Pharmacological Knock-down of the Presenilin 1 Heterodimer by a Novel γ-Secretase Inhibitor

IMPLICATIONS FOR PRESENILIN BIOLOGY

Received for publication, April 6, 2001, and in revised form, August 28, 2001
Published, JBC Papers in Press, September 26, 2001, DOI 10.1074/jbc.M103075200

Dirk Beher‡§, Jonathan D. J. Wrigley‡, Alan Nadin‡, Geneviève Evin‡, Colin L. Masters‡, Timothy Harrison‡, José L. Castro‡, and Mark S. Shearman‡

From the ‡Departments of Biochemistry & Molecular Biology and §Medical Chemistry, Merck Sharp & Dohme Research Laboratories, The Neuroscience Research Centre, Terlings Park, Harlow, Essex CM20 2QR, United Kingdom and the ¶Department of Pathology, University of Melbourne, and Neuropathology Laboratory, Mental Health Institute of Victoria, Parkville, Victoria 3010, Australia

Alzheimer’s disease (AD)1 is a progressive neurodegenerative disorder of the central nervous system characterized by an extracellular deposition of amyloid-β peptide (Aβ) (1, 2) in parenchymal senile plaques. Systematic genetic studies have led to the identification of three genes causative for familial forms of the disease, leading to increased production of a longer Aβ peptide species, Aβ(1–42), which is more prone to aggregation than the shorter and more predominant species, Aβ(1–40) (for review, see Refs. 3 and 4). The first gene identified was the β-amyloid precursor protein (βAPP) (5–7) from which Aβ is generated by two sequential proteolytic cleavages mediated by β- and γ-secretases. An alternative processing pathway involves the cleavage of βAPP within the Aβ sequence by α-secretase and prevents amyloid formation (8). γ-Secretase cleavage appears to be mediated by members of the disintegrin and metalloprotease family TACE (9) and ADAM-10 (10). Recently, a membrane-bound aspartyl protease has been cloned and characterized as β-secretase (BACE, Asp-2) (11–15). Novel evidence has highlighted a critical role of presenilin as the most likely candidate for γ-secretase itself. Mutations in the presenilin 1 and 2 (PS1 and PS2) genes are known to account for the majority of familial Alzheimer’s disease cases (16–19). Studies performed with neurons from PS1-knock-out embryos have shown that PS1 is essential for γ-secretase activity (20). Presenilins are polytopic membrane proteins that undergo endoproteolytic processing within their putative loop region, yielding N-terminal and C-terminal polypeptides (PS1-NTF and PS1-CTF) thought to consist of six and two transmembrane domains, respectively (21). Mutagenesis of either of two aspartate residues in transmembrane domains 6 or 7 of PS1 inactivates γ-secretase and blocks endoproteolysis (22, 23). Because these aspartates are critical for PS1 function, it has been proposed that PS1 is either a novel membrane-bound aspartyl protease or an essential di-aspartyl cofactor for the enzyme (23). This is further supported by the findings that PS1 is linked with γ-secretase activity in the detergent-solubilized state (24) and that photoactivated derivatives of 1,685,458, a highly specific and potent aspartyl protease transition state analogue inhibitor of γ-secretase (25), bind selectively and directly to PS1 fragments (26). Others have reported similar covalent labeling of affinity probes to presenilins using either a transition-state mimic (27) or a novel peptidomimetic γ-secretase inhibitor (28). Moreover, evidence has been generated that full-length PS1 (PS1-FL) might be a zymogen (26), which could directly to PS1 fragments (26). Others have reported similar covalent labeling of affinity probes to presenilins using either a transition-state mimic (27) or a novel peptidomimetic γ-secretase inhibitor (28). Moreover, evidence has been generated that full-length PS1 (PS1-FL) might be a zymogen (26), which could be activated by a putative autocleavage event within its loop region (29). One prediction of this model is that an inhibitor of γ-secretase that binds to PS1 should interfere with its endoproteolysis. We report that a novel γ-secretase inhibitor, CBAP, not only blocks PS1 endoproteolysis, but upon chronic treat-

Received, January 25, 2000, and in revised form, August 17, 2000.
Published, JBC Papers in Press, October 23, 2000, DOI 10.1074/jbc.M003075200

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: AD, Alzheimer’s disease; βAPP, β-amyloid precursor protein; Aβ, amyloid-β peptide; PS1/2, presenilin 1/2; PS1-FL, full-length presenilin 1; NTF, N-terminal fragment; CTF, C-terminal fragment; NICD, Notch intracellular domain; HEK293, human embryonic kidney 293; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; CHAPSO, 3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate; CBAP, [1S-benzyl-4R-[1-(5-cyclohexyl-2-oxo-2,3-dihydro-1H-benzo[e][1,4]diazepin-3(R,S)-ylcarbamoyl)-S-ethylcarbamoyl]-2R-hydroxy-5-phenyl-pentyl]-carbamic acid tert-butyl ester; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride; HOBT, 1-hydroxybenzotriazole hydrate; TBS, Tris-buffered saline.

This paper is available on line at http://www.jbc.org

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Antibodies—Monoclonal and polyclonal antibodies were obtained from the following sources and diluted for Western blot analyses as indicated: anti-βAPP (22C11, Roche Molecular Biochemicals, 0.5 μg/ml, anti-calnexin (StressGen, 1:2,500), anti-β-catenin (Transduction Laboratories, 1:1,000), anti-c-Myc (9E10, Calbiochem, 1:400), anti-β-COP (Sigma, 1:500), biotinylated anti-αβ 4G8 (Senetek), polyclonal anti-αGal-Gal, horseradish peroxidase-conjugated polyclonal goat anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech, 1:5,000), and polyclonal antiserum R7343 (mouse serum 98/1 raised against residues 1–200 of PS1, black flag mouse serum, 1:10) provided by M. Kounnas (Merck Research Laboratories, San Diego, CA). β-Cellulin (StressGen, 1:2,500), anti-calnexin (StressGen, 1:2,500), anti-αCOP (Sigma, 1:500), biotinylated anti-Aβ 4G8 (Senetek), polyclonal anti-rat Grp 78 (BiP, Calbiochem, 1:4,000), anti-c-Myc (9E10, Calbiochem, 1:400), anti-calnexin (StressGen, 1:2,500), anti-catenin (Transduction Laboratories, 1:2,500), anti-αGal-Gal (Transduction Laboratories, 1:2,500), and anti-rabbit IgG (Zymed, 1:1,000) were used. The secondary antibodies were used at the following dilutions: anti-rabbit IgG (1:4,000) and anti-mouse IgG (1:2,500). Quantification of Aβ was done using the bicinchoninic acid assay (34) in a 96-well plate format according to the manufacturer's instructions.

Experimental Procedures

Membrane Preparation and Solubilization of Membrane Proteins for Analysis of PS1 Polypeptides—The cells were broken by trituration in 1 ml of TBS (50 mM Tris-Cl, pH 7.4, 150 mM NaCl) and membranes were sedimented 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× EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals) by trituration, and after incubation for 30 min on ice, insoluble debris was removed by centrifugation for 10 min at 20,000 × g, 4°C. Protein concentrations of the supernatants were determined using the bicinchoninic acid assay (34) in a 96-well plate format according to the manufacturer’s instructions (Pierce & Warriner).

Extraction of Protein from Whole Cells for the Analysis of Notch Expression—Cell extracts from whole cells were prepared by sonication for 5 min in TBS, 1% Triton X-100, 0.5% Nonidet-P40, 0.2% SDS, 1 mM EDTA, 1× EDTA-free protease inhibitor mixture after incubation for 30 min at 4°C. Insoluble debris was removed by centrifugation for 10 min at 20,000 × g and protein levels normalized as described above.

Western Blot Analysis—Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Probing of the membranes was carried out with various antibodies, as indicated: the figure legends, using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). Quantitation of bands using a computerized image analysis system (MCID, Imaging Research Inc.) was performed as described previously (35). Quantification of Aβ Peptides in Conditioned Cell Culture Media—Aβ peptides secreted into the media were quantified by an ECL assay as described (34) in an analogous 96-well plate format (Ref. 36 and 37; Origen M-Series™ analyzer, Igen).
Inhibition of PS1 Endoproteolysis by γ-Secretase Inhibitors

4R-(tert-butyldimethylsilyl oxy)-6-phenylhexanoic acid (44) (53 mg, 0.1 mmol), EDC (22 mg, 0.11 mmol), and HOBT (0.11 mmol) in dimethylformamide was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate; washed with citric acid solution, sodium bicarbonate solution, and brine; dried (MgSO₄); filtered; and evaporated in vacuo. The crude product was dissolved in tert-n-butylammonium fluoride (1.0 M solution in tetrahydrofuran, 1 ml) and stirred overnight at room temperature. The reaction mixture was dissolved in ethyl acetate, washed with water, dried (MgSO₄), filtered, and evaporated in vacuo. Purification by column chromatography (silica, eluant: ethyl acetate) gave CBAP (31 mg, 44%) as a white solid. Compounds 7 (CBAP) and 8 are both mixtures of diastereoisomers at the benzodiazepine C-3 position. All new compounds gave satisfactory analytical and spectroscopic data.

RESULTS

Structure-Activity Relationship for Inhibition of PS1 Endoproteolysis and γ-Secretase Activity—Following our initial observation that treatment of SH-SY5Y cells for 16 h with 10 μM amount of the specific γ-secretase inhibitor L-685,458 results in an increase in PS1-FL steady-state levels (Fig. 1A), we compared a variety of derivatives for their potential to inhibit PS1-FL endoproteolysis with their ability to inhibit βAPP γ-secretase activity, monitored by inhibition of βA peptide production (Fig. 1). Most importantly, compounds that were inactive in regard to the inhibition of γ-secretase, such as the diastereoisomer L-682,679 or the desbenzyl derivative (compound 1) did not block the cleavage of PS1-FL (Fig. 1A). Compounds 2 and 3 are also effective inhibitors of PS1-FL endoproteolysis and γ-secretase activity, indicating that, for both activities, changes to the backbone amino acid side chains of L-685,458 are well tolerated. Surprisingly, compound 4 with an extension to the dipeptidyl moiety of L-685,458 is inactive in the PS1 cleavage assay (Fig. 1B), but still a potent inhibitor of γ-secretase activity. Compounds 5 and 6 do not contain the hydroxyethylene isostere but have been described as very potent inhibitors of γ-secretase activity (28). Both compounds increase PS1-FL polypeptide levels, suggesting that structurally unrelated inhibitors can cause an inhibition of PS1-FL cleavage. Compound 7 (CBAP), a chimeric molecule containing the L-685,458 isostere and a benzodiazepine group, was the only compound identified of a large number of inhibitors (n ~ 70; Fig. 1 and data not shown) that, under the standard experimental protocol, produced a strong increase in PS1-FL levels while concomitantly causing a detectable reduction in the levels of PS1-NTF (Fig. 1B). The specificity of this effect was further confirmed because the derivative compound 8 (Fig. 1B), lacking the alanine residue neighboring the isostere, is inactive for both inhibition of βA peptide production and PS1-FL cleavage.

It is noteworthy that, in the cellular assay system used, all compounds analyzed inhibited both βA(40) and βA(42) peptide production with almost identical potencies. Using a colorimetric cell proliferation assay, no overt cytotoxicity was observed in the cells for any of the compounds tested.

Pharmacological Knock-down of PS1 Fragments by CBAP—Following a chronic 7-day treatment of SH-SY5Y cells with CBAP, a stable increase in PS1-FL accumulation was observed, together with an almost complete inhibition of PS1 fragment formation (Fig. 2, A and B). Only very weak PS1-NTF immunoreactivity was detectable (Fig. 2A), and, when corresponding immunoblots were probed with an anti-lop antiseraum, PS1-CTF immunoreactivity was absent (Fig. 2B; this slight discrepancy reflects the differing affinities of the antiseraus used). As controls, cells were treated with vehicle L-685,458, or compound 5 or 6 (Fig. 2), but only CBAP was able to reduce PS1 fragment levels beyond the limit of detection for the antibody directed against the PS1-CTF. We describe this unique effect of CBAP as a pharmacological knock-down of PS1 fragments. As an added control, all samples were immunoblotted for the un-related β-COP Golgi protein (Fig. 2C). Identical immunoreactivities were observed in all samples, which excludes variations in the protein normalization procedure and nonspecific effects of the γ-secretase inhibitors on the cells.

Time Course and Dose Response of Inhibition of PS1 Endoproteolysis and γ-Secretase Activity—L-685,458 and CBAP appear to exert an immediate inhibitory action on both PS1 endoproteolysis and βAPP γ-secretase activity, as seen by an increase in PS1-FL and inhibition of βA peptide secretion after only 2 h (the earliest time point when βA was measurable; Fig. 3, A and B). βA peptide release reaches a maximum after 6–8 h in the vehicle-treated cells, whereas the inhibitor-treated cells do not release any detectable quantities of βA peptide at any time point (Fig. 3B). The accumulation of PS1-FL continues during the period analyzed, because it requires the re-synthesis of PS1, which is the critical rate-limiting step for the stabilization experiments (Fig. 3A). Taken together with the results of the chronic treatment study (Fig. 2), these data indicate that CBAP does not act as a γ-secretase inhibitor simply by blocking PS1 endoproteolysis; γ-secretase enzyme activity is completely inhibited almost immediately after addition of the compound to the cells, whereas a significant knock-down of the PS1 fragments, which are believed to represent the active enzyme, requires treatment for several days. Mechanistically, inhibition of γ-secretase activity and PS1 endoproteolysis by CBAP appear to occur simultaneously. This inhibition is dose-dependent (Fig. 3, C and D), but the potency for βA inhibition is not equipotent with inhibition of PS1 endoproteolysis. The accumulation of βAPP α-CTF (Fig. 3C) and inhibition of βA peptide production (Fig. 1) occur at concentrations lower than that needed to cause an accumulation of PS1-FL (Fig. 3C). ED₅₀ values for PS1-FL accumulation for both L-685,458 and CBAP are in the low micromolar range (Fig. 3D), whereas both inhibit βA peptide production at nanomolar concentrations (Fig. 1).

CBAP Binds Directly to PS1—L-852,646, a photoaffinity ligand derivative of L-685,458, can be covalently cross-linked to PS1-NTF (26). Because CBAP was able to induce a pharmacological knock-down of PS1 fragment formation by stabilizing PS1-FL, we wished to determine whether this compound was directed to the same binding site as L-852,646. In the absence of a competing ligand, PS1-NTF was labeled, whereas, when added to the reaction in 100-fold excess, both CBAP and L-685,458 specifically inhibited the photolabeling of PS1-NTF (Fig. 4). Based on the displacement of the photoprobe by both inhibitors, we conclude that they bind to the same or overlapping binding sites on the PS1-NTF.

γ-Secretase Inhibitors and Presenilin Knock-out Phenotypes—Because of the intimate relationship between γ-secretase and presenilins, we investigated whether γ-secretase inhibitors are able to recapitulate aberrant phenotypes associated with the inactivation or knock-out of PS1. Our main aim was to discriminate PS1/γ-secretase-mediated substrate cleavage from PS1 functions associated with membrane protein trafficking. Release of the Notch intracellular domain (NICD) upon activation of the Notch receptor requires presenilins (45) and is blocked by weak γ-secretase inhibitors, such as the peptide aldehydes MDL28170 and MG132, and the difluoroketone peptide analogue MW167 (45). Western blot analyses of c-Myc-tagged NICD from HEK293 βAPP/APP/NotchΔE cells that were exposed to increasing concentrations of inhibitors for 16 h demonstrated that all compounds tested in this assay inhibited the formation of NICD in a dose-dependent manner (Fig. 5A). This inhibition led to an accumulation of the NotchΔE protein, the substrate for this γ-secretase-like cleavage reaction. Furthermore, in the same
cells, this effect coincided with a dose-dependent accumulation of βAPP-CTFs (Fig. 5B) and inhibition of Aβ(40) and Aβ(42) production (Fig. 5, C and D). Upon treatment with the inhibitors, an accumulation of mainly the βAPP α-CTF was detected (Fig. 5B) as verified by immunoprecipitation Western blotting using monoclonal antibodies 4G8 and W0–2 (data not shown).

A predominant α-secretase processing pathway for βAPP in the chosen cell line was anticipated, as similar data have been described for the HEK293 βAPPε95 cell line (25), which was used as a starting point to generate the HEK293 βAPPε95/NotchΔE cell line.

The above findings confirm that this class of compounds acts
mechanistically as inhibitors of the γ-secretase cleavage of two substrates: βAPP and Notch. The IC₅₀ values obtained (Fig. 5F) reveal that for each compound potencies for inhibition of βAPP and Notch γ-secretase cleavage are closely comparable. Compound 6 was the most potent inhibitor analyzed and acts as a subnanomolar inhibitor for both cleavages (0.24–0.37 nM, Fig. 5F).

Deficits in Trk receptor maturation have been reported in neurons from PS1 knock-out animals (39). To determine whether presenilin/γ-secretase activity was involved in this process and whether CBAP was able to induce similar deficits, a pulse-chase analysis of rat primary cortical neurons was performed. Under vehicle control conditions (Fig. 6) after a 30–120-min chase period, the majority of immature Trk receptors migrating at ~110 kDa underwent maturation to higher glycosylated species, as seen by a shift of the apparent molecular mass to ~120–130 kDa. In CBAP-treated neurons, the mobility pattern observed in the gel and the time course were identical to the vehicle control (Fig. 6). Thus, we conclude that γ-secretase inhibition does not impair Trk receptor maturation and that this process requires presenilin expression and function independent of protease activity.

Complex Formation and Sorting of PS1-FL—It is known that, in PS1-transfected cells, both cleavage and sorting of PS1 are rate-limited, causing an accumulation of PS1-FL in the ER (46, 47). Furthermore, PS1 fragments are known to be incorporated into high molecular weight complexes, whereas in transfected cell lines wild-type PS1-FL is detected in complexes of lower molecular weight (48). Because CBAP gave us the opportunity to increase the ratio of PS1-FL versus fragments without changing the endogenous expression levels of PS1, we aimed to determine whether endoproteolysis of PS1 serves as a signal for assembly of high molecular weight complexes or trafficking of the fragments into the Golgi compartment.

Glycerol velocity gradient centrifugation of ChAPS-solubilized PS1 was performed under conditions where inhibitor treatment resulted in an increase in PS1-FL, but with significant amounts of fragments remaining (serving as an internal control). Western blot analysis of the gradient fractions essentially showed an identical distribution of all PS1 polypeptides, as well as the marker proteins δ-catenin. PS1-NTF and PS1-CTF were co-enriched in the Golgi fractions, containing the Golgi-specific polypeptide β-COP (49) (fraction 5; Fig. 7C) and higher glycosylated,
classes of proteases, including aspartyl proteases such as HIV-1 and cathepsin D (25), and structural modifications that abolish γ-secretase inhibition concomitantly lead to a loss of an inhibition of PS1 processing (e.g. compounds 1 and 8). Accordingly, for endoproteolytic cleavage to be mediated by a molecularly distinct presenilinase, this enzyme would require a pharmacological profile very similar to γ-secretase itself, an explanation that as yet would appear unlikely.

Two of the above possibilities require active site-directed transition state analogue inhibitors to bind to PS1-FL, considered to be a zymogen. We have no direct biochemical evidence to date that any of our inhibitors bind to PS1-FL, and previous work has shown that PS1-FL could not be cross-linked in vitro to specific photoprobes derived from L-685,458 (26) using membranes from cells overexpressing PS1. It still remains to be seen whether photoprobes may be able to cross-link to PS1-FL in cells. If untransfected cells are used, where PS1 trafficking and complex formation should resemble the in vivo situation, the minute amounts of holoprotein present in these cells will make this an extremely difficult task. Hence, it cannot be ruled out completely that, at high concentrations, certain inhibitors bind to PS1-FL in vivo. The compounds described herein have been identified and selected based on their ability to inhibit Aβ peptide production, and it is therefore not surprising that the potencies for Aβ inhibition do not necessarily mirror the inhibition of PS1 endoproteolysis. Furthermore, it is likely that processing of PS1-FL into its fragments results in a conformationally distinct entity. Complexes containing PS1-FL and those containing its fragments are therefore likely to constitute independent binding sites, and only compounds with a reasonable affinity for the former would be able to block the endoproteolysis of PS1. This could explain why some potent inhibitors of Aβ formation, such as compound 4 and others (data not shown), were not able to block PS1-FL cleavage and why higher concentrations of compounds are needed to inhibit PS1 endoproteolysis compared with γ-secretase inhibition.

Using CBAP as a tool to shift the steady-state levels of PS1 fragments toward the full-length polypeptide in vivo, we could address the question of accumulation of PS1-FL in the ER of transfected cell lines and the structural requirements for PS1/γ-secretase complex formation. PS1 fragments are known to be components of a high molecular weight complex, which may include β-catenin (48), the recently cloned transmembrane glycoprotein nicastrin (52), and possibly others. Analyses of cells overexpressing PS1 suggested that under this paradigm exogenous wild-type PS1-FL is present in a different complex of lower molecular weight (48). When solubilized PS1 from CBAP-treated cells was analyzed by rate-zonal glycerol velocity gradient centrifugation, we observed that both endogenous wild-type PS1-FL and its fragments are incorporated in a complex of identical molecular mass (~350 kDa). We explored this further by determining the structural requirements for trafficking of PS1 from the ER to Golgi compartments. Subcellular fractionation of organelles from CBAP-treated cells provided evidence that both PS1 fragments and PS1-FL follow the same sorting pathway. This is again in contrast to the clear separation of PS1 fragments and PS1-FL described previously in transfected cell lines (47). Accordingly, our data argue that the accumulation of PS1-FL in the ER fraction of transfected cells is not caused by the lack of cleavage but must be a direct result of the artificial overexpression of the polypeptide. Taken together these data support the hypothesis of a "limiting stoichiometric cofactor" (46), which would be necessary for the protein to exit the ER compartment. Because PS1-FL is incorporated into a high molecular weight complex in inhibitor-treated cells, it is likely that this factor binds to PS1-FL immediately after its

![Diagram of L-685,458 and CBAP binding to PS1-NTF](http://www.jbc.org/)

**Fig. 4. γ-Secretase inhibitors L-685,458 and CBAP bind to PS1-NTF.** After UV-cross linking in the presence of the biotinylated, photoreactive γ-secretase inhibitor L-852,646 and streptavidin bead precipitation, samples were probed with polyclonal 98/1 antiserum for PS1-NTF. Only in the absence of competing compounds was PS1-NTF precipitated by streptavidin beads (lane 1), whereas 100-fold excess of either L-685,458 (lane 2) or CBAP (lane 3) abolished the precipitation.

|   | 1 | 2 | 3 |
|---|---|---|---|
| - | + | - | L-685,458 (2 μM) |
| - | - | + | CBAP (2 μM) |
| + | + | L-852,646 (20 nM) |

**Table:**

|   |   |   |
|---|---|---|
| 1 | 2 | 3 |
| - | + | - |
| - | - | + |
| + | + | L-852,646 (20 nM) |

**Table 1:**

|   |   |   |
|---|---|---|
| 1 | 2 | 3 |
| - | + | - |
| - | - | + |
| + | + | L-852,646 (20 nM) |

The compounds described herein have been identified and selected based on their ability to inhibit Aβ peptide production, and it is therefore not surprising that the potencies for Aβ inhibition do not necessarily mirror the inhibition of PS1 endoproteolysis. Furthermore, it is likely that processing of PS1-FL into its fragments results in a conformationally distinct entity. Complexes containing PS1-FL and those containing its fragments are therefore likely to constitute independent binding sites, and only compounds with a reasonable affinity for the former would be able to block the endoproteolysis of PS1. This could explain why some potent inhibitors of Aβ formation, such as compound 4 and others (data not shown), were not able to block PS1-FL cleavage and why higher concentrations of compounds are needed to inhibit PS1 endoproteolysis compared with γ-secretase inhibition.

**DISCUSSION**

Our data demonstrate that certain potent and structurally diverse γ-secretase inhibitors either identified by ourselves such as L-685,458, or others (compounds 5 and 6; Ref. 28) cause a cellular accumulation of PS1-FL. This effect is immediate, indicating that as soon as one of these compounds enters cells it inhibits separate processing events simultaneously: presenilin/γ-secretase cleavage of βAPP and Notch and PS1-FL endoproteolysis.

Detailed structure-activity relationship analyses of these two processes led to the discovery of a novel L-685,458 derivative, CBAP, which enabled us to determine the principle mechanism underlying our initial observations. The accumulation of PS1-FL could be explained by interfering nonspecifically with its degradation by the proteasome system. This system has been shown to degrade PS1-FL rapidly (50), leading to half-lives of ~1.5 h for the full-length polypeptide in PS1-transfected cells (51). However, the knock-down of PS1 fragments, observed exclusively upon chronic treatment of cells with CBAP, unambiguously demonstrates that the inhibitors directly block the endoproteolytic cleavage of the PS1-FL polypeptide into its fragments. Mechanistically, this could be explained in a number of ways: (i) direct inhibition of the putative enzyme responsible for the cleavage of PS1-FL ("presenilinase"), (ii) indirect inhibition of presenilinase cleavage as a consequence of binding to and changing the conformation of PS1-FL, and (iii) binding to PS1-FL and interfering with intramolecular autoproteolysis. Although our data do not provide conclusive evidence to determine which of these possibilities is correct, we have made several key observations. First, L-685,458 shows greater than 100-fold selectivity over other
synthesis, enabling the protein to be shuttled to the Golgi compartment. Cleavage into its fragments might be facilitated by the factor, or it may occur during the subsequent transport from the ER to the Golgi compartment, but our data clearly demonstrate that it is neither a structural requirement for trafficking nor for complex assembly.

We have used the inhibitors described herein to determine which aberrant phenotypes in PS1 inactivated or deleted systems are related to a loss of protease function, against those caused by the physical absence of the PS1 polypeptide. All inhibitors analyzed were found to block the intramembranous cleavage of Notch− polypeptide at the S3 site, thereby inhibiting NICD formation. These results support previous findings (45) and strengthen the view that PS1 forms an essential component of the active site of protease. Moreover, neither a preferential inhibition of one of the APP− cleavages at position 40 or 42 nor the Notch S3 cleavage was observed. Inhibition of APP− cleavage without affecting Notch processing has been reported for a class of inhibitors containing an isocoumarin core, characteristic of chymotrypsin inhibitors (53). The compounds described in that study, however, were 10,000−200,000-fold less potent inhibitors than the ones described here. It will be interesting to see in the future if selectivity of the isocoumarin-type inhibitors can be maintained over a larger concentration range and if derivatives can be identified that are several orders of magnitude more potent. In contrast to the Notch data, treatment of cortical neurons with APP− secretase inhibitors did not recapitulate the impairment in Trk receptor maturation, another aberrant phenotype that has been described in PS1 knock-out animals (39). This indicates that inhibition of protease activity does not affect this function of presenilin, which points toward a role for PS1 in membrane protein trafficking (39).

The discovery of novel properties of γ-secretase inhibitors related to secretase activity and presenilin endoproteolysis provides highly specific tools, which can be utilized to obtain a better understanding of the biological impact of inhibiting presenilin, βAPP, and Notch processing. Resolving this issue is of utmost interest, as specific γ-secretase inhibitors that block PS1-associated protease functions are expected to be valuable therapeutic agents to combat AD pathology.

**FIG. 5. γ-Secretase inhibitors block Notch S3 and βAPP γ-cleavage with similar potencies.** HER293 cells stably transfected with βAPP(99) and mNotchΔE(M1727V) and NICD using anti-c-Myc antibody 9E10 (A) or for βAPP C-terminal fragments and transmembrane βAPP using antiserum R7334 (B). C and D, Aβ40 (C) and Aβ42 (D) peptides in the corresponding conditioned media were quantified and compared (E) with the inhibition of NICD formation determined by densitometric analysis of shorter exposures of the immunoblots shown in A. All data are expressed as percentage of Me2SO control values. F, IC50 values for inhibition of Aβ40, Aβ42, and NICD generation were calculated by nonlinear regression fit analysis of the graphs shown in C−E using GraphPad Prism™ software (conc., concentration).

**FIG. 6.** Trk receptor maturation is not affected by γ-secretase inhibitors. Primary rat cortical neurons were pulse-labeled with [35S]methionine (20 min) and chased in the presence of an excess of nonradioactive methionine for 30, 60, and 120 min. The experiment was performed either in the presence of vehicle Me2SO (DMSO, lane 1) or CBAP (10 μM, lane 2). Mature and immature Trk polypeptides immunoprecipitated from cell lysates are indicated by arrows.
FIG. 7. Incorporation of PS1-FL and its fragments into identical complex and sorting of PS1-FL. SH-SY5Y cells were treated with vehicle Me2SO (A) or CBAP (10 μM) (B) for 2 days and PS1 complexes analyzed by glycerol velocity gradient centrifugation. Acetone precipitates of each fraction were immunoblotted for βAPP, PS1-CTF (00/2), PS1-FL, and PS1-NTF (98/1) as indicated. Following antibody stripping, the βAPP filter was re-probed for β-catenin. Marker proteins separated on identical gradients are indicated by arrows. C, SH-SY5Y cells were treated with CBAP (10 μM) for 3 days and cellular organelles separated by sucrose density gradient centrifugation. Aliquots of individual fractions were immunoblotted for PS1-FL and -NTF as indicated. Following antibody stripping, the same filter was re-probed for calnexin.

Acknowledgments—We thank Janetta Culvenor for 98/1 and 00/2 antisera, Maria Kounnas for the R7334 antiserum, Sangram Sisodia for providing the anti-PS1 loop antiserum, Christine Haldon for tissue culture expertise, Jamie Bilsland and Sarah Harper for preparation of primary neurons, Adrian Smith and Joseph Neduvelil for the synthesis of compound 2 andL-852,646, respectively, and Ian Churcher for compound 5 and 6. We are grateful to David Williams for technical assistance and to Neil Wilkie and Scott Pollack for providing their protocol for Trk receptor immunoprecipitation.

REFERENCES
1. Glenner, G. G., and Wong, C. W. (1984) Biochem. Biophys. Res. Commun. 120, 885–890
2. Masters, C. L., Simms, G. G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4245–4249
3. Hardy, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2095–2097
4. Hardy, J. (1997) Trends Neurosci. 20, 154–159
5. Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) Nature 325, 733–736
6. Chartier-Harlin, M. C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., and Hardy, J. (1991) Nature 353, 844–846
7. Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., and James, L. (1991) Nature 349, 704–706
8. Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., and Ward, P. J. (1990) Science 248, 1122–1126
9. Buxbaum, J. D., Liu, K. N., Luo, Y., Slack, J. L., Stocking, K. L., Peschon, J. J., Johnson, R. S., Castner, B. J., Cerretti, D. P., and Black, R. A. (1998) J. Biol. Chem. 273, 27765–27767
10. Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasonovski, M., Haase, C., and Fahrenholz, F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3922–3927
11. Vassar, R., Bennett, B. D., Babu-Bhan, S., Kahn, S., Mendiz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loof, R., Luo, Y., Fisher, S., Fuller, J., Edens, S., Lile, J., Jarossinski, M. A., Bier, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) Science 286, 735–741
12. Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C., and Christie, G. (1999) Mol. Cell Neurosci. 14, 419–427
13. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1456–1460
14. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Cacavella, R., Davis, D., Doan, M., Doviet, H. F., Frigon, N., Hong, J., Jacobson-Crouk, K., Jewett, N., Keim, P., Krops, J., Lieberburg, I., Power, M., Tan, H., Tatsumo, G., Tung, J., Schenk, D., Seubert, P., Suemenns, M., Wang, S., Walker, D., and John, V. (1999) Nature 402, 537–540
15. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brasier, J. R., Stratman, N. C., Mathews, W. R., Bubl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L., and Gurney, M. E. (1999) Nature 402, 533–537
16. Levy-Lahad, E., Waseo, W., Pookraj, P., Romano, D. M., Oshima, J., Pettigell, W. H., Yu, C. E., Jondro, P. D., Schmidt, S. D., and Wang, K. (1995) Science 269, 973–977
17. Levy-Lahad, E., Wijisman, E. M., Nemens, E., Anderson, L., Goldberg, R. A., Weber, J. L., Bird, T. D., and Schellenberg, G. D. (1995) Science 269, 970–973
18. Rogae, F. J., Sherrington, B., Rogae, E. A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., and Tsuda, T. (1995) Nature 376, 775–778
19. Sherrington, R., Rogae, E. I., Lin, Y., Roque, A. M., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., and Holman, K. (1995) Nature 375, 754–760
Inhibition of PS1 Endoproteolysis by γ-Secretase Inhibitors

20. De Strooper, B., Saftig, P., Craessaerts, K., Vandersteichele, H., Guhde, G., Annanet, W., Von Figura, K., and Van Leuven, F. (1998) Nature 391, 387–390.

21. Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1996) Neuron 17, 181–190.

22. Kimberly, W. T., Xia, W., Rahmati, T., Wolfe, M. S., and Selkoe, D. J. (2000) J. Biol. Chem. 275, 3173–3178.

23. Wolfe, M. S., De Los, A. J., Miller, D. D., Xia, W., and Selkoe, D. J. (1999) Biochemistry 38, 11223–11230.

24. Li, Y. M., Lai, M. T., Xu, M., Huang, Q., DImuzio-Mower, J., Sardana, M. K., Shi, X. P., Yin, K. C., Shafier, J. A., and Gardell, S. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6138–6143.

25. Shearman, M. S., Beher, D., Clarke, E. E., Lewis, H. D., Harrison, T., Hunt, P., Nadin, A., Smith, A. L., Stevenson, G., and Castro, J. L. (2000) Biochemistry 39, 8689–8704.

26. Li, Y. M., Xu, M., Lai, M. T., Huang, Q., Castro, J. L., DImuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Nedevell, J. G., Register, R. B., Sardana, M. K., Shearman, M. S., Smith, A. L., Shi, X. P., Yin, K. C., Shafier, J. A., and Gardell, S. J. (2000) Nature 405, 689–694.

27. Edler, W. P., Kimberly, W. T., Ostaszewski, B. L., Diehl, T. S., Moore, C. L., Tsai, Y. J., Rahmati, T., Xia, W., Selkoe, D. J., and Wolfe, M. S. (2000) Nat. Cell Biol. 2, 428–434.

28. Seiffert, D., Bradley, J. D., Rominger, C. M., Rominger, D. H., Yang, F., Meredith, J. E., Jr., Wang, Q., Roach, A. H., Thompson, L. A., Spitz, S. M., Higaki, J. N., Prakash, S. R., Combs, A. P., Copeland, R. A., Arneric, S. P., Hartig, P. R., Robertson, D. W., Cordell, B., Stern, A. M., Olson, R. E., and Zacsek, R. (2000) J. Biol. Chem. 275, 34086–34091.

29. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) Nature 399, 513–517.

30. Evin, G., Sharples, R. A., Weidemann, A., Reinhard, F. B., Carbone, V., Culvenor, J. G., Holsinger, R. M., Sernee, M. F., Beyreuther, K., and Masters, C. L. (2001) Biochemistry 40, 8359–8368.

31. Culvenor, J. G., Evin, G., Cooney, M. A., Wardan, H., Sharples, R. A., Maher, F., Reed, G., Diehlmann, A., Weidemann, A., Beyreuther, K., and Masters, C. L. (2001) Cell 105, 192–206.

32. Kopan, R., Schroeter, E. H., Weintrob, C., and Nye, J. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1683–1688.

33. Dyrks, T., Dyrks, K., Monning, U., Urmonen, B., Turner, J., and Beyreuther, K. (1993) FEBS Lett. 355, 89–93.

34. Hill, H. D., and Straka, J. G. (1988) Anal. Biochem. 170, 203–208.

35. Beher, D., Elle, C., Underwood, J., Davis, J. B., Ward, R., Karran, E., Masters, C. L., Beyreuther, K., and Multhaup, G. (1999) J. Neurochem. 72, 1564–1573.

36. Khorkova, O. E., Patel, K., Heroux, J., and Sahasrabudhe, S. (1998) J. Neurosci. Methods 82, 159–166.

37. Yang, H., Leland, J. K., Yost, D., and Massey, R. J. (1994) Bio/Technology 12, 193–194.

38. Bilsland, J. G., and Harper, S. J. (1998) J. Neurosci. Methods 84, 121–130.

39. Naruse, S., Thinakaran, G., Luo, J. J., Kusiak, J. W., Tomita, T., Iwatsubo, T., Qian, X., Ginty, D. D., Price, D. L., Borchelt, D. R., Wung, P. C., and Sisodia, S. S. (1998) Neuron 21, 1213–1221.

40. deSols, S. J., Giuliani, E. A., Guare, J. P., Vacca, J. P., Sanders, W. M., Graham, S. L., Wiggins, J. M., Darke, P. L., Sigal, I. S., and Zugay, J. A. (1991) J. Med. Chem. 34, 2852–2857.

41. Chambers, S. B., Hoheisel, S. C., Fletcher, S. R., Matassa, V. G., Mitchell, P. J., Watt, A. P., Baker, R., Freedman, S. B., Patel, S., and Smith, A. J. (1993) Bioorg. Med. Chem. Lett. 3, 1919–1924.

42. Katritzky, A. R., Urogdi, L., and Mayen, E. (1990) J. Org. Chem. 55, 2206–2214.

43. Sherrill, R. G., and Sugg, E. E. (1995) J. Org. Chem. 60, 730–734.

44. Nadin, A., Sanchez Lopez, J. M., Nedevell, J. G., and Thomas, S. R. (2001) Tetrahedron 57, 1861–1864.

45. De Strooper, B., Anaeert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) Nature 398, 518–522.

46. Thinakaran, G., Harris, C. L., Ratovitski, T., Davenport, F., Slunt, H. H., Price, D. L., Borchelt, D. R., and Sisodia, S. S. (1997) J. Biol. Chem. 272, 28415–28422.

47. Zhang, J., Kang, D. E., Xia, W., Okoshi, M., Mor, H., Selkoe, D. J., and Koo, E. H. (1998) J. Biol. Chem. 273, 12436–12442.

48. Yu, G., Chen, F., Levesque, G., Nishimura, M., Zhang, D. M., Levesque, L., Rogaeva, E., Xu, D., Liang, Y., Dunn, M., George-Hyslop, P. H., and Fraser, P. E. (1998) J. Biol. Chem. 273, 16470–16475.

49. Duden, R., Griffiths, G., Frank, R., Argos, P., and Kreis, T. E. (1991) Cell 64, 649–665.

50. Steiner, H., Capelli, A., Pesold, B., Citron, M., Klieptel, P. M., Selkoe, D. J., Romig, H., Mendla, K., and Haas, C. (1998) J. Biol. Chem. 273, 32322–32331.

51. Ratovitski, T., Slunt, H. H., Thinakaran, G., Price, D. L., Sisodia, S. S., and Borchelt, D. R. (1997) J. Biol. Chem. 272, 24536–24541.

52. Yu, G., Nishimura, M., Arawaks, S., Levitan, D., Zhang, L., Tandon, A., Song, Y. Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. H., Sato, C., Rogaeva, E., Smith, M., Janus, C., Zhang, Y., Abersold, R., Faber, L. S., Surbi, S., Bruni, A., Fraser, P., and George-Hyslop, P. (2000) Nature 407, 48–54.

53. Petit, A., Bibel, M., Alves da Costa, C., Pourquié, O., Checler, F., and Kraus, J. L. (2001) Nat. Cell Biol. 3, 507–511.

54. Clarke, E. E., and Shearman, M. S. (2000) J. Neurosci. Methods 102, 61–68.
