 30 min at 180,000 × g. Membrane proteins were solubilized in 1% (w/v)
CHAPSO, 50 mM MES-NaOH, pH 6.0, 1 mM EDTA, 0.15 mM NaCl, 5 mM MgCl2, 1× EDTA-free protease inhibitor mixture (Roche Applied
Science). Insoluble debris was removed by centrifugation at 180,000 × g
for 30 min, and the resulting supernatant (solubilized γ-secretase) was
adjusted with the same buffer without CHAPSO to give a final deter-
genent concentration of 0.5% CHAPSO (w/v).

Inhibitor Affinity Captures—Endogenous biotinylated proteins were
removed by adding streptavidin-coupled magnetic beads (Dynal) to
CHAPSO-particulated enzyme and centrifugation for 2 min at 20,000 × g
after incubation for 30 min at 4 °C. For specific capture, the pre-
cleared solubilized preparation (1.8 ml; 0.6–0.7 mg/ml protein) was
incubated for 90 min at 37 °C with

1 mM EDTA, 0.15 M NaCl, 5 mM MgCl2, 1× EDTA-free protease inhibitor mixture) at
2.5% of the input before capture.

Quantification of Cell-free Production of Aβ Peptides—Membranes
from the different HEK cell lines were prepared essentially as described
for the affinity precipitations above and membranes stored in PBS, 5% glycerol
were collected by centrifugation for 30 min at 180,000 × g. Membrane proteins were solubilized in 1% (w/v) CHAPSO, 50 mM MES-NaOH, pH 6.0, 0.15 mM NaCl, 5 mM MgCl2, 1× EDTA-free protease
inhibitor mixture (Roche Applied Science). Insoluble debris was removed
by centrifugation at 180,000 × g for 30 min, and the resulting supernatant (solubilized γ-secretase) was adjusted with the same buffer without
CHAPSO to give a final detergent concentration of 0.5% CHAPSO (w/v).

For in vitro generation of Aβ peptides, equal amounts of
CHAPSO-solubilized protein were incubated for 90 min at 37 °C with
6.6 μg of recombinant C100FLAG in 20 mM HEPES, pH 7.3, 2 mM
EDTA, 0.1% bovine serum albumin, 0.5% CHAPSO in a 100-μl final
volume. Aβ peptides were quantified by an electrochemiluminescence
assay in a 96-well plate format (Origen M-Series™ analyzer, Igen) as
described (39) by using 20 μl of the reaction for Aβ(40) and 50 μl for Aβ(42),
despite specific background signals, including pre-existing Aβ was
defined by the signal obtained when the assay was performed in the
presence of 10 μM of the potent γ-secretase inhibitor Merck A. For
detection of γ-secretase activity in size exclusion chromatography, anti-
FLAG immunoaffinity extraction and lipid reconstitution experiments
Aβ levels were quantified by using 25 μl of the reaction in an analogous
assay. This method utilizes avidin-coated 96-well plates with carbon
electrodes on the base for capture of the antibody-antigen complexes
and the Meso Scale Discovery™ instrument (Sector Imager 6000) for
electrochemiluminescence reading.

Size Exclusion Chromatography—Solubilized membrane prepara-
tions (1 ml, 2–2 mg of solubilized protein) obtained from γ-NRC-F8-
secretase cell lines were centrifuged for 15 min at 10,000 × g to remove
potentially aggregated material that may have formed as a result of
lowering the CHAPSO concentration to 0.5%. The resulting superna-
tant was loaded onto a Superdex 200 10/300 GL column (Amersham
Biosciences) using an AKTA fast performance liquid chromatography
system (Amersham Biosciences). Fractionation was performed in buffer
A (0.5% (w/v) CHAPSO, 50 mM MES-NaOH, pH 6.0, 1 mM EDTA, 0.15
M NaCl, 5 mM MgCl2, 1× EDTA-free protease inhibitor mixture) at
a flow rate of 0.5 ml/min, 0.5-ml fractions being collected for analysis
(γ-secretase enzyme activity and Western blot).

Anti-FLAG Immunoaffinity Extraction—EZview™ anti-FLAG M2
Affinity Gel (Sigma) was initially washed four times with deionized
water and equilibrated with an equal volume of buffer A prior to use. All
washing and elution steps in this batch purification procedure were
performed by suspension of the resin in the appropriate buffer and
subsequent centrifugation (3300 × g, 30 s). Approximately 200 μl of
the resin was then incubated on a rolling platform for 2 h at room
temperature with a solubilized membrane preparation derived from ~20 mg
of γ-NRC-F8) membranes. Separate aliquots of this enzyme preparation
were retained for analysis. Following incubation with the enzyme, the
resin was washed four times with buffer A. Elution steps were
performed by incubating the resin with 100 μg/ml FLAG peptide
(Sigma) in buffer A. For the initial elution, the resin was incubated
for 15 min at room temperature and for all subsequent elutions for 5 min.

Lipid Reconstitution/Cholesterol Treatment—Brain extract type VII
(Core了自己的 brain) was suspended in chloroform:methanol (2:1)
to give a stock concentration of 6.6 mg/ml. A stock concentration of
this preparation was subsequently vacuum-dried and suspended in buffer A
to give a working stock of 10 mg/ml. Water-soluble cholesterol (choles-
terol-charged methyl-β-cycloextrin) (Sigma) was prepared as a 2.5 mM
stock in H2O and diluted as appropriate to yield a final concentration of
5 μM when added to solubilized γ-secretase. Methyl-β-cycloextrin was
prepared as a 50 mg/ml stock in H2O and utilized at a final concentra-
tion of 10 μM.

RESULTS

Generation of the γ-(NRC-F8)-Secretase Cell Line—In order to
achieve the functional overexpression of γ-secretase in a human HER293 cell line, we chose a strategy that enabled the
sequential addition of PS1 and nicastrin followed by simulta-
nous introduction of Aph-1aL and Pen-2 to a cell line that
already stably overexpresses the substrate precursor βAPP695.
Epitope-tagged variants of Aph-1aL and Pen-2 were used be-
cause specific antibodies recognizing these polypeptides were
not commercially available at the time. It is noteworthy that in
our hands only the C-terminally VS-hexahistidine epitope-tagged
version of Aph-1aL gave the anticipated results for this polypeptide.
The corresponding C-terminally FLAG-tagged versions (data not shown) tended to aggregate and migrated exclusively as dimers and oligomers on SDS-polyacrylamide
geis. The splice variant Aph-1aL-CHO was chosen for this study
because it has been characterized in detail (46). A comparison
of the protein expression of individual cell lines generated (Fig.
1) indicated that co-expression of all four γ-secretase complex
components PS1, nicastrin, Aph-1aL, and Pen-2 changed the
post-translational processing of both nicastrin and PS1. Primary
transfection with nicastrin led to an accumulation of its
immature form (Mv – 98 × 10^3), whereas the introduction of
Aph-1aL and Pen-2 strongly increased the expression of the
mature form of nicastrin migrating at a higher relative molecu-
lar mass (Mv – 105 × 10^3). Concomitantly, the levels of both
PS1-NTF and PS1-CTF were increased in the γ-(NRC-F8)-
secretase cell line compared with the founder cell lines. This
was, however, not reflected by a decrease in PS1 full-length
polypeptide immunoreactivity, suggesting that functional over-
expression of the complex can both lead to an enhanced endo-
proteolytic processing of PS1 and an increase in the stability of
PS1-FL.
PS1, Nicastrin, Aph-1aL, and Pen-2 Form γ-Secretase—To investigate whether all of the overexpressed PS1 interactors are incorporated into the active enzyme complex, we performed affinity precipitation studies using the well characterized biotinylated aspartyl protease transition state inhibitor Merck C (39). The results show (Fig. 2) that the PS1 heterodimer, exclusively mature nicastrin, Aph-1aL, and Pen-2, specifically bound to the affinity probe. This implies an incorporation of all of the overexpressed PS1 interactors into the functional enzyme complex. Note that β-catenin, which is a known PS1 ligand (27), was not found to be associated with the enzyme complex even in a situation where the enzyme is overexpressed. When the relative enzyme activities normalized to the founder cell line were determined for each of the cell lines in a cell-free γ-secretase assay (Fig. 3), only co-expression of all known complex components lead to a strong increase in γ(40)- and γ(42)-enzyme activity. Both increases were within a similar range (14.3-fold for γ(40) and 14.6-fold for γ(42)), indicating that the same molecular entity generates Aβ(40) and Aβ(42) peptides.

Cellular Aβ Peptide Production—Most surprisingly, when the cellular Aβ production was analyzed a continuous decrease of Aβ secretion into the conditioned media was observed (Fig. 4A), which is in sharp contrast to the rise in cell-free enzyme activity. In this regard, it is noteworthy that because all of the cell lines that have been generated overexpress βAPPswe (which is only a substrate precursor), conversion by γ-secretase is still dependent on BACE cleavage. BACE cleavage of βAPP itself appears to be a rate-limiting step because the KM of the Swedish familial AD mutation (47), which converts βAPP into a better substrate for this enzyme (1), leads to a strong overproduction of Aβ peptides (48). To circumvent this rate-limiting step upstream of γ-secretase processing, we transiently transfected the different cell lines with SPA4CT (45). This polypeptide is converted into a direct γ-secretase substrate by constitutive signal peptidase cleavage upon its biosynthesis. Western blot analysis of the cell lysates demonstrated a successful overexpression (Fig. 4B) of SPA4CT as seen by the strong increase in the immunoreactivities for the β-CTF (C99) and α-CTF (C83), the latter resulting from α-secretase cleavage. Consistently, Aβ production was increased in all cell lines upon SPA4CT transfection compared with the mock controls. A continuous increase of cellular Aβ production was observed upon SPA4CT transfection, which followed the sequence of exogenous overexpression of the individual complex components with the strongest secretion obtained from γ(NRC-F8)-secretase cells (Fig. 4C).

Subcellular Localization of the γ-Secretase Complex Components—To investigate the subcellular localization of the individual polypeptides constituting the γ-secretase enzyme complex, we performed confocal immunofluorescence microscopy. Homogeneous and substantial overexpression of the γ-secretase subunits was detected in the corresponding cell lines (Fig. 5, A–D). This is in good accordance to protein expression data obtained by probing the corresponding cell lysates by Western blotting (Fig. 1). As expected, a weak staining was obtained for endogenous PS1 (Fig. 5A) and nicastrin (Fig. 5, A and B). In contrast, because epitope tag-specific antibodies were used for Aph-1aL and Pen-2 detection, only the exogenous polypeptide was stained (Fig. 5D).

Triple immunostainings using overlapping combinations of primary antibodies (Fig. 5, A and B) indicate a co-localization of the individual subunits in the same subcellular compartments. This was manifested by a continuous perinuclear staining (indicative of Golgi localization) and the appearance of various patches on the cell surface. After failing to detect co-localization of nicastrin (which was used as representative marker for the γ-secretase complex) with an endoplasmic reticulum (ER) (chaperone BiP), ER-Golgi intermediate compartment (COP-I coatamer β-COP), or early endosome marker (early endosome

**Fig. 1.** Comparison of the protein expression in the individual HEK cell lines. A comparative Western blot analysis of the cell extracts prepared from the HEK cell lines generated for this study is shown. Individual polypeptides were immunostained using ECL as indicated. Note that endogenous Aph-1aL and Pen-2 are not detected because antibodies specific for the epitope tag of the exogenous polypeptides were used for their detection. The cytoskeletal protein α-tubulin served as a control for equal loading. Overexpression of PS1 (lane 2) in the βAPPswe cell line (lane 1) leads to an accumulation of the full-length peptide without major changes in endoproteolytic fragment levels. Similarly, overexpression of nicastrin leads to an accumulation of the immature polypeptide (lane 3), whereas introduction of Aph-1aL and Pen-2 (lane 4) promotes nicastrin maturation and PS1 endoproteolytic processing.

**Fig. 2.** The PS1 heterodimer, nicastrin, Aph-1aL, and Pen-2 bind to an active site-directed γ-secretase inhibitor. Solubilized γ-secretase from γ(NRC-F8)-secretase cells was incubated with 0.1 µM of the biotinylated γ-secretase inhibitor Merck C in the absence or presence of a 100-fold excess (10 µM) of Merck A and precipitated with streptavidin-coupled beads. CHAPSO-solubilized membranes before ligand addition (Input, 2.5% of total) were compared with the captured fraction in the presence (Bound, Non-spec., 100% of total) or absence (Bound, Spec., 100% of total) of the competing nonbiotinylated inhibitor by Western blot analysis. Individual polypeptides were detected by infrared imaging using Alexa Fluor 680-conjugated goat anti-rabbit or anti-mouse Fab’γ fragments (Molecular Probes) and a LI-COR® Odyssey infrared imager (LI-COR Biosciences Inc.) as indicated. Precipitation of the PS1 heterodimer, nicastrin, Aph-1aL, and Pen-2 is abolished in the presence of competing inhibitor. β-Catenin, which serves as a control, is not captured under any condition.
antigen 1, EAA1), we utilized the 58K Golgi protein to address a potential Golgi association. 58K Golgi protein is a microtubule-binding protein associated with the cytoplasmic surface of the Golgi apparatus (49) and a commonly used marker for this compartment. A strong co-immunostaining of 58K Golgi protein with either PS1 or nicastrin was observed (Fig. 6, C and D). Similar results were obtained with syntaxin 6, which is an alternative Golgi/trans-Golgi network marker protein (data not shown). Taken together, these findings suggest a predominant co-localization of the enzyme complex subunits in the Golgi apparatus. To address the potential plasma membrane localization of γ-secretase subunits a Pan-cadherin antibody was used as a marker (50). The known predominant cell surface localization of these intercellular adhesion molecules was used to investigate the apparent change of the subcellular localization of PS1 upon reconstitution of the γ-secretase complex (Fig. 6, E and F). The results show a lack of PS1 and cadherin co-localization in the PS1-overexpressing cell line (Fig. 6E), whereas in the γ(NRC-F8)-secretase cell line both polypeptides are present on the cell surface (Fig. 6F).

Effect of γ-Secretase Overexpression on βAPP Maturation and Trafficking—A close inspection of the βAPP immunoblots shown in Fig. 1 suggested changes in the maturation of this protein in the different cell lines because the ratios of mature and immature βAPP appeared to be changing. To investigate this further, another set of lysates was prepared that was run on large Tris-glycine gels for a better separation of the individual bands. The Western blot data (Fig. 7A) confirm a decrease of the immature form of the overexpressed βAPP695 (M, ~95 × 10^3) and an increase of the mature form (M, ~100 × 10^3) upon the subsequent reconstitution of the γ-secretase complex. To investigate whether this is also reflected at a cellular level, βAPP695 expression in the founder cell line (only overexpressing βAPP695) and the γ(NRC-F8)-secretase cell line were compared by confocal immuno-fluorescence microscopy. A distinct change of the βAPP staining pattern was observed, which was confirmed by a variety of antibodies recognizing either the cytosolic C terminus or the ectodomain of βAPP (Supplemental Material). Whereas a predominant diffuse, perinuclear stain was observed in the founder cell line, the γ(NRC-F8)-secretase cell line was characterized by a punctate staining suggesting the direction of βAPP695 trafficking into larger intracellular vesicles.

In order to characterize the underlying mechanisms further, we chose to pharmacologically separate γ-secretase protease activity from other functions possibly associated with the PS1-γ-secretase complex by using specific inhibitors of the enzyme. βAPP695-expressing cells were either treated with MeSO vehicle (Fig. 7C), the transition state analogue inhibitor Merck A (39, 51) (Fig. 7E), or the compound CBAP (Fig. 7G), which apart from being a potent γ-secretase inhibitor is able to block PS1 endoproteolytic processing (28). These cells were compared with γ(NRC-F8)-secretase cells treated under identical conditions (vehicle, Fig. 7D; Merck A, Fig. 7F; CBAP, Fig. 7H). The subcellular localization of βAPP did not change in γ-secretase inhibitor-treated γ(NRC-F8)-secretase cells (and the founder cell line), which implies a protease-independent role of the functional γ-secretase complex in the regulation of the matu-

![Fig. 3. γ-Secretase enzyme activity is strongly increased in solubilized enzyme preparations obtained from γ(NRC-F8)-secretase membranes.](image)

![Fig. 4. Effects of γ-secretase overexpression on cellular Aβ peptide generation. A, Aβ40 peptide secretion from each of the individual cell lines was quantified. The signal obtained in the Origen electrochemiluminescence assay was expressed relative to the βAPP695 founder cell line. B, each of the cell lines was either transiently transfected with SPA4CT (+) or an empty vector mock control (−) (two dishes for each treatment). Equal amounts of solubilized membrane protein were separated by SDS-PAGE and immunoblotted for βAPP-CTFs using the C-terminal polyclonal rabbit antiserum R7334. Individual polypeptides were detected by infrared imaging using Alexa Fluor 680-conjugated goat anti-rabbit Fab’2 fragments (Molecular Probes) and a LI-COR® Odyssey infrared imager (LI-COR Biosciences Inc.) as indicated. A representative Western blot obtained from one set of the duplicate transfections is shown. C, Aβ40 peptide secretion into the corresponding media was quantified using the Origen electrochemiluminescence assay, and the values are displayed as relative levels compared with the corresponding mock controls. Error bars indicate the mean ± S.E. of the combined values from the duplicate transfections with quadruplicate Aβ measurements for each.](image)
Fig. 5. Cellular expression of γ-secretase complex subunits. The protein expression in individual clones generated was analyzed by immunocytochemistry and confocal imaging. The individual cell lines βAPPswy (A), βAPPswy/PS1 (B), βAPPswy/PS1/nicastrin (C), and γNRC-F8γ-secretase (D) were immunostained for the individual γ-secretase complex components as indicated (green color). Nuclei were stained with TOTO-3 iodine (blue). The data reveal the intracellular localization of individual components of the γ-secretase complex. Note that in βAPPswy (A) and βAPPswy/PS1 (B) endogenous PS1 and nicastrin (NCT), respectively, can be detected. Because epitope tag-specific antibodies were used for detection of Pen-2 and Aph-1aL, the endogenous protein is not stained by the respective antibodies. All overexpressed components of the γ-secretase complex appear to be associated with intracellular membranes and possibly the plasma membrane.

**Evidence That Lipids Are a Prerequisite for γ-Secretase Activity**—To investigate further whether an unidentified cofactor has been partitioned from the complex, we attempted to reconstitute activity by supplementing all fractions with a sample of the γ-secretase-enriched peak 1. Addition of peak 1 can be seen to yield an ~30% recovery in activity in the fractions corresponding to peak 2, data that were corroborated by the reciprocal experiment, supplementing all fractions with a sample of peak 2 (Fig. 9A). As expected, a similar result was obtained, with a partial recovery of activity, solely in the peak 1 fractions. Because none of the known subunits of γ-secretase are enriched in the second peak (Fig. 8C), these data are indicative of a novel cofactor of γ-secretase, which is essential for enzyme activity, being present in peak 2.

In order to determine whether this cofactor is proteinaceous, we performed a heat denaturation step on both peaks 1 and 2, prior to their recombination. As expected, denaturation of peak 1, which is known to contain the protein components of the γ-secretase complex, completely abolished the ability of the enzyme to reconstitute activity upon recombination of the two peaks (Fig. 9B). In contrast, heat denaturation of peak 2 was seen to have no effect on the reconstitution of activity, implying that the novel cofactor is not a protein.

Because this cofactor appears not to be proteinaceous, and γ-secretase is an integral membrane protein, we reasoned that lipids may represent the missing factor. We therefore chose to investigate the ability of brain lipids to reconstitute the lost enzyme activity in peak 1. Varying amounts of a brain lipid fraction containing the major phospholipids and glycolipids from bovine brain (brain extract type VII) were combined with peak 1 and were seen to lead to a maximum recovery of ~25% of enzyme activity (Fig. 9C). This effect was dose-proportional up to 500 µg/ml lipid concentration, which was found to be the optimum for recovery of enzyme activity. Any further increase in the lipid concentration caused a decrease in enzyme activity. Because the maximum recovery of activity is comparable with that achieved by the addition of peak 2, this has led us to postulate that lipids are the most likely candidate for the essential cofactor eluted in peak 2. Note that addition of brain
lipoysis at the optimal concentration (500 μg/ml) to solubilize the enzyme prior to SEC (load fraction) did not modulate γ-secretase activity (data not shown).

We chose to investigate the contribution of lipids to γ-secretase activity further because differing effects of cholesterol on γ-secretase activity have been reported previously (52, 53). The cholesterol content of a CHAPSO-solubilized γ-secretase preparation

**Fig. 6. Co-localization of the subunits of the γ-secretase complex.** In order to investigate whether all components of the γ-secretase complex (PS1, nicastrin, Aph-1αL, and Pen-2) are co-localized to the same subcellular compartments, triple immunostainings of γ(NRC-F8)-secretase cells were performed. The following antibody combinations were tested. A, nicastrin/Pen-2/Aph-1αL (green/red/blue); B, PS1/Pen-2/Aph-1αL (green/red/blue). The results obtained demonstrate a prominent intracellular co-localization of all components (white color) in perinuclear compartments (arrow). In order to characterize this perinuclear staining pattern and to determine the main cellular compartment where all γ-secretase complex subunits are co-localized, a co-localization study using different organelle-specific markers was performed. Positive co-immunostaining (yellow color; the inset shows a higher magnification) of nicastrin (green) (C) and PS1 (green) (D) with the Golgi marker 58K Golgi protein (red) suggests that all components of the γ-secretase complex co-localize in the Golgi compartment (arrow). Identical results were obtained using an alternative Golgi marker, syntaxin-6 (data not shown). Apart from the Golgi compartment, PS1 and other γ-secretase complex co-factors appear to be localized to the plasma membrane. To confirm this observation, the pattern of PS1 expression was characterized by co-immunostaining of PS1 with a plasma membrane marker protein Pan-cadherin that resides at the cell surface. To investigate changes in the subcellular localization of PS1, which are introduced by the successful overexpression of all γ-secretase complex components, βAPPsh/PS1 (E) and γ(NRC-F8)-secretase (F) cells were compared. Our results demonstrate that in βAPPsh/PS1 cells, PS1 (green) shows an exclusive perinuclear staining and does not co-localize with Pan-cadherin (red; the latter indicated by the arrow). In contrast, in γ(NRC-F8)-secretase cells, a discrete portion of PS1 (green) (arrow) is trafficked to the cell membrane as demonstrated by its co-localization with Pan-cadherin (red). The co-localization is seen by the yellow color (arrow), and the inset shows a higher magnification. In all images nuclei were stained with TOTO-3 iodine (blue).

**Fig. 7. Altered βAPP maturation and trafficking in γ(NRC-F8)-secretase cells.** A, Western blot analysis of βAPP expression in the different cell lines generated for this study. Equal amounts of protein (50 μg) were loaded and probed with the C-terminal R7334 antibody. Lane 1, βAPPsh; lane 2, βAPPsh/PS1; lane 3, βAPPsh/PS1/nicastrin, and lane 4, γ(NRC-F8)-secretase cell lysates. Note the increase in mature βAPPsh and the corresponding decrease in immature βAPPsh immunoreactivity in the γ(NRC-F8)-secretase cell lysates. B, immunofluorescence staining of βAPPsh (22C11 antibody) in untransfected HEK293 wild-type cells serving as a control. No positive staining was obtained in the absence of exogenous βAPP expression. When the γ(NRC-F8)-secretase cells were compared with the βAPPsh founder cell line, a change in the staining pattern for βAPPsh was observed. The pattern was changed from a diffuse, perinuclear staining to a discrete, punctate staining in the γ(NRC-F8)-secretase cells, which was confirmed with a panel of four different βAPP-specific antibodies (Supplemental Material). This was clearly detected in the γ(NRC-F8)-secretase cells, although a trend was visible in the intermediate cell lines (data not shown). To investigate whether this effect is the result of increased enzymatic activity of γ-secretase, βAPPsh cells were treated for 16 h with vehicle Me2SO (C), 10 μM γ-secretase inhibitors Merck A (E), or CBAP (G) and compared with (NRC-F8)-secretase cells treated under identical conditions with vehicle Me2SO (D), 10 μM Merck A (F), or 10 μM CBAP (H). Essentially, the immunofluorescence staining of βAPPsh (green, 22C11 antibody) was not changed upon γ-secretase inhibitor treatment in any of the cell lines (nuclei stained with TOTO-3 iodine in blue). This indicates that a pharmacological inhibition of γ-secretase is not able to reverse the phenotype observed by the γ(NRC-F8)-secretase cell line to that observed by the βAPPsh founder cell line. The addition of cholesterol to the culture medium (100 μg/ml) modulated a decrease in mature βAPPsh and a corresponding increase in immature βAPPsh levels. This effect was modulated by either sequestering cholesterol with MCD or raising cholesterol concentrations by addition of cholesterol-charged MCD (“water-soluble cholesterol”). The use of the latter circumvents the experimental problems associated with the poor water solubility of cholesterol. Supplementation with cholesterol-charged MCD was observed to yield a 2-fold increase in γ-secretase activity, whereas the reduction of free cholesterol by treatment with free MCD almost completely abolished enzyme activity (Fig. 9D). These results indicate that cholesterol is both required for and is able to enhance the activity of γ-secretase.

**Partitioning of γ-Secretase from a Lipid Fraction Is Common to Alternative Separation Techniques**—To determine whether...
confirmed the presence of nicastrin, Aph-1aL, Pen-2, and both PS1 fragments, in the first eluate (Fig. 10B, E1), indicative of preservation of the γ-secretase complex under the same conditions as the previous SEC.

Because of the obvious similarities to data obtained with the SEC approach, we attempted to reconstitute the lost activity by the addition of either the brain lipid extract or peak 2 from an SEC separation to the first eluate from the FLAG affinity chromatography approach. Again, both SEC peak 2 and brain extract type VII were seen to result in the partial recovery of similar proportions of activity (Fig. 10C). Furthermore, the level of activity recovered in the affinity chromatography fractions (−15%) is almost 50% of the recovery attained with the SEC fractions (−30%), which correlates well with the apparent loss of 50% of the initial enzyme activity in the affinity chromatography flow-through.

**DISCUSSION**

The successful generation of a cell line that overexpresses the human γ-secretase enzyme complex provided the foundation for subsequent in vitro study of this unusual aspartyl-type protease. This goal was achieved by co-expression of the known subunits PS1, nicastrin, Aph-1aL, and Pen-2. Because two PS homologues and three forms of Aph-1 (the splice variants Aph-1aL and Aph-1aS and the homologue Aph-1b) are found in the human genome, this predicts the presence of more than one distinct γ-secretase enzyme complex (54). By choosing PS1 and Aph-1aL, it is likely that we have generated a cell line that appears to contain the most abundant form of the γ-secretase complex (55, 56). Functional overexpression of the enzyme was confirmed by an increase in relative γ(40)- and γ(42)-enzyme activities (~14–15-fold), which was observed in our cell-free enzyme assay. In good agreement with Ref. 25, we found that all of the overexpressed subunits can be isolated with an aspartyl protease transition state analogue inhibitor (39) that targets the catalytic aspartates in PS1 (38). This finding confirms that nicastrin and both PS1 endoproteolytic fragments, Aph-1aL and Pen-2, are not only required for the biogenesis of γ-secretase but are also essential components of the active enzyme complex. It is noteworthy that various reports describing the overexpression of γ-secretase have been published while the final experiments for this paper were performed (23–25, 40). However, the lack of any quantitative data on relative enzyme activities (including the analysis of the main γ(40)- and γ(42)-enzyme activities) in those previous reports makes a comparison of the relative degrees of overexpression achieved difficult.

In addition to the observed boost in γ-secretase activity, a substantial increase in the steady state levels of PS1 endoproteolytic fragments, which are the catalytic subunits of the enzyme, was detected. This was accompanied by a change in the glycosylation pattern of nicastrin, yielding to a greater abundance of the complex glycosylated form of this polypeptide. Because this mature form of nicastrin is exclusively found in the active enzyme complex (38), (Fig. 2) combined with the change in PS1 endoproteolysis, this is indicative of functional interaction of the individual components at a molecular level during their biogenesis.

One issue that remained contentious in the reconstitution studies described so far (40) was that although all previous reports agreed on the increase of γ-secretase enzyme activity in a cell-free assay utilizing a recombinant substrate, none of the groups had observed a substantial increase in Aβ peptide secretion from the cells. We have obtained similar findings and even observed a trend of a decrease of Aβ peptide secretion upon sequential transfection of the founder cell lines. This decrease is likely to be a direct result of a reduced cell growth.

**FIG. 8.** SEC of solubilized γ-secretase activity results in loss of enzyme activity. To estimate the molecular mass of γ-secretase, enzyme activity in the SEC fractions was quantified by detecting Aβ(40) peptide generated from a recombinant substrate. A, sample (40 μl) of the initial solubilized enzyme preparation from γ(NRC-Fs) membranes (Load (L)) was assayed, and the relative enzyme activities in aliquots of individual SEC fractions (same volume; 40 μl) are displayed as a percentage of this value. Error bars indicate the mean ± S.E. of quadruplicate Aβ measurements from each sample. Arrows indicate the position of molecular mass standards separated under identical conditions: dextran blue (2000 kDa; void volume), thyroglobulin (669 kDa), apofer-ritin (443 kDa), β-amylase (200 kDa), and bovine serum albumin (66 kDa). Closer analysis of the remaining γ-secretase activity in the SEC fractions (B) demonstrates the presence of two individual peaks (referred to as peak 1 and 2) comprising fractions 14–18 and 26–28, respectively. Note that these account only for a very small fraction of the total activity applied to the column. C, Western blot detection of the known γ-secretase complex components in the SEC fractions, individual polypeptides are immunostained as indicated. Nicastrin, Aph-1aL, Pen-2, the PS heterodimer, and PS1-FL co-elute in fractions correlating to peak 1 but not peak 2. Note that although all components are abundant in peak 1, the overall distribution patterns are not identical, and in particular mature (mat.) and immature (immat.) nicastrin are separated.

The loss of enzyme activity observed upon SEC is a common result associated with the purification of γ-secretase, we exploited immunoaffinity purification by utilizing an anti-FLAG antibody resin. This utilizes the FLAG epitope tag that had been engineered onto the N terminus of Pen-2 for isolation of antibody resin. This utilizes the FLAG epitope tag that had been engineered onto the N terminus of Pen-2 for isolation of antibody resin.
that was visible after plating of the cells (data not shown). This might be caused by the cellular stress associated with the use of the various selection antibiotics or the strong overexpression of exogenous proteins. The final γ(NRC-F8)-secretase enriched cell line, for example, had to be incubated in the presence of four different antibiotics to maintain the expression of five different exogenous polypeptides.

By directly transfecting the cell lines with the constitutive γ-secretase substrate SPA4CT, we attempted to address this lack of an increase in cellular Aβ production by circumventing endogenous, rate-limiting BACE cleavage of the substrate precursor βAPP. Although these data showed a consistent trend with an increasing cellular Aβ secretion upon reconstitution of γ-secretase (γ(NRC-F8)-secretase > βAPP/PS1/nicastrin > βAPP/PS1 > βAPP cell line), this did not exactly match the data obtained for enzyme activity in the cell-free enzyme assay that showed a strong increase of γ-secretase activity in the γ(NRC-F8)-secretase cell line compared with the others. By comparing the cellular and cell-free data carefully, it appears that only the γ(NRC-F8)-secretase is the outlier because the general trend of increased enzyme activity upon the introduction of nicastrin appears to be preserved in both paradigms. The actual rise in cell-free and cellular Aβ production upon the introduction of nicastrin may be a result of a subtle influence of the overexpression of this essential γ-secretase component on the formation of enzyme complexes, because an increased concentration of one of the rate-limiting subunits might slightly shift the equilibrium toward complex formation.

However, the discrepancy observed upon transient expression of SPA4CT in the γ(NRC-F8)-secretase cell line can possibly explain some of the issues raised in the literature. It suggests that even in cells overexpressing a direct βAPP-derived γ-secretase substrate trafficking or other cellular mechanisms could regulate the access of the substrate to the overexpressed enzyme. Because in the cell-free assay the substrate is exogenously added to the CHAPSO-solubilized enzyme, the substrate amount accessible to the enzyme is experimentally controlled. Therefore, this assay lacks the complexity of a cellular system and is a direct readout for enzyme activity. In this context, the 10-fold increase in Aβ secretion in the βAPP695-expressing founder cell line upon SPA4CT transfection compared with mock control indicates that this might be the limit for the endogenous γ-secretase enzyme.

A confocal immunofluorescence microscopy study of the subcellular localization of the overexpressed polypeptides indicated that in good agreement with Ref. 58, all γ-secretase subunits can be found to be co-localized already in the Golgi compartment. An interesting observation was the change of
trafficking of PS1 from intracellular membranes to the cell surface upon reconstitution of γ-secretase. This finding can explain at least partially the past debates on the subcellular localization of PS1. Original reports claimed a predominant ER/Golgi localization upon overexpression of PS1 or PS2 in various cell lines (59), and in contrast to this, biochemical fractionation of rat brain indicated the presence of PS1 in post-Golgi membranes such as synaptosomal membranes and neurite growth cone membranes (60). The sudden change of PS1 trafficking observed in the γ(NRC-F8)-secretase cell line suggests that the overexpressed protein is only transported to the cell surface when the enzyme complex is correctly assembled.

In cell lines overexpressing a single subunit of the complex the other co-factors become rate-limiting as postulated early on (61). This leads to an accumulation of the overexpressed polypeptide in early biosynthetic compartments such as ER/Golgi. Indeed, a cell surface localization of PS1 has been detected in a recent study (62) that used the relatively low expression of a green fluorescent protein-tagged PS1 variant.

Apart from the change in relative enzyme activity, both the maturation and subcellular localization of βAPP were apparently altered in the γ(NRC-F8)-secretase cell line compared with all three different founder cell lines. The particular strength of these data lies in the fact that these findings were obtained by using independent methods. The increase in βAPP maturation suggests that the observed change from a diffuse perinuclear staining (ER/Golgi-like) to punctate staining reflects the enhanced accumulation of βAPP in late biosynthetic compartments such as the trans-Golgi network or intracellular transport vesicles. Although this altered trafficking is only observed in the γ(NRC-F8)-secretase cell line (suggesting it is dependent on functional γ-secretase complex assembly), the lack of any phenotypic changes upon γ-secretase inhibitor treatment argues that this is associated with a protease-independent function of the γ-secretase complex. Historically, changes of protein trafficking that were observed upon inactivation of PS1 by genetic knock-out (63) or mutagenesis (64) were interpreted as evidence against the model that PS1 is the catalytic subunit of γ-secretase. It is conceivable, however, that at least three different scenarios need to be carefully considered. First, a genetic deletion of presenilins will have considerable knock-on effects on the other components of the complex and further interacting proteins in the entire pathway. These loss-of-function phenotypes can be separated pharmacologically from the inactivation of enzyme activity with specific inhibitors. Examples include the maturation of the Trk receptor (28) and the regulation of β-catenin stability (65), which remain unaffected by γ-secretase inhibitor treatment.

Second, the inactivation of the enzyme may lead to changes in the trafficking of its substrates via an indirect mechanism. This could potentially explain the changes observed in βAPP trafficking upon mutagenesis of the two critical transmembrane aspartates in PS1 (62, 70, 71). Because inhibition of the enzyme leads to an accumulation of the corresponding CTFs (28), the relative γ-secretase activity in eluate 1 (E1) alone from a separate SEC run or brain lipid extract type VII. Values are displayed as a percentage of the activity obtained by using independent methods. The increase in βAPP maturation suggests that the observed change from a diffuse perinuclear staining (ER/Golgi-like) to punctate staining reflects the enhanced accumulation of βAPP in late biosynthetic compartments such as the trans-Golgi network or intracellular transport vesicles. Although this altered trafficking is only observed in the γ(NRC-F8)-secretase cell line (suggesting it is dependent on functional γ-secretase complex assembly), the lack of any phenotypic changes upon γ-secretase inhibitor treatment argues that this is associated with a protease-independent function of the γ-secretase complex. Historically, changes of protein trafficking that were observed upon inactivation of PS1 by genetic knock-out (63) or mutagenesis (64) were interpreted as evidence against the model that PS1 is the catalytic subunit of γ-secretase. It is conceivable, however, that at least three different scenarios need to be carefully considered. First, a genetic deletion of presenilins will have considerable knock-on effects on the other components of the complex and further interacting proteins in the entire pathway. These loss-of-function phenotypes can be separated pharmacologically from the inactivation of enzyme activity with specific inhibitors. Examples include the maturation of the Trk receptor (28) and the regulation of β-catenin stability (65), which remain unaffected by γ-secretase inhibitor treatment.

Third, the complex appears to have a protease-independent function in the trafficking of βAPP, which is not sensitive to γ-secretase inhibitor treatment. The underlying mechanism is unknown, but a successful reconstitution of the active γ-secretase enzyme complex is required for these changes to occur. Therefore, it is likely that this is a function of the active enzyme and not a particular subunit on its own.

For the successful development of an effective AD therapy based around manipulation of γ-secretase activity, it is essential to gain a comprehensive understanding of the γ-secretase complex organization and molecular architecture. A critical development toward achieving this aim will be the identification of a suitable approach for the purification of the active enzyme. With these aims in mind, we initiated experiments to attempt to determine the size of the active γ-secretase complex
by using size exclusion chromatography. Several previous investigations have led to a range of reported molecular masses for the complex varying from 250 kDa to $2 \times 10^5$ kDa (29, 30, 35, 72), although it has not been demonstrated previously that the retained activity is comparable with the activity present in the starting material, and therefore representative of the major active species. To our knowledge this is the first study that has reported the relative recovery of enzyme activity, and surprisingly, our data indicate an almost complete loss of enzyme activity following SEC. Because of the very low levels of activity that were detectable, care had to be taken with interpretation of the data. However, upon close examination of the retained activity, it appears to be segregated between two individual peaks, each comprising ~1% of the initial activity. This led us to postulate that a novel cofactor may have been separated from the complex, which subsequently elutes in peak 2. Recombination of the two peaks resulted in recovery of appreciable levels of activity, and although peak 1 was found to contain all known components of the complex, the majority of these was not detectable in peak 2. Therefore, it appears that peak 1 is composed of the majority of the γ-secretase complex, with a small proportion of this unidentified cofactor, and conversely that peak 2 contains the majority of this cofactor, with only minimal amounts of the complex subunits, which remain at or below the level of the detection of several of our antibodies. This would explain the minimal levels of activity in each peak, and how, upon recombination of the peaks, this essential cofactor re-associates with the complex, leading to a partial recovery of enzyme activity. Peak 2 was insensitive to heat denaturation (in contrast to peak 1), which suggested that the missing factor is of a nonproteinaceous nature.

Our empirical search for a molecule that would possess the same characteristics (nonproteinaceous and able to reconstitute activity) led to the discovery that a brain lipid extract, which contains the major phospholipids and glycolipids normally found in brain tissue, is able to replicate the effects of cholesterol-charged MCD avoided solubility problems, which are critical steps in the race to delineate the structure and mechanism of this critical enzyme.

Indeed, comparison of the SEC elution profile with known molecular mass standards suggests that the γ-secretase complex has a molecular mass of ~800 kDa. This corroborates recent evidence that suggests a size of >670 kDa (72) and would correspond to the presence of a tetrameric formation of the complex. The enzyme would therefore be present in a very intricate scenario with 72 transmembrane domains traversing the lipid bilayer. However, the finding that this potentially tetrameric complex is almost completely inactive raises the possibility that the removal of the lipids forces γ-secretase to assemble into this stable but inactive oligomeric species. Generally, it cannot be ruled out that the apparent mass of the complex may be altered by the recombination with lipids (or peak 2), and therefore the exact size of the active enzyme still has to be considered elusive to date.

In an attempt to generate an enriched preparation of active γ-secretase, we evaluated the use of immunooaffinity extraction as a suitable approach. This approach gave a notable finding, because the use of the FLAG epitope fused to Pen-2 for immunooaffinity isolation led to the specific capture of all known cofactors. Subsequent determination of enzyme activity indicated the capture of intact but catalytically inactive enzyme complexes. This indicates that the loss of γ-secretase enzyme activity is a general problem associated with common separation techniques. Again, supplementation of the γ-secretase-enriched eluate from this separation with either peak 2 from the SEC separation or the brain lipid extract yielded a partial recovery of enzyme activity. Most interestingly, it appears that with either experimental approach (SEC or immunooaffinity isolation) we are only able to reactivate around a third of the enzyme activity theoretically present in the fractions, and although the reason for this remains unknown at present, several scenarios can be envisaged. For example, the lipid extract used may not be representative of the membrane environment in which the native complex resides. Furthermore, the nature of the lipid fraction that is likely to be isolated in peak 2 may have been disturbed by chromatographic fractionation in a manner that does not allow the native situation to be completely reconstituted. In particular, it has been reported that the γ-secretase complex resides within lipid rafts (53, 75) and once disturbed may not reform, resulting in only partial enzyme activity. Alternative explanations are that the removal of lipids may result in a degree of irreparable rearrangement of the protein components of the γ-secretase complex, or simply that the native conditions for formation of the active complex may not be replicated sufficiently in our experiments.

Therefore, in order to develop a strategy for the successful purification of γ-secretase, care must be taken to ensure that not only is the complex enriched in an intact form but also that such a species possesses significant amounts of enzyme activity. Without these advances, our data have obvious implications for the purification and crystallization of γ-secretase, which are critical steps in the race to delineate the structure and mechanism of this critical enzyme.

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