Alzheimer’s disease is characterized by the deposits of the 4-kDa amyloid β peptide (Aβ). The Aβ protein precursor (APP) is cleaved by β-secretase to generate a C-terminal fragment, CTFβ, which in turn is cleaved by γ-secretase to generate Aβ. Alternative cleavage of the APP by α-secretase at Aβ16/17 generates the C-terminal fragment, CTFα. In addition to Aβ, endoproteolytic cleavage of CTFα and CTFβ by γ-secretase should yield a C-terminal fragment of 57–59 residues (CTFγ). However, CTFγ has not yet been reported in either brain or cell lysates, presumably due to its instability in vivo. We detected the in vitro generation of Aβ as well as an ~6-kDa fragment from guinea pig brain membranes. We have provided biochemical and pharmacological evidence that this 6-kDa fragment is the elusive CTFγ, and we describe an in vitro assay for γ-secretase activity. The fragment migrates with a synthetic peptide corresponding to the 57-residue CTFγ fragment. Three compounds previously identified as γ-secretase inhibitors, pepstatin-A, MG132, and a substrate-based difluoroketone (β-butoxycarbonyl-Val-Ile-(S)-4-amino-3-oxo-2,2-difluoropentanoyl-Val-Ile-OMe), reduced the yield of CTFγ, providing additional evidence that the fragment arises from γ-secretase cleavage. Consistent with reports that presenilins are the elusive γ-secretases, subcellular fractionation studies showed that presenilin-1, CTFα, and CTFβ are enriched in the CTFγ-generating fractions. The in vitro γ-secretase assay described here will be useful for the detailed characterization of the enzyme and to screen for γ-secretase inhibitors.

The Aββ that is invariably deposited in Alzheimer’s disease

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age of APP (32–34). However, tumor necrosis factor-α-convert- 
ing enzyme was shown to be specific for the phosphol ester- 
induced α-secretase activity (33). Although ADAM 10 appeared 
to be involved in both constitutive and inducible pathways, 
inhibition of this activity by a dominant-negative mutation 
indicated that it also plays a more important role in the induc- 
able α-secretase pathway (34).

The final step in Aβ biogenesis is the γ-secretase cleavage and is 
the activity responsible for generating CTFγ in vivo. This cleav- 
age is particularly interesting as the scissile bond lies within 
the transmembrane domain (35, 36). Since the discovery that 
a PS1 knockout mutant mouse was deficient in γ-secretase ac-
tivity, the possibility was raised that PS1 and PS2 are the 
identical to human allowing the use of human-specific reagents 
for the activity responsible for generating CTFγ.

To develop such an assay, we incubated membranes from guinea 
pig brain and cow brains as well as from cultured cells to look for 
production of Aβ and CTFγ. The CTFγ fragment was detected 
in all systems tested, but guinea pig brain was used because 
the entire APP sequence is known, and the sequence of Aβ is 
identical to human allowing the use of human-specific reagents 
for its analysis. After appropriate fractionation, we observed a 
consistent time-dependent generation of a putative CTFγ frag-
ment from these membranes. In addition to describing a useful 
robust cell-free assay for γ-secretase, this study presents the 
initial characterization of CTFγ from brain membranes.

**EXPERIMENTAL PROCEDURES**

**Materials**—All buffers and reagents were obtained from VWR Scien-
tific, Sigma, Life Technologies, Inc., or Fisher Scientific unless other-
wise stated. Guinea pig brain and cow brain were obtained from Co-
calio Biologics Inc. Fetal calf serum and newborn calf serum were 
obtained from HyClone. Bis-Tris precast gels, MES buffer concentrate, 
and molecular weight markers were obtained from Invitrogen Corp. Maleimide-
activated keyhole limpet hemocyanin (KLH), Supersignal West Pico 
substrate, the BCA protein assay kit, and Brij 35 were from Pierce. The 
previously reported substrate-based difluoroketone inhibitor (t-
butoxyxycarbonyl-Val-Ile-(S)-4-amino-3-oxo-2,2-difluoropentanoyl-Val-
Ile-Ome) of γ-secretase that we are calling Wolfe-1 (42) was synthesized 
by Dr. Ziani-Cherif at the Mayo Clinic Chemical Core Facility. Addi-
tional reagents such as peptatin A (Peptides International), 1,10-
phenanthroline (PNT; J. T. Baker Co.), thiorphan (ICN), phosphatidy-
linositol-specific phospholipase C (PI-PLC; Molecular Probes), human 
recombinant caspase-3, Z-Val-Ala-Asp(OMe)-CH2F (Z-VAD-FMK), 
MG132, phenylmethylsulfonyl fluoride, E64, leupeptin, 
N-acetyl-Leu-Leu-Leu-Nle-CHO (ALLN), CHAPSO, and Big CHAP (Calbiochem) 
were purchased as indicated. The Chinese hamster ovary (CHO) cell line, 
G9PLAP, expressing the human placental alkaline phosphatase (PLAP) 
cDNA, a kind gift from Dr. Victoria Stevens (Emory University, At-
lanta, GA), was maintained in F12HAMS medium supplemented with 
10% fetal calf serum and 200 μg/ml geneticin (31).

**Antibodies, Peptides, and Standards**—Antibodies against marker 
proteins syntaxin-6 (43) (Transduction Laboratories) and synapto-
phycin (44) (Roche Molecular Biochemicals) were kind gifts from Drs. 
Svlevler (Mayo Clinic, Jacksonville, FL) and Lahiri (Indiana Univer-
sity, IN). The anti-cross-reacting determinant (anti-CDR) antibody (Ox-
ford Glycosciences) reacts with a neeptide generated after the cleav-
ge of GPI-anchored proteins by PI-PLC. P1998, a rabbit polyclonal 
antibody against the juxtamembrane cytoplasmic domain of APP (726– 
744 of AP770) and the synthetic C-terminal 57 residues of APP (CTFγ- 
57) were kind donations from Pfizer. CTFγ-57 includes 10 residues 
from the human membrane domain of APP, the entire 47-residue cytoplasmic 
domain of APP and corresponds to one of the predicted CTFγ fragments.

The monoclonal antibodies BNT77, BA27, and BC05 utilized for the 
quantification of Aβ40 and Aβ42 were kind gifts of Dr. Nobu Suzuki 
and have been described previously (12). The rabbit antibody O443 was raised against a maleimide-activated KLH-conjugated synthetic peptide (CKMQQQNYENVT) prepared 
at the Mayo Clinic Protein Core Facility, which corresponds to the 
C-terminal 20 residues of APP. The animal care, injections, and 
bleeds were carried out by Cocalico Biologicals, Inc. (Animal approval 
number A3669-01), and the sera were characterized in our laboratory. 
The antibody detects less than 0.1 fmol (0.6 pg) of synthetic CTFγ by 
Western blot analysis. The anti-PS1 antibody was a copy of that re-
ported by Duff et al. (45) and was prepared against a KLH-conjugated 
synthetic peptide corresponding to residues 2–13 (CRKTELPLPFLSY).

The LC99 construct in the pCEP4 vector consisting of the signal 
sequence of APP fused to its C-terminal 99 residues, starting at the Aβ 
sequence, was transfected into CHO cells (CHO C99) for use as a 
standard for CTFγ.

**In Vitro Generation of CTFγ**

The final step in Aβ biogenesis is the γ-secretase cleavage and is 
the activity responsible for generating CTFγ in vivo. This cleav-
age is particularly interesting as the scissile bond lies within 
the transmembrane domain (35, 36). Since the discovery that 
a PS1 knockout mutant mouse was deficient in γ-secretase ac-
tivity, the possibility was raised that PS1 and PS2 are the 
elusive γ-secretases (37). The finding that PS1 and PS2 show 
conserved and essential aspartate residues within their respec-
tive transmembrane domains led to the suggestion that they 
are unusual aspartyl proteases with active sites within or close 
to the membrane (38). Recently, an *in vitro* assay for γ-secretase 
was described, and PS1 was found to be a part of the active 
protease (39). By using this assay, a biotinylated inhibitor was 
cross-linked to PS1 and PS2, showing that the presenilins are 
indeed the active subunits of γ-secretase (40, 41). However, the 
active enzyme was shown to be a large complex, presumably 
with many unidentified subunits, of which some may be also 
esential for γ-secretase activity. To understand this interest-
ing cleavage event, it is important to tease out each component of 
the γ-secretase and examine its individual role in detail by a 
combination of biochemical and genetic methods. To address 
these issues, it is necessary to have a robust *in vitro* γ-secretase 
assay in both the membrane-associated and soluble states. To 
develop such an assay, we incubated membranes from guinea 
pig and cow brains as well as from cultured cells to look for 
production of Aβ and CTFγ. The CTFγ fragment was detected 
in all systems tested, but guinea pig brain was used because 
the entire APP sequence is known, and the sequence of Aβ is 
identical to human allowing the use of human-specific reagents 
for its analysis. After appropriate fractionation, we observed a 
consistent time-dependent generation of a putative CTFγ frag-
ment from these membranes. In addition to describing a useful 
robust cell-free assay for γ-secretase, this study presents the 
initial characterization of CTFγ from brain membranes.
FIG. 1. Major APP processing pathways. The APP holoprotein is a type I integral membrane protein with a large N-terminal extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. αβ is depicted as a gray box and partly embedded within the membrane (12–14 residues). Antibodies O443 and PF998 are against the final 20 residues and 19 juxtamembrane residues, respectively. APP is cleaved to sAPPα and CTFα by α-secretase or sAPPβ and CTFβ by β-secretase. The membrane-bound CTFα and CTFβ are further cleaved within the transmembrane domain by γ-secretase (green arrow) to generate P3 and αβ, respectively. Most (90%) αβ and P3 end at residue 40 and a small fraction (5–10%) after residue 42 of the αβ sequence. In addition, γ-secretase cleavage should yield a C-terminal fragment, CTFγ, of 57–59 residues.

experiments, treated samples were compared with controls adjusted to 100%. αβ was measured as reported earlier by a specific and sensitive sandwich ELISA using a monoclonal antibody BNT77 against αβ 11-28 for capture and horseradish peroxidase-labeled end-specific antibodies BA27 (αβ 40) or BC05 (αβ 42) for detection (12, 31).

RESULTS

Generation of a Novel C-terminal APP Fragment from Guinea Pig Brain Membranes—To assay for γ-secretase activity, guinea pig brains were homogenized and fractionated as described under "Experimental Procedures" into F1P, F2P, and F3P and resuspended in equal volumes of buffer B. Since the yield of membranes from CHO cells was low, these membranes were only fractionated into 10,000 and 100,000 × g pellets (F2P and F3P). Equal volumes of each fraction were incubated for 0 and 2 h at 37 °C, chilled on ice, and centrifuged at 100,000 × g to remove the membranes. Supernatants were examined by Western blotting with the O443 antibody raised against αβ 11-28 for capture and horseradish peroxidase-labeled end-specific antibodies BA27 (αβ 40) or BC05 (αβ 42) for detection (12, 31).

The capacity to immunoprecipitate the putative CTFγ with the in vitro findings that αβ 40 constitutes 5–10% of the secreted αβ (12). The increase in αβ 40 was highly significant (p = 4.7 × 10−12) but αβ 42 was not (p = 0.18), presumably due to the large variance in background levels (two-tailed t test).

To determine the stability of CTFγ, the supernatant obtained after removal of membranes from the incubation mix was incubated further for 2 and 4 h in the absence of membranes. The CTFγ fragment was degraded by over 50% after 2 h and by over 90% after 4 h at 37 °C. Addition of PNT, a metalloprotease inhibitor, partially protected the fragment from degradation after 4 h (Fig. 4). In addition, PNT increased the yield of the C-terminal fragment by 2-fold, suggesting that one or more metalloproteases in the preparation degrade the fragment and/or the enzyme responsible for its generation (Fig. 4).

Although several protease inhibitors including phenylmethylsulfonyl fluoride, E64, leupeptin, and ALLN were tested, protection was only seen by EDTA and PNT, making metalloproteases the only identified class important for degrading the released CTFγ in vitro (data not shown). The pH optimum of the observed γ-secretase activity was between pH 7 and pH 7.5, but activity was detected in a broad range from pH 5 to pH 9 (Fig. 5). This pH optimum is similar to that recently reported for γ-secretase activity using membranes purified from HeLa cells (39).

We attempted to determine the cleavage site(s) that generate CTFγ in collaboration with Dr. Rong Wang (Rockefeller University, New York). Presumably due to its low levels and un-
FIG. 4. PNT increases the yield of the putative CTFγ and partially protects it from degradation. Western blots probed with O443 (A) show F2P membranes incubated in the absence of PNT for 0 h (lane 1) or 2 h (lane 2) or in the presence of PNT for 0 h (lane 11) and 2 h (lane 12). Supernatants generated after a 2-h incubation without PNT were incubated further in the absence (lanes 3–6) or presence of PNT (lanes 7–10) for an additional 2 h (lanes 3, 4, 7, and 8) and 4 h (lanes 5, 6, 9, and 10). Note the higher CTFγ recovery after 4 h in the presence of PNT (lanes 9 and 10) than in its absence (lanes 5 and 6). Also note the 5-fold higher yield of CTFγ with PNT (lane 15) than without PNT (lane 2). The graphical data shown in B represent the mean quantified by densitometry ± S.D. from three independent experiments.

FIG. 5. The yield of CTFγ is optimal at close to neutral pH. The generation of CTFγ at each pH was determined as an increase in the fragment yield as detected by Western blotting with O443 after a 2-h incubation compared with 0 h as blank.

favorable flying characteristics, the exact size of CTFγ could not be detected by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy. However, the fragment comigrates with a 57-residue synthetic peptide corresponding to one of the predicted CTFγ fragments on polyacrylamide gels (Fig. 6). Judging from the separation of CTFα from CTFβ and the molecular weight markers, small changes of over five residues (0.5 kDa) should be readily detected in this size range. Thus, the cleavage size is likely to be close to the C-terminal end of the Aβ sequence and occur within the transmembrane domain of APP as predicted for γ-secretase activity (Fig. 6).

Caspase Inhibitor Does Not Reduce CTFγ Production—Previous studies have shown that caspase can cleave within the cytoplasmic domain of full-length APP in cells undergoing apoptosis (25, 47–49). This cleavage resulted in an ~3-kDa fragment and was inhibited by a broad spectrum caspase inhibitor, Z-VAD-FMK (49). It is unlikely that the observed 6-kDa fragments are generated by caspase, as the fragment is much larger than reported, and this cleavage occurs in the membrane pellet fraction after washing away the cytoplasm. However, we cannot rule out that some caspase is bound to the membrane pellet. To rule out the possibility that the CTFγ-like fragment is a product of caspase cleavage, we included Z-VAD-FMK, a broad spectrum caspase inhibitor, in our incubations. Z-VAD-FMK did not inhibit the generation of the putative CTFγ (Fig. 6). We also incubated the 57-residue synthetic CTFγ as well as the brain membrane fraction with caspase ± Z-VAD-FMK.

Caspase-3 cleaved the synthetic CTFγ as shown by the reduction in band intensity, and Z-VAD-FMK blocked this reduction, indicating that the inhibitor was active under the conditions used (Fig. 6). Similarly, reduction in the yield of the 6-kDa CTFγ was also observed when brain membranes were spiked with purified caspase, which was restored in the presence of Z-VAD-FMK (Fig. 6).

γ-Secretase Inhibitors Reduce CTFγ—A number of protease inhibitors were tested to determine the class of proteases involved in the generation of the putative CTFγ and to understand the role of γ-secretase in generating this fragment. Previous reports have already identified several inhibitors that lower γ-secretase activity as defined by a reduction in the production of Aβ and by the increase in CTFα and CTFβ levels in cell lysates (2). Three of these inhibitors are pepstatin-A, MG132, and Wolfe-1 (a γ-secretase substrate-based difluoroketone inhibitor). F2P membranes treated with the inhibitors pepstatin-A (Fig. 7A, lanes 5–8), Wolfe-1 ((42) Fig. 7A, lanes 9–12), and MG132 (Fig. 7B, lanes 7–12) showed a dose-dependent reduction in the yield of the 6-kDa fragment generated from brain membranes in vitro. These data provide additional evidence, indicating that the putative CTFγ fragment is a product of γ-secretase cleavage.

CTFγ Is Generated in a Fraction Enriched in CTFα, CTFβ, and Presenilin—CTFα and CTFβ are the postulated substrates for γ-secretase activity, and PS1/PS2 are reported to be an integral part of purified γ-secretase (39). Furthermore, reports show that PS1 binds APP and its C-terminal fragments, consistent with its role in γ-secretase activity (50–52). To examine the role of PS1 in the generation of CTFγ, we analyzed brain membrane fractions. As described earlier, we initially obtained three membrane pellet fractions F1P, F2P, and F3P generated after sequential centrifugation. The fractions were probed with antibodies against APP and PS1 and marker proteins as described under “Experimental Procedures”. The cell-surface GPI-anchored proteins (53) and the Golgi-marker syntaxin-6 (43) were preferentially found in F3P, which also had the highest concentration of protein (Fig. 8). In contrast, most of the γ-secretase activity together with CTFα and CTFβ was in F2P, suggesting that γ-secretase activity was not enriched in the plasma membrane or in Golgi vesicles. Although the N-terminal fragment of PS1 was observed in all three fractions, its mobility was somewhat retarded in F3P. The reason for this shift in mobility is not known but may be due to post-translational modification such as phosphorylation of PS1 (54).

The active F2P was further fractionated by centrifugation to equilibrium in a sucrose step gradient ranging from 20 to 40% sucrose as described above. The eight fractions were examined for γ-secretase activity by incubating membrane pellets as described earlier. In addition, the membranes were analyzed on Western blots for APP, CTFα, CTFβ, and PS1 (Fig. 9). The peak
In Vitro Generation of CTFγ

FIG. 7. Known γ-secretase inhibitors reduce the yield of CTFγ. F2P from guinea pig brain was incubated in the presence of 5 mM PNT and the indicated protease inhibitors and supernatants were analyzed on Western blots with the O443 antibody as described under “Experimental Procedures.” A compares CTFγ in PNT alone for 0 (C-0) and 2 h (C-2) with PNT + 10 μM (P-1) and 100 μM (P-2) pepstatin-A, or 0.2 mM (W-1) and 1 mM (W-2) W-2. B shows samples incubated in the presence of PNT alone for 0 (C-0) and 2 h (C-2), and PNT + 50 μM (M-1) or 200 μM (M-2) MG132 for 2 h. The relative yield of CTFγ from the various conditions is summarized in C, with each bar representing the mean and S.E. of 8–10 independent incubations from three separate experiments.

FIG. 8. CTFγ is generated in a fraction distinct from the cell surface and the Golgi apparatus. Guinea pig brain fractions F1P, F2P, and F3P were analyzed on Western blots stained using reagents shown on the right side of each panel. Note that full-length APP is enriched in F3P, whereas CTFα, CTFγ, and some of the CTFβ are enriched in F2P. The N-terminal fragment of PS1 at ~30 kDa was seen in all fractions. However, the fragment was slightly shifted up in F3P. The anti-CRD antibody against PI-PLC-cleaved GPI-anchored proteins detected two major groups of bands in the 38- and 62-kDa range primarily in F3P. Each of these groups presumably represents several GPI-anchored proteins in these size ranges. The Golgi marker, syntaxin-6 at 31 kDa, was also primarily in F3P. The synaptic vesicle marker, synaptophysin, was primarily detected in F1P and F2P indicating that most of it is pelleted at 10,000 × g, although a sizable quantity is also seen in F3P. A total protein stain (Ponceau S) detected the strongest signal in F3P, although Fig. 2 shows that most of the CTFγ is generated in F2P.

FIG. 9. The peak of PS1, CTFα, and CTFβ coincide with the peak of CTFγ generation. F2P from guinea pig brain was further fractionated on sucrose density gradients, and eight fractions were collected from the top of the tube as described under “Experimental Procedures,” and equal volumes of each fraction were analyzed by Western blotting with O443 (top three panels) and anti-PS1 N terminus (bottom panel). Fractions 1 and 2 were mixed and loaded in the 1st lane, and the remaining fractions were loaded in individual lanes. The top panel shows full-length APP, the 2nd panel CTFα, and the 3rd panel CTFγ generated after incubation for 2 h. CTFβ can be detected as a faint band above CTFα in the 2nd panel. The bottom panel shows the ~30-kDa PS1-NTF. Note that PS1-NTF, APP, CTFα, CTFβ, and generation of CTFγ peak in fraction 5.

of γ-secretase activity as determined by the relative intensity of the CTFγ fragment generated was in fraction 5. Interestingly, PS1, CTFα, and CTFβ are all enriched in this fraction. However, it is important to note that PS1 was distributed in several fractions with low γ-secretase activity (Figs. 8 and 9). Recent findings show that most of the PS1 is in the endoplasmic reticulum and intermediate compartments with small amounts in the Golgi and cell surface (55). PS1 was also detected in detergent-resistant glycosphingolipid-enriched membranes (DIGs (56)), which was also enriched in cell-associated Aβ (57). Our preliminary observations2 show that PS1, BACE, and the ADAM 10 protease (Kuzbanian) are all enriched in detergent-insoluble glycosphingolipid-enriched membranes isolated from guinea pig brain.3 Since we did not detect γ-secretase activity in detergent-insoluble glycosphingolipid-enriched membranes prepared by flotation of Triton X-100-extracted membranes, we homogenized guinea pig brain in carbonate buffer to strip peripheral proteins, and generated a membrane fraction enriched in caveolin as described by Lisanti and coworkers (58). The γ-secretase activity was enriched in the caveolin-rich fractions suggesting that it may be present in DIG-related membranes.3

Solubilization of the Active γ-Secretase—The in vitro γ-secretase activity is lost when either F2P or membrane fraction 5 purified by sucrose density gradients is extracted with several detergents such as Triton X-100, methyl β-cyclodextrin, digitonin, Nonidet P-40, CHAPS, and octyl β-glucoside (data not shown). The data presented compare γ-secretase activity in fraction 5 in the presence of Brij 35, Big CHAP, and CHAPSO (Fig. 10A). γ-Secretase activity, determined as an increase in CTFγ production, appeared equivalent to the detergent-free control in CHAPSO, reduced in Brij 35, and almost absent in Big CHAP. Thus, the detergent extraction profile of this in vitro activity is similar to the in vitro assay recently reported by Li et al. (39). Since dissolving the activity is essential for its further purification and characterization, we removed the CHAPSO-insoluble membranes by centrifugation at 100,000 × g for 60 min and compared γ-secretase activity in the supernatant with activity in the mixture (Fig. 10B). Our data indicate that most of the activity was extracted from the sucrose gradient purified fraction 5 in this detergent, as judged by the approximately similar intensities of the CTFγ band obtained by incubation of equivalent amounts of the CHAPSO-solubilized supernatant alone and the CHAPSO-treated membrane mix. Solubilization of the activity is necessary for further biochemical analysis of γ-secretase in vitro.

2 I. Pinnix, U. Musunuru, H. Tun, A. Sridharan, T. Golde, C. Eckman, C. Ziani-Cherif, L. Onstead, and K. Sambamurti, unpublished observations.

3 H. Tun, I. Pinnix, and K. Sambamurti, manuscript in preparation.
In Vitro Generation of CTFγ

**DISCUSSION**

The current study reports a novel γ-secretase assay from brain membranes and is the initial description of CTFγ, a previously undescribed fragment predicted as a product of γ-secretase cleavage of APP. The CTFγ fragment is normally not detected in cell lysates and brain homogenates, probably due to its instability in vivo. A possible exception is a report of a faint 5.8-kDa CTF in human brain homogenates (22). However, CTFγ is readily detected in an in vitro γ-secretase assay based on fractionated brain membranes or cell homogenates. The in vitro detection of CTFγ strongly suggests that γ-secretase cleavage is due to a specific endoproteolytic cleavage and not random degradation of the cytoplasmic tail domain of APP as suggested previously (59).

An in vitro assay for γ-secretase has been recently reported by looking for Aβ as a product using a purified substrate consisting of the C-terminal 99 residues of APP (39). In the presence of thiorphan, an inhibitor of Aβ degradation in the brain (60), we were also able to detect the generation of Aβ40 (Fig. 3) as well as Aβ42 in our in vitro assay. Since a pool of CTFγ is not already present in brain or cell lysates at detectable levels, the low background allows the characterization of γ-secretase activity from fractionated membranes more easily than the measurement of Aβ. Based on the reports that β-secretase accounts for <10% of the secretory processing of APP (22), using CTFγ as an end point for γ-secretase activity may be 10 times more sensitive than the measurement of Aβ.

In addition, the measurement of CTFγ is not complicated by aggregation like Aβ or by the presence of alternative fragments such as P3 (Fig. 1).

Subcellular fractionation studies based on the in vitro generation of CTFγ suggest that γ-secretase activity is localized in fractions enriched in CTFα and CTFβ. PS1 is apparently enriched in these fractions, consistent with the currently favored hypothesis that presenilins include the active site of γ-secretase. However, high levels of PS1 are still seen in fractions showing little in vitro γ-secretase activity. Thus, if PS1 is indeed the active subunit of γ-secretase as suggested, only a small subset of it, presumably in a special compartment, is involved in γ-secretase activity. The restriction of the activity may be due to either the observed enrichment of the substrate CTFα and CTFβ in this compartment, limitations in essential components of the γ-secretase complex other than presenilins, or unfavorable conditions in the isolated organelle.

Although high levels of Aβ are secreted by several cell lines in culture, CTFγ is not readily detected in cell lysates, indicating that it is either rapidly degraded in the cytoplasm or sequestered in a manner that prevents its detection. Similar fragments from other membrane proteins such as Notch and SREBP are transported into the nucleus where they act as transcription factors, are rapidly degraded, and are difficult to detect (61, 62). The C-terminal tail of APP includes a sequence (KFFEQQ) that resembles a motif present in soluble proteins that are rapidly degraded in lysosomes (63). It will be useful to determine whether this sequence is responsible for rapid CTFγ turnover in cells and to identify alternative cellular pathways for its degradation, if any.

Recent reports show that PS1 specifically binds a γ-secretase inhibitor indicating that the active site of γ-secretase lies within the transmembrane domains of PS1 and PS2 (40, 41). It was proposed that presenilins are unusual aspartyl proteases, but this is not definitive as the aspartyl protease inhibitor, pepstatin A, can inhibit other classes of proteases at high concentrations (64). In addition, other γ-secretase inhibitors are known to inhibit several classes of proteases including serine proteases, cysteine proteases, and the proteasome (2). The data showing γ-secretase activity in fractions enriched in PS1 agree with studies using knockout mice deficient in PS1/PS2 that fail to generate Aβ and accumulate CTFα and CTFβ (37, 65, 66). The detection of CTFγ provides another powerful tool for the analysis of γ-secretase activity in vitro and for identifying mechanisms involved in its regulation and for developing drugs that block this activity.

Although presenilins have been identified as being essential for γ-secretase activity and are shown to contain the active sites, it is probably not sufficient, as it has not been possible to reconstitute the activity with pure PS1/PS2 and purified γ-secretase is a multisubunit complex (39). The mechanism of the endoproteolytic cleavage of the membrane-spanning domains of proteins may identify the role of each component. For example, it has been suggested that the cleavage is initiated by the sliding of the peptide out of the membrane into the cytoplasm (67). However, the energy required for this sliding reaction is likely to be very high. It is also likely that the reaction is mediated by a water molecule within the active site of the enzyme for the hydrolytic reaction. Thus, a water molecule may be held between the two transmembrane aspartate residues on PS1/PS2 that serve to hydrolyze within the transmembrane domain. It is possible that some of the unidentified subunits are involved in either transport of water or the APP for hydrolysis. The mechanisms for maintaining a supply of the water molecules in the membrane for the hydrolysis may provide useful insights into the mechanisms of γ-secretase cleavage and identify additional therapeutic targets for inhibiting this activity. A similar intramembrane proteolytic cleavage has been described for the sterol regulatory element binding protein (SREBP). The protease that cleaves SREBP has been identified as S2P by genetic complementation of a mutant cell line. The predicted active site of this enzyme resembles that of a metalloprotease with the exception that hydrophobic residues flank the domain, suggesting its intramembranous location (62). The development of an in vitro assay for S2P may be useful for comparison with γ-secretase and also provide useful insights into intramembrane proteolysis.

Since the generation of CTFγ is closely tied to the generation of Aβ, the mutations that increase the production of Aβ42 should also increase the 57-residue CTFγ starting at residue 43 of the Aβ sequence. Since FAD mutations that increase Aβ by preventing its degradation have not been reported, the correlation between CTFγ-57 and AD is as strong as Aβ42. In addition, CTFγ includes a peptide sequence that is reported to

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**Figure 10.** The in vitro γ-secretase activity can be solubilized in select detergents. Membranes from fraction 5 shown in Fig. 9 were diluted and collected by centrifugation as described under "Experimental Procedures." A, the fraction was incubated in pairs in the absence (lanes 1 and 2) and presence (lanes 3–14) of the indicated detergents for 0 and 2 h. Activity was recognized as an increase in the CTFγ level at 2 h (even lanes) over 0 h (odd lanes) for each condition. Note that CTFγ levels did not increase in the presence of Big CHAP (lanes 7–10). B shows that CTFγ is generated in the CHAPSO-soluble fraction. CHAPSO-resistant membranes were removed by spinning at 100,000 χ g for 1 h, and the supernatants were incubated for 0 (lanes 1 and 4) and 2 h (lanes 2 and 5). The CHAPSO-treated membranes were incubated for 2 h (lanes 3 and 6) as in A. Note that removal of CHAPSO-resistant membranes did not reduce the yield of CTFγ. The CHAPSO-resistant membrane pellet did not generate any CTFγ (data not shown).
induce apoptosis in neurons as discussed in the Introduction. The localization of the enzyme, the biological activity of the fragment, the cleavage site involved in its production, the mechanism of its turnover, and its role in AD are important unanswered questions that will be facilitated by this initial description of CTFγ.

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