In Vitro Characterization of the Presenilin-Dependent γ-Secretase Complex Using a Novel Affinity Ligand

Dirk Beher,*,‡ Michael Fricker,‡ Alan Nadin,§ Earl E. Clarke,‡ Jonathan D. J. Wrigley,‡ Yue-Ming Li,∥,⊥ Janetta G. Culvenor,# Colin L. Masters,# Timothy Harrison,§ and Mark S. Shearman‡

Departments of Biochemistry & Molecular Biology and Medicinal Chemistry, Merck Sharp & Dohme Research Laboratories, The Neuroscience Research Centre, Terlings Park, Harlow, Essex CM20 2QR, United Kingdom, Department of Biological Chemistry, Merck Research Laboratories, West Point, Pennsylvania, and Department of Pathology, University of Melbourne, and Neuropathology Laboratory, Mental Health Institute of Victoria, Parkville, Victoria 3010, Australia

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ABSTRACT: γ-Secretase is the enzyme activity releasing the amyloid-β peptide from membrane-bound processing intermediates derived from the β-amyloid precursor protein. Cellular release and subsequent aggregation of the amyloid-β peptide is thought to be causative for the pathogenesis of Alzheimer's disease. γ-Secretase performs an unusual intramembranous cleavage and has been closely linked to a macromolecular complex containing presenilins. To generate a molecular probe for γ-secretase, we have developed a novel biotinylated affinity ligand which is based on a specific inhibitor containing a hydroxyethylene dipeptide isostere, known to serve as a transition state analogue for aspartic proteinases. Using this probe we confirmed the presence of the presenilin heterodimer and mature nicastrin in the active enzyme complex and, furthermore, that substrate binding site(s) and active center(s) are spatially separated. Affinity precipitations suggest that only a discrete fraction of cellular presenilin is present in the active γ-secretase complex and that both γ(40)- and γ(42)-activities are mediated by the same molecular entity. This was also reflected by a co-distribution of both enzyme activities in subcellular fractions enriched for *trans*-Golgi network membranes.

The generation of amyloid-β (Aβ)[1] peptides thought to be causative agents in Alzheimer's disease (AD) requires sequential processing of the β-amyloid precursor protein (βAPP) (*1*) by β- and γ-secretase enzymes. An alternative processing pathway leads to the release of the βAPP ectodomain as secretory βAPP by cleavage within the Aβ domain, thereby excluding Aβ peptide formation (*2*). Members of the disintegrin and metalloprotease family (ADAM) such as ADAM 10 and TACE appear to mediate this alternative cleavage, termed α-secretase cleavage (*3*, *4*). β-Site βAPP-cleaving enzyme 1 (BACE1, Asp-2) (*5*−*9*) is the major β-secretase responsible for the generation of Aβ peptides by neurones (*10*) and has been shown to generate the membrane-bound βAPP C-terminal fragment (C99) intermediate, which is a prerequisite for the release of Aβ peptide by γ-secretase. Its homologue BACE2 (Asp-1) (*8*, *11*, *12*), however, appears to function as an alternative α-secretase (*13*) since it cleaves preferentially near the α-secretase site after residues 19 and 20 of the Aβ peptide sequence (*14*). Whereas BACE has been characterized in detail using overexpressing cells lines (*15*) and purified recombinant enzyme (*16*), γ-secretase, the critical enzyme releasing the Aβ peptide from membrane-bound βAPP processing intermediates, has been much more elusive. This enzyme appears to have an extremely loose substrate specificity (*17*) and cleaves rather at a specific position relative to the membrane bilayer (*18*). γ-Secretase itself displays characteristics of an aspartyl protease, as it is inhibited by structurally diverse inhibitors that can mimic the transition state for this class of protease. These include compounds with moderate affinities such as substrate-based difluoroketone analogues (*19*) or potent inhibitors containing a hydroxyethylene dipeptide isostere (*20*).

A variety of Aβ peptides with C- and N-terminal truncations can be detected in cell culture media (*21*) and the brain (*22*). Their generation depends on γ-secretase (*23*), indicating

* Corresponding author. Address: Department of Biochemistry & Molecular Biology, Merck Sharp & Dohme Research Laboratories, The Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, U.K. Tel.: +44-1279-440478. Fax: +44-1279-440712. E-mail: dirk_beher@merck.com.

‡ Department of Biochemistry & Molecular Biology, Merck Sharp & Dohme Research Laboratories.

§ Department of Medicinal Chemistry, Merck Sharp & Dohme Research Laboratories.

∥ Merck Research Laboratories.

⊥ Present address: Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, New York 10021.

University of Melbourne and Mental Health Institute of Victoria.

[1] Abbreviations: AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; Aβ, amyloid-β peptide; CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate; DCM, dichloromethane; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; ECL, electrochemiluminescence; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; ER, endoplasmic reticulum; HOBT, 1-hydroxybenzotriazole hydrate; MES, 2-[N-morpholino]ethanesulfonic acid; TBAF, tetrabutylammonium fluoride; TFA, trifluoroacetic acid; TGN, *trans*-Golgi network; THF, tetrahydrofuran; PS1/2, presenilin 1/2; PS1-FL, full-length presenilin 1; NTF, N-terminal fragment; CTF, C-terminal fragment; PAGE, polyacrylamide gel electrophoresis; SAR, structure−activity relationship; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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that the same enzyme could cleave at multiple positions. This property appears to be critical for the disease mechanisms since an 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 *24* and *25*], is linked to all known mutations causing an autosomal dominant inherited variant of AD (familial AD, FAD). These mutations are either found in genes encoding βAPP itself or the presenilins 1 and 2 (PS1 and PS2), polytopic membrane proteins which have been linked closely to γ-secretase. Presenilins themselves undergo endoproteolytic processing within their putative loop region, yielding N-terminal and C-terminal polypeptides (PS1-NTF and PS1-CTF) thought to consist, respectively, of six, and two membrane domains (*26*). Presenilin expression is absolutely required for γ-secretase activity, as inactivation of PS1 in neurones leads to a drastic reduction of γ-secretase activity and an accumulation of the corresponding substrates, βAPP CTFs (*27*). This has been substantiated by the observation that in PS1/PS2 double knock-out models γ-secretase activity is abolished completely (*28*, *29*). A further link of presenilins to γ-secretase activity was established by results obtained from mutagenesis studies showing that substitution of either of two conserved aspartate residues in transmembrane domains 6 or 7 of PS1 (D275 or D385) inactivates γ-secretase and diminishes PS1 endoproteolysis (*30*). Since 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 (*30*). This model is substantially supported by the observation that affinity probes derived from γ-secretase inhibitors selectively label the fragments of PS1 (*31*−*33*). Various potent γ-secretase inhibitors do not only act as inhibitors of enzyme activity but are also able to block the endoproteolytic processing of PS1 into its fragments (*34*) further highlighting a close relationship of PS1 to the enzyme. Moreover, two transmembrane aspartates in opposing transmembrane domains appear to constitute a characteristic motif which is also found in a signal peptide peptidase which has been recently cloned and characterized as a presenilin-type aspartyl protease (*35*).

γ-Secretase activity does not solely depend on presenilins but appears to require the formation of a high-molecular weight complex with nicastrin (*36*), and two other proteins identified in functional screens in *Caenorhabditis elegans* termed aph-1 and pen-2 (*37*, *38*). Coprecipitation studies suggest that these proteins interact physically with presenilins (*39*−*41*) and interference with their RNA expression abolishes γ-secretase activity (*38*). To study further the biochemical identity of the γ-secretase enzyme and to discriminate PS1 interactions in the enzyme complex from enzyme-independent interactions, we have developed a novel biotinylated affinity ligand based on a well characterized aspartyl protease transition state analogue γ-secretase inhibitor. Affinity precipitation studies revealed a specific capture of PS1-NTF, PS1-CTF, the substrate β-CTF (C99), and mature nicastrin in the enzyme complex. The selective presence of mature nicastrin suggests that formation of the active enzyme complex requires trafficking of its components to a late compartment of biosynthesis. Accordingly, a peak for γ-secretase enzyme activity was found in a subcellular fraction enriched for *trans*-Golgi network (TGN) membranes but absent in fractions containing early biosynthetic compartments such as the endoplasmic reticulum (ER). Further evidence suggests that only a discrete pool of total cellular PS1 is present in the active enzyme complex which mediates both γ(40)- and γ(42)-secretase activities.

EXPERIMENTAL PROCEDURES

Materials. Monoclonal antibodies and polyclonal antisera were obtained from the following sources and diluted for Western blot analyses as indicated: anti-βAPP (22C11, Chemicon, 0.5 μg/mL), anti-calnexin (StressGen, 1:2,500), anti-β-catenin (BD Transduction Laboratories, 1:1,000), anti-β−COP (Sigma, 1:500), anti-syntaxin 6 (BD Transduction Laboratories, 1:500), anti-rab6 (Autogen Bioclear, 1:500), biotinylated anti-Aβ 4G8 (Senetek), HRP-conjugated polyclonal goat anti-mouse and anti-rabbit antibodies (Amersham, 1:5,000), and polyclonal rabbit antiserum R7334 (raised against residues 659−694 of βAPP$_{695}$, 1:750). PS1-FL and its fragments were detected using the polyclonal rabbit antisera 00/2 raised against the loop peptide 301−317 (*42*) (1:2,000) and 98/1 raised against residues 1−20 of PS1 (1:2,500) (*43*). Polyclonal rabbit antiserum 00/19 against nicastrin was raised against a commercially synthesized peptide comprising the C-terminal residues 691−709 of human nicastrin which was coupled to diphtheria toxoid prior to the immunizations.

Western Blot Analyses. 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 in the figures, using the enhanced-chemiluminescence system (ECL, Amersham). Quantitation of bands using a computerized image analysis system (MCID, Imaging Research Inc.) was performed as described previously (*44*).

Membrane Preparation. Membranes from human SH-SY5Y neuroblastoma cells were prepared essentially as described previously (*34*). In short, 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, and 10 mM KCl and sedimented by centrifugation for 10 min at 1,000 g. Cells were homogenized in 20 mM HEPES-HCl, pH 7.3, and 90 mM KCl and nuclei and cellular debris removed by centrifugation for 10 min at 1000*g*. Cellular membranes were sedimented by centrifugation for 1 h at 45 000 rpm (50.2 Ti rotor, Beckman), resuspended in PBS and 5% glycerol, and stored at −80 °C prior to further use.

Subcellular Fractionation and Sucrose Density Gradient Centrifugation. Human SH-SY5Y neuroblastoma cell membranes were prepared as described above and separated on a linear continuous sucrose gradient (0.2−2M) according to ref *34*. Fractions were collected (17 × 1.0 mL) from the bottom, diluted into 5 mM HEPES-HCl, pH 7.3, and membranes were sedimented by centrifugation at 45 000 rpm (50.2 Ti rotor, Beckman) for 1 h. The final pellets were homogenized in 400 μL PBS and 5% glycerol (v/v) and stored at −80 °C prior to immunoblot analysis or in vitro γ-secretase assays.

CHAPSO-Solubilization of Active γ-Secretase and Inhibitor Affinity Precipitations. SH-SY5Y 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 M NaCl, 5 mM $MgCl_2$, 1× EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals). Insoluble debris was removed by centrifugation at 180 000*g* for 30 min and the resulting supernatant (solubilized γ-secretase) adjusted with the same buffer without CHAPSO to give a final detergent concentration of 0.5% CHAPSO (w/v). Endogenous biotinylated proteins were removed by adding streptavidin-coupled magnetic beads and centrifugation for 2 min at 20 000*g* after an incubation for 30 min at 4 °C. For specific capture the precleared solubilized preparation (1.8 mL; 0.6−0.7 mg/mL protein) was incubated for 2 h at room temperature with the biotinylated aspartyl transition state analogue inhibitor Merck C at the concentrations indicated in the figure legends. Nonspecific binding was analyzed either by omitting the biotinylated affinity ligand or adding a 100-fold excess of the nonbiotinylated inhibitor Merck A. γ-Secretase−inhibitor complexes were captured by addition of 250 μL (10 mg/mL) of streptavidin-coupled magnetic beads (Dynal) and incubation for 0.5 h at room temperature. Enzyme−inhibitor complexes were precipitated by centrifugation, the beads washed three times with corresponding buffer (0.5% CHAPSO) and subjected to immunoblotting as described (*34*) after addition of SDS-PAGE sample buffer. Note that routinely the precipitate was split into three samples for Western blot analysis. By considering these individual samples as 100%, the precipitate (100%) was compared to 2.5% of either the input before capture or the remaining unbound fraction after capture.

Exogenous Substrate γ-Secretase Assays. For determination of the potencies of Merck A and B, membranes from SH-SY5Y membranes stored in PBS, 5% glycerol were collected by centrifugation for 30 min at 180 000*g*. For in vitro generation of Aβ peptides, 80 μg of membranes were incubated with 7.5 μg of recombinant C100Flag (*45*) in 20 mM HEPES, pH 7.3, 2 mM EDTA, 0.1% bovine serum albumin, 0.5% CHAPSO in 100 μL final volume similar to described methods (*45*) in the presence of increasing compound concentrations. Aβ peptides were quantified by an electrochemiluminescence assay in a 96-well plate format [Origen M-Series analyzer, Igen] as described (*34*) using 35 μL of the reaction for Aβ(40) and 50 μL for Aβ(42) detection. Nonspecific background was defined by the signal obtained when the assay was performed in the presence of 10 μM of Merck A. For determination of γ-secretase activity in the sucrose gradient fractions, 20 μL of each fraction was incubated with 10 μg of recombinant C100Flag and processed further as above.

Synthetic Chemistry. A solution of $BOCNH(CH_2)_5NHCO(CH_2)_5NH_2$ (1.1 g), $HO_2C(CH_2)_5NHCO(CH_2)_5NHFMOC$ (1.5 g), EDC (0.86 g), and HOBT (0.61 g) in DMF (20 mL) was stirred at room temperature for 5 days. The reaction mixture was diluted with ethyl acetate, and washed with citric acid solution, sodium bicarbonate solution, and brine. A white precipitate formed, which was collected by filtration and washed with water and ether several times and dried in vacuo to give the tripeptide (1.9 g, 77%). The resulting tripeptide (1.0 g) was dissolved in 10% TFA-DCM and stirred overnight. The reaction mixture was evaporated in vacuo and purified by column chromatography to give the amine (1.0 g, ca. 100%) as a white powder. This was dissolved in DMF (15 mL) and treated with Leu-Phe-NH_2 (0.82 g), EDC (0.48 g), HOBT (0.34 g) and stirred for 72 h. The reaction mixture was diluted with ethyl acetate and brine. A white precipitate formed, which was collected by filtration and washed with water and ether and dried in vacuo to give the corresponding pentapeptide (0.8 g). This was dissolved in 20% TFA-DCM and stirred for 2 h. The reaction mixture was evaporated in vacuo and purified by flash column chromatography to give the amine (500 mg, 31%) as a white solid. This was dissolved in DMF (10 mL) and treated with 2*R*-benzyl-5*S*-*tert*-butoxycarbonylamino-4*R*-(*tert*-butyldimethylsilanyloxy)-6-phenyl-hexanoic acid (*46*) (0.31 g), EDC (134 mg), and HOBT (95 mg) and stirred overnight. The reaction mixture was diluted with ethyl acetate and washed with citric acid solution, sodium bicarbonate solution, and brine, dried ($MgSO_4$), filtered, and evaporated in vacuo. Purification by flash column chromatography gave the hexapeptide (700 mg, 90%) as a white powder. The hexapeptide (350 mg) was dissolved in TBAF (1.0 M in THF, 3 mL) and stirred for 2 days. The reaction mixture was evaporated in vacuo, purified by column chromatography on silica and further by reverse-phase HPLC to give Merck B (50 mg).

A solution of Merck B (10 mg) was treated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce) (1 equiv) and triethylamine (1 equiv) and stirred overnight. Addition of water and ether caused formation of a precipitate, which was filtered and washed with ether and water to give Merck C (9.6 mg, 78%) as a white solid. The synthesis of Merck A has been described previously (*20*).

RESULTS

Development of a Biotinylated γ-Secretase Affinity Ligand. To develop a novel soluble γ-secretase affinity ligand, we synthesized Merck B, a derivative of our well-characterized affinity probes (*31*) with an extended linker and a free amine functionality allowing further derivatization (Figure 1a). Essentially, this compound represents an extended version of Merck A, a potent and selective γ-secretase inhibitor (*20*) containing a hydroxyethylene dipeptide isostere. This group is known to mimic one of the two hydroxyl groups of the *gem*-diol transition state of aspartyl protease substrates. The inhibitor potencies of Merck B and its parent compound Merck A were compared for inhibition of γ(40)- and γ(42)-secretase activities in the exogenous substrate enzyme assay (*45*) (Figure 1b,c). The data reveal that Merck A and Merck B are both potent inhibitors of γ(40)- and γ(42)-secretase activities with IC_{50} values in the low nanomolar range. In good accordance with previous observations using a variety of specific inhibitors in cell-based assays (*34*), the actual potencies for inhibition of both γ-secretase activities are comparable. Merck B was used as an intermediate to synthesize the biotinylated affinity ligand Merck C by coupling of its primary amine to a thiol-cleavable linker with a biotin moiety attached. Since the detection of the Aβ peptides generated in the γ-secretase enzyme assay depends on their capture by a biotinylated antibody, it was predictable that Merck C would compete in this assay and therefore we did not evaluate this derivative under these conditions. The structure−activity relationship (SAR) for this inhibitor series, however, indicates clearly that C-terminal extensions at the peptide moiety are well tolerated, as demonstrated by the

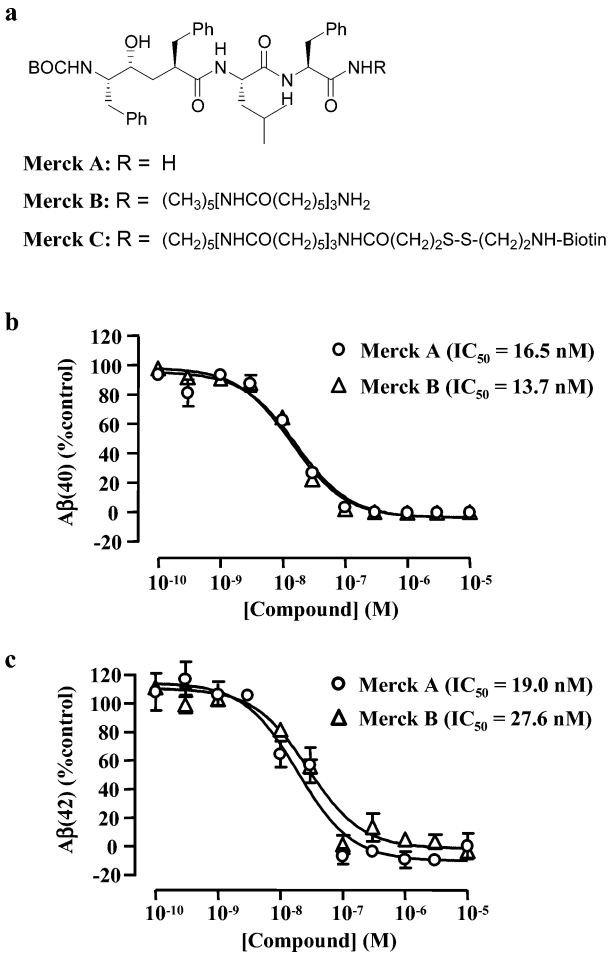

FIGURE 1: γ-Secretase inhibitors synthesized for this study and their potencies for inhibition of γ(40)- and γ(42)-secretase activities. (a) Structures of the aspartyl protease transition state analogue Merck A (*20*) and its extended derivatives Merck B and Merck C. IC_{50} values for inhibition of the in vitro generation of (b) Aβ(40) and (c) Aβ(42) in the exogenous substrate assay utilizing SH-SY5Y membranes as source of γ-secretase enzyme and recombinant C100Flag as substrate. Note that the assay is performed in the presence of 0.5% CHAPSO, which is the optimum detergent concentration for γ-secretase activity in these membranes (D. Beher, unpublished observation). The reduction of Aβ generation was measured relative to Me_2SO-treated controls, and error bars indicate the standard error of the mean from triplicate experiments.

high potencies of Merck B (Figure 1) and similar biotinylated photoaffinity probes described previously (*31*).

Merck C Captures the PS1 Heterodimer and Mature Nicastrin. To determine the potential of Merck C to capture solubilized γ-secretase, we optimized the parameters for capture of PS1 fragments which, according to current knowledge, are the most likely candidate for the active site of the enzyme (*31*, *47*, *48*). Inhibitor binding was expected to be optimal under solubilization conditions where the enzyme remains in a catalytically active conformation. Accordingly, human SH-SY5Y neuroblastoma cell membranes were solubilized in CHAPSO since this detergent has been shown to provide the best means for preserving γ-secretase activity in a solubilized state (*45*) and has been used in almost all studies to date [e.g., refs *31* and *49*].

CHAPSO-solubilized γ-secretase was incubated with the biotinylated affinity ligand Merck C followed by precipitation of enzyme−inhibitor complexes with streptavidin-coupled magnetic beads. Polypeptides present in the precipitates ("Bound"), and the corresponding supernatants ("Unbound") were characterized by Western blotting. Optimal capture conditions were determined empirically, as those yielding the most efficient captures when γ-secretase was solubilized at pH 6.0 and precipitated by streptavidin-coupled magnetic beads (as these beads appeared to show the lowest nonspecific binding when compared to alternative matrices such as streptavidin-agarose beads; D. Beher, unpublished observation). Further analysis revealed that by combining an incubation of solubilized γ-secretase at an affinity ligand concentration of 0.1 μM followed by capture using 2.5 mg of magnetic beads (Figure 2a,b), an optimal specific precipitation of PS1 fragments and nicastrin was observed. Omission of the affinity ligand (Figure 2a,b) abolished the precipitation of all these polypeptides. Using the optimized conditions, neither β-catenin nor βAPP was captured specifically (Figure 2), since these proteins were essentially undetectable in precipitates and only observed in the corresponding input and unbound fractions. To provide additional proof of the capture specificity, a similar affinity capture experiment was performed using the affinity ligand Merck C (0.1 μM) in the presence or absence of a 100-fold excess Merck A (10 μM) (Figure 3a). Again, both PS1 fragments and nicastrin were specifically precipitated, whereas β-catenin was not detected in these fractions, only being found in the input and unbound fractions. In the presence of an excess of Merck A, PS1-NTF and PS1-CTF and nicastrin immunoreactivities were virtually undetectable in the bound fractions (Figure 3a, "bound nonspec."). This result implies that either omission of the affinity ligand or addition of an excess of Merck A can serve as an appropriate control for nonspecific capture. Furthermore, a selective precipitation of mature nicastrin was observed as highlighted in Figure 3b and seen essentially in all capture experiments (Figures 2a−5b). Whereas two nicastrin bands migrating at ~108 and ~100 kDa were detected in the input fraction, only the higher molecular weight band (~108 kDa) was captured specifically by the affinity ligand. It is noteworthy that recent studies indicate that both mature and immature forms of nicastrin carry *N*-linked oligosaccharides and differences in the migration could be caused by differential maturation of the *N*-glycan chains (*39*, *50*).

Simultaneous binding Of Merck B and the Substrate to the γ-Secretase Enzyme. Recent affinity precipitations studies using an immobilized inhibitor (*49*) suggest that substrate binding site(s) and active center(s) of the enzyme are not identical. To test this further, we repeated the capture experiments using membranes prepared from SH-SY5Y cells stably overexpressing the γ-secretase substrate SPA4CT, an artifical β-CTF (C99) variant (*51*). Again, a specific capture of PS1 fragments and nicastrin was observed (Figure 4). β-CTF accumulates as the predominant γ-secretase substrate in these membranes, and consequently β-CTF was precipitated specifically by the affinity ligand. It is noteworthy that the affinity ligand did not bind to PS1-FL (Figure 4) as seen by its complete absence in the bound fraction.

Only a Discrete Fraction of PS1 is Captured with Active γ-Secretase. Considering the results obtained with the affinity ligand (Figures 2−4), there appears to be a low overall capture efficiency. Using this approach neither an obvious depletion of PS1-NTF and PS1-CTF nor nicastrin immunoreactivities was observed in the corresponding supernatants

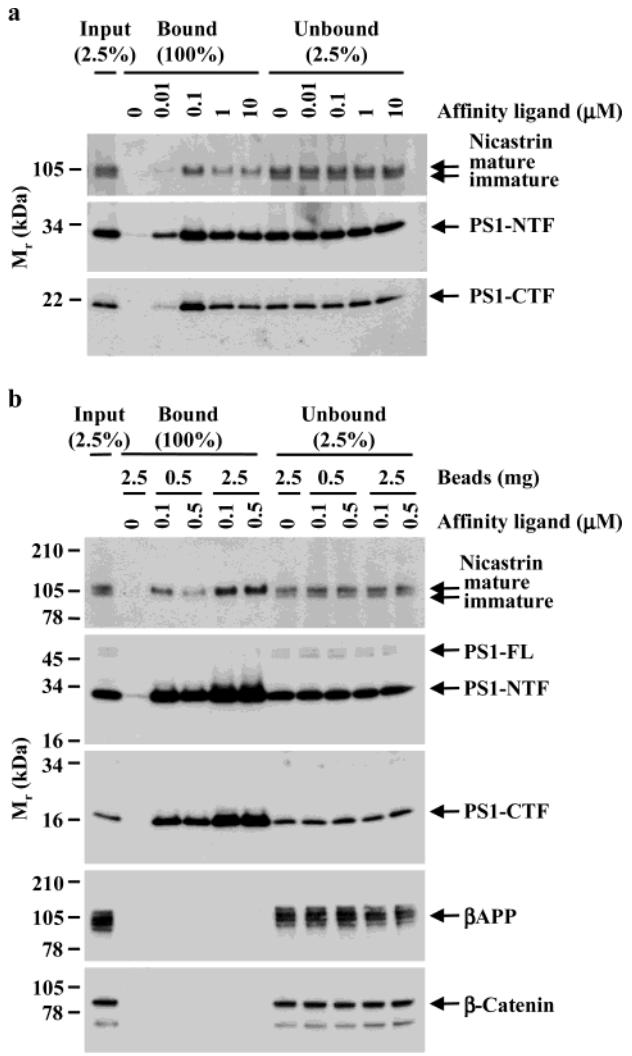

FIGURE 2: Affinity capture of PS1 heterodimer and nicastrin by the biotinylated inhibitor Merck C. (a) Solubilized γ-secretase from SH-SY5Y membranes was incubated with increasing concentrations of the biotinylated affinity ligand Merck C and precipitated with a constant amount of streptavidin-coupled beads (0.5 mg beads). CHAPSO-solubilized membranes before ligand addition (input: 2.5% of total) were compared to the captured fraction (bound: 100% of total) and the corresponding supernatant after capture (unbound: 2.5% of total) by Western blot analysis. Individual polypeptides were immunostained as indicated. (b) Solubilized γ-secretase from SH-SY5Y membranes was incubated with different concentrations of the affinity ligand (0.1 μM and 0.5 μM as indicated) and precipitated with increasing amounts of streptavidin-coupled beads (0.5 and 2.5 mg of beads, respectively). Again, CHAPSO-solubilized membranes before ligand addition (input: 2.5% of total) were compared to the captured fraction (bound: 100% of total) and the corresponding supernatant after capture (unbound: 2.5% of total) by Western blot analysis. Individual polypeptides were immunostained as indicated.

of the captures ("unbound") when compared to the original input. Furthermore, considering that the Western blot analyses compare 100% of the bound fractions to 2.5% of the input/unbound fractions, there appears to be a specific capture representing ~10% of total PS1 (densitometry of PS1-NTF and PS1-CTF lanes; mean of all experiments shown in Figures 2b−4). To investigate this further, we sought to quantitate the depletion of γ-secretase enzyme activity in the corresponding supernatants following affinity precipitation. After formation of enzyme−inhibitor complexes, the samples were treated with a total of eight repeated additions

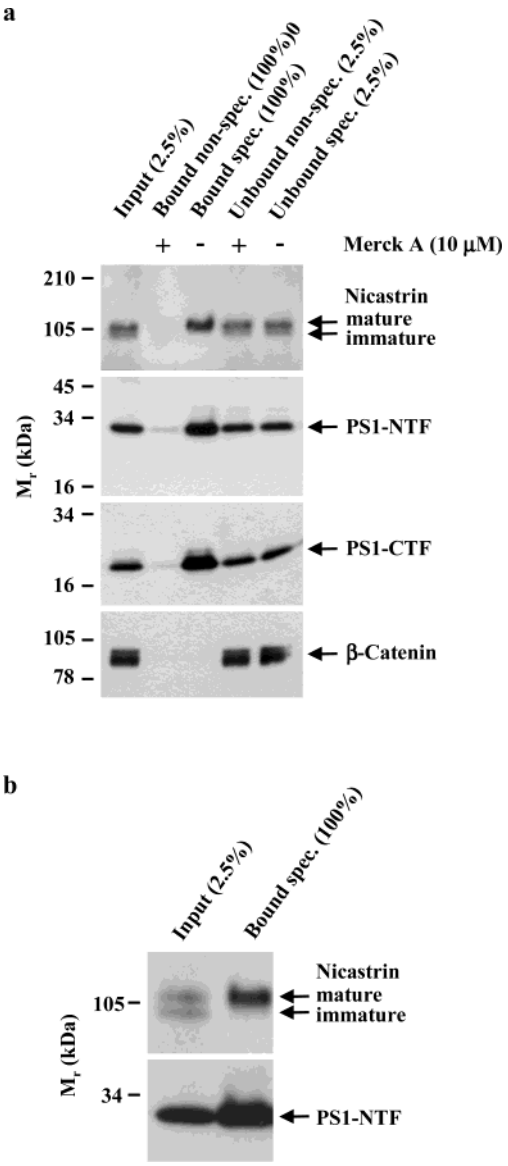

FIGURE 3: The binding of PS1 heterodimer and nicastrin to the biotinylated affinity probe is competed by the nonbiotinylated inhibitor Merck A. (a) Solubilized γ-secretase from SH-SY5Y membranes was incubated with 0.1 μM of the affinity ligand Merck C in the absence or presence of a 100-fold excess (10 μM) of Merck A and precipitated with streptavidin-coupled beads (2.5 mg). CHAPSO-solubilized membranes before ligand addition (input: 2.5% of total) were compared to the captured fraction in the presence (bound nonspec.: 100% of total) or absence (bound spec.: 100% of total) of the competing inhibitor Merck A and the corresponding supernatants after capture (unbound nonspec. and unbound spec.: 2.5% of total) by Western blot analysis. Individual polypeptides were immunostained as indicated. In the presence of a 100-fold excess of competing inhibitor Merck A, the precipitation of the PS1 heterodimer and nicastrin is abolished. β-Catenin, however, is not captured under any condition. (b) Solubilized γ-secretase from SH-SY5Y membranes was incubated with 0.1 μM of the affinity ligand Merck C and precipitated with streptavidin-coupled beads (2.5 mg). CHAPSO-solubilized membranes before ligand addition (input: 2.5% of total) were compared to the specifically captured fraction (bound spec.: 100% of total) by Western blot analysis after an extended gel run. A clear separation of mature and immature nicastrin polypeptides is visible in the input and only the band with a higher Mr representing mature nicastrin is captured by the affinity ligand.

of streptavidin beads followed by precipitation to remove any free affinity ligand. As shown (Figure 5a,b) with an

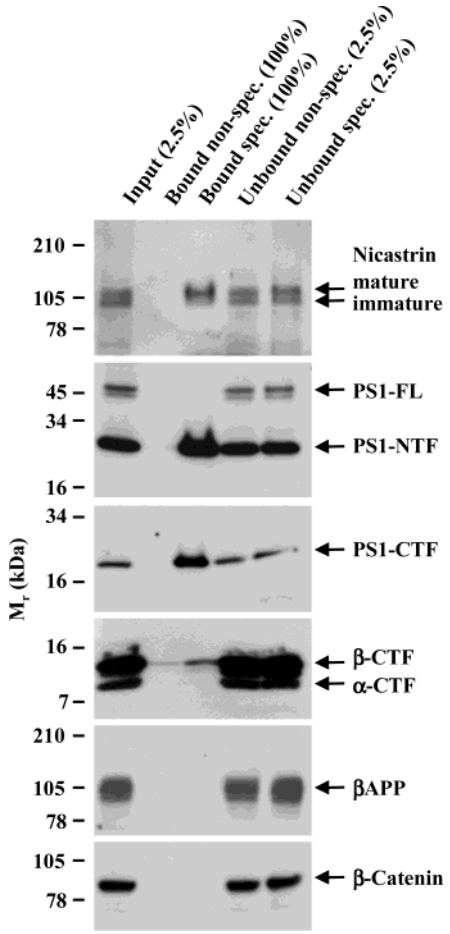

FIGURE 4: Simultaneous binding of the substrate and a transition state analogue inhibitor to γ-secretase. γ-Secretase was solubilized using membranes prepared from SH-SY5Y cells stably overexpressing a γ-secretase substrate β-CTF (C99) variant. The solubilized membranes were incubated either in the presence of Merck A or the affinity probe Merck C (0.1 μM each) followed by precipitation with streptavidin-coupled beads (2.5 mg). Merck A was added to the control sample without the affinity ligand to ensure that both samples contain a γ-secretase inhibitor as a precaution to prevent any turnover of the β-CTF by γ-secretase. Solubilized membranes before ligand addition (input: 2.5% of total) were compared to the captured fraction in the absence (bound nonspec.: 100%) or presence (bound spec.: 100% of total) of the affinity ligand and the corresponding supernatants after capture (unbound nonspec. and unbound spec.: 2.5% of total each) by Western blot analysis. Individual polypeptides were immunostained as indicated.

increased number of repeated bead additions PS1-NTF, PS1-CTF, and nicastrin immunoreactivities decreased in the bound fraction to undetectable levels. This indicates the efficient removal of the affinity ligand. When the final supernatant (after eight repeated captures) was assayed for γ-secretase enzyme activity under conditions identical to the affinity capture, a ~70% decrease of both γ(40)- and γ(42)-secretase activity was observed (Figure 5c). Taken together, these data imply that ~10% of the total PS1 captured appears to account for ~70% of the total γ-secretase enzyme activity. This indicates that only a discrete fraction of total cellular presenilin and nicastrin molecules are present in the active enzyme complex. Furthermore, as expected from the inhibitor profiling data (Figure 1), a single specific affinity ligand binds both to γ(40)- and γ(42)-secretase, which suggests that these activities are mediated by the same molecular entity.

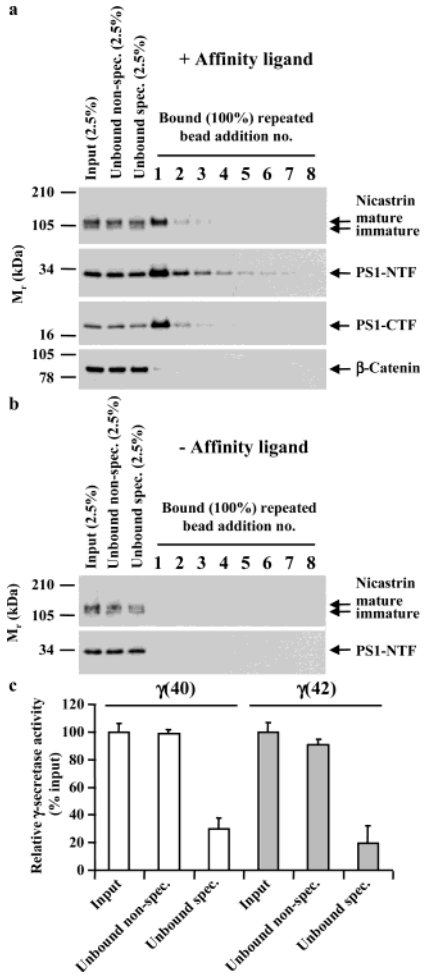

FIGURE 5: Quantitation of the depletion of γ-secretase activity from solubilized membranes by binding to the biotinylated affinity probe. Solubilized γ-secretase from SH-SY5Y membranes was incubated (a) in the presence (0.1 μM; + affinity ligand) or (b) absence of the biotinylated affinity ligand Merck C (− affinity ligand). For capture of γ-secretase and free ligand, streptavidin-coupled beads (2.5 mg) were added eight times repeatedly (without any further affinity ligand addition). For each individual streptavidin-bead addition, a 30 min capture incubation was followed by subsequent precipitation of the beads by centrifugation. The resulting supernatant was subjected to the next repeated streptavidin bead capture and the corresponding bead pellet washed three times in CHAPSO-buffer. (a) Solubilized γ-secretase before ligand addition (input: 2.5% of total) and corresponding supernatants after eight repeated bead captures in the absence (unbound nonspec.: 2.5% of total) or original presence of the affinity ligand (unbound spec.: 2.5% of total) were compared by Western blot analysis. Polypeptides captured by each individual bead addition (100% of total: + affinity ligand) are shown as indicated. (b) The results of the corresponding experiment performed in the absence of the affinity ligand are shown. Solubilized γ-secretase before ligand addition (input: 2.5% of total) and corresponding supernatants after eight repeated bead captures in the absence (unbound nonspec.: 2.5% of total) or original presence of the affinity ligand (unbound spec.: 2.5% of total) were compared to the material captured by each individual bead addition in total absence of the affinity ligand (100% of total: − affinity ligand). (c) γ(40)- and γ(42)-secretase activities present in the solubilized membranes before ligand addition (input) and after eight repeated bead captures in the absence (unbound nonspec.) or original presence of the affinity ligand (unbound spec.) were compared using an exogenous substrate assay (10 μg C100Flag, 40 μL of solubilized enzyme each reaction). The samples used for this assay were from the same experiments shown in panels a and b. Error bars indicate the standard error of the measurements obtained from quadruplicate in vitro reactions. The results shown in panels a−c are representative of three independent experiments.

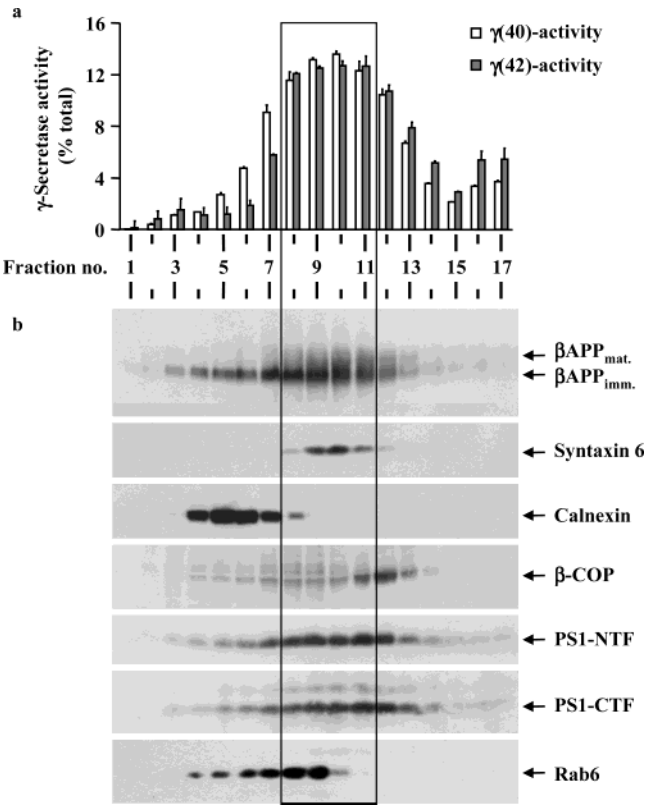

FIGURE 6: Subcellular fractionation of γ-secretase. Membranous organelles from SH-SY5Y cells were separated by sucrose density gradient centrifugation. (a) Equal aliquots of individual fractions were analyzed for γ(40)- and γ(42)-secretase activities using an exogenous substrate assay and the relative enzyme activity in each fraction was plotted in the graph. (b) Equal aliquots of the same fractions were immunoblotted for individual polypeptides as indicated. The graph in panel a shows the average of duplicate measurements, and the data shown in panels a and b are representative of three independent experiments. The box highlights four fractions containing the highest γ(40)- and γ(42)-secretase activities (fractions 8−11).

Subcellular Distribution of γ(40)- and γ(42)-Secretase Activity. To investigate whether the above finding is supported by the subcellular distribution of both γ-secretase activities, γ(40) and γ(42), membranous organelles from SH-SY5Y neuroblastoma cells were separated by sucrose equilibrium density gradient centrifugation. The distribution of γ(40)- and γ(42)-secretase activity across the gradient was measured by de novo production of Aβ peptides from the recombinant substrate C100Flag in individual fractions and directly compared to known marker proteins (as detected by Western blotting) (Figure 6a,b). β-COP is a COP-I coatamer component required for transport between ER and Golgi (*52*) and resides mainly in the ER-Golgi intermediate compartment (ERGIC) (*53*, *54*) and early Golgi compartments such as *cis*-Golgi (*52*). Rab6 is a ubiquitous small GTPase associated with the membranes of *medial*- and *trans*-Golgi (*55*, *56*) and has been implicated in *intra*-Golgi transport (*57*) and a novel Golgi/ER retrograde pathway (*58*, *59*). Although PS1-NTF and PS1-CTF immunoreactivities (Figure 6b) and γ(40)- and γ(42)-secretase enzyme activity (Figure 6a) could be detected across the gradient, the actual peaks were dissimilar to those of the marker proteins of the earlier biosynthetic organelles such as the ER marker calnexin, ERGIC marker β-COP, and *medial*/*trans*-Golgi marker rab6. In contrast to this, the main peaks for γ(40)- and γ(42)-secretase activity and PS1 fragments tracked the distribution of syntaxin 6 (Figures 6a and b, fractions 8−11, highlighted box). Overall this suggests an enrichment of γ-secretase enzyme activity in the *trans*-Golgi network (TGN) since syntaxin 6 is localized mainly to this organelle (*60*, *61*) and has been implicated in the clathrin-coated vesicle trafficking from the TGN to endosomes (*62*). Most interestingly, γ-(40)- and γ(42)-secretase activity were not separable as demonstrated by an identical co-distribution across the entire gradient.

DISCUSSION

Since their original discovery as causative genes for FAD (*63*, *64*), much experimental evidence generated has linked presenilins intimately to the γ-secretase enzyme responsible for the intramembrane cleavage of the βAPP-CTFs and other type I transmembrane proteins. To generate a molecular probe for the characterization of this enzyme, we have developed a novel biotinylated γ-secretase inhibitor. In contrast to alternative approaches such as co-immunoprecipitation experiments, this inhibitor-based strategy provides a means to discriminate catalytically active enzyme complexes from alternative complexes formed by individual components. Using conditions which preserve enzyme activity, the affinity ligand specifically captured both the PS1 heterodimer and mature nicastrin. In addition to isolation of these polypeptides, we observed simultaneous binding of γ-secretase and a βAPP-derived substrate, with a distinct preference for the binding of β-CTF, compared to the alternative α-CTF (C83) substrate. This could be either a result of the greater abundance of β-CTF in the membrane source used for this experiment (cells overexpressing a variant of β-CTF), assuming a competition of both βAPP-CTFs for the enzyme, or, alternatively, an indication of different affinities of the enzyme for these individual substrates. With respect to the co-isolation of γ-secretase and its substrate, similar findings have been reported using an immobilized γ-secretase inhibitor (*49*). However, in that study α-CTF (C83) was exclusively captured since it was the only βAPP-derived γ-secretase substrate detectable in the untransfected cell line used as a source of enzyme. On the basis of this finding, it was suggested that substrate binding site(s) and catalytic center(s) of the enzyme are spatially discrete (*49*). The observation that common γ-secretase inhibitors, including the parent compound used for the development of the affinity ligand, act as linear, noncompetitive inhibitors (*65*) supports this model even further.

It is noteworthy that it was known previously that inactivation of γ-secretase either by mutagenesis of the critical transmembrane aspartates or γ-secretase inhibitor treatment promotes the interaction with βAPP-CTFs (*66*). This finding can be explained in retrospect knowing that substrate binding and inhibitor binding to PS1 can occur simultaneously—in the presence of an inhibitor (or the inactivating mutations), the enzyme is prevented from cleaving the substrate but still binds the substrate which consequently can be coprecipitated with PS1.

In good agreement with ref *49*, we fail to observe any capture of either the well-described PS1 ligand β-catenin (*67*) or βAPP, which was reported to form stable complexes with PS1 when both proteins are overexpressed together in cells

(*68*, *69*). This clearly indicates that although PS1 can interact with these polypeptides under certain conditions, these interactions are functionally irrelevant with respect to γ-secretase function. In this context, it is noteworthy that it has been recently reported that PS1 regulates β-catenin stability independent of its associated proteolytic function (*70*). Similar conclusions have been drawn previously regarding the potential role of PS1 in intracellular trafficking and especially the maturation of the Trk receptor (*34*). Multiple functions and diverse interactions of PS1 could also explain our finding that only a discrete fraction of cellular PS1 appears to be associated with γ-secretase activity. It is unclear at present what exactly discriminates the pool of PS1 captured by the affinity probe from the unbound fraction lacking γ-secretase-associated activity. It could be either PS1 molecules involved in various other interactions (such as the PS1/β-catenin interaction) or, potentially, the existence of different conformational states of PS1 and accessory components of the γ-secretase enzyme. The selective capture of mature nicastrin in the complex [published also by Kimberly et al. during the preparation of this manuscript (*71*)] has major implications for the biogenesis of the enzyme complex. Nicastrin is essential for γ-secretase function regarding γ-cleavage of βAPP and S3 cleavage of the Notch receptor (*36*, *72*−*74*). Since extensive processing of glycan chains occurs during trafficking through Golgi and TGN compartments, this indicates that the assembly and maturation of the functional enzyme complex takes place in these compartments. This conclusion is substantiated further by the observation of a complete absence of γ-secretase activity in membranes co-fractionating with marker proteins of early biosynthetic compartments and enrichment of this activity in a fraction containing TGN membranes. In good accordance with the enzyme activity profiling data, a similar enrichment of the Aβ(40) and Aβ(42) γ-secretase products in a TGN membrane fraction has been reported by others using iodixanol gradients for separation (*75*). This was confirmed independently using the sucrose gradient system described herein (*76*).

Some evidence in the literature suggests that γ-secretase activities generating Aβ(40) and Aβ(42) may be pharmacologically distinct (*77*, *78*). These conclusions were based on data obtained from mutagenesis studies or the pharmacology of less specific inhibitors which increase Aβ(42) production when used at lower concentrations. Our data, however, clearly argue that both activities are being carried out by the same enzyme. We observed a remarkable co-distribution of γ(40)- and γ(42)-enzyme activities across the entire sucrose gradient. Our potent transition state analogue inhibitors inhibited the generation of both peptides with identical potencies in vitro, and most importantly, the affinity probe depleted both enzyme activities from solubilized membranes. This indicates the molecular entities captured—the PS1 heterodimer, nicastrin, and potentially the recently cloned transmembrane proteins aph-1 and pen-2 (*37*, *38*)—represent the machinery capable of producing Aβ(40) and Aβ(42). It is likely, that these conclusions can be extended even to the γ-secretase activities producing further C-terminally truncated peptides [such as Aβ(38)] since a variety of γ-secretase inhibitors block their production in cell-based systems (*23*).

Taken together, these data strengthen the current hypothesis that the PS1 heterodimer constitutes a critical component of a novel intramembrane-cleaving aspartic protease. The biotinylated affinity probe can be expected to serve as an important novel tool to study the structural requirements for the formation of this macromolecular enzyme complex. It will be of importance to discriminate PS1 interactions with cofactors critical for the actual γ-secretase entity from those either downstream/upstream of the enzyme complex formation or completely unrelated to the activity. Since γ-secretase is a prime target for the development of therapeutic agents to treat AD, a greater understanding of the enzyme could accelerate the discovery of novel drugs.

NOTE ADDED AFTER ASAP POSTING

This paper was inadvertently posted on the Web on 06/20/03. The cell line was improperly identified in line 29 of the Discussion. The correct version was posted 06/20/03.

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