Transition-State Analogue γ-Secretase Inhibitors Stabilize a 900 kDa Presenilin/Nicastrin Complex†

Geneviève Evin,* Louise D. Canterford, David E. Hoke, Robyn A. Sharples, Janetta G. Culvenor, and Colin L. Masters

Department of Pathology, The University of Melbourne, Parkville 3010, and The Mental Health Research Institute, Parkville 3052, Australia

Received August 24, 2004; Revised Manuscript Received November 23, 2004

ABSTRACT: γ-Secretase mediates the final step, which generates Alzheimer's disease Aβ amyloid protein, by cleaving the transmembrane domain of the amyloid-β protein precursor. Four gene products, presenilin, nicastrin, APH-1, and PEN-2, are required for γ-secretase activity that is contained within a high molecular mass complex. To further characterize γ-secretase, we probed membranes from human neuroblastoma SH-SY5Y cells with γ-secretase inhibitor biotin derivatives of L-685,458, pepstatin A, and the difluoro alcohol 1-Bt. These inhibitor derivatives bound and precipitated PS1 fragments from membrane CHAPSO extracts. Analysis of PS1 complexes by blue native gel electrophoresis and western blotting indicated that the CHAPSO extracts contained complexes of ∼900, 500, and 400 kDa. With this technique, derivatives of the three inhibitors were detected only in association with the 900 kDa species. Size-exclusion chromatography showed that 13% of PS1 immunoreactivity extracted with CHAPSO was comprised within a ≥900 kDa species with the remaining eluting in fractions of 669−250 kDa but that most enzymatic activity was associated with the 900 kDa fractions. After treatment with L-685,458 inhibitor, 49% PS1 immunoreactivity was eluted in the 900 kDa fraction, supporting evidence that the inhibitor stabilized this complex. Subcellular fractionation of SH-SY5Y cells indicated that the 900 kDa complex was formed as PS1 and NCT matured through the secretory pathway and that enzymatic activity correlated with complex maturation. From these observations, we propose a model for the structure of active γ-secretase that would consist of dimerization of 400−500 kDa subunits and be consistent with the apparent molecular mass of the complex.

Aβ represents a fragment of the type I integral protein, amyloid protein precursor (APP)[1] (*1*), that is produced by the sequential action of β-secretase and γ-secretase and deposits as amyloid plaques in Alzheimer's disease affected brain. In a first proteolytic step, β-secretase (identified as BACE) releases the large APP ectodomain from the membrane and generates a membrane-associated, C-terminal fragment that becomes subsequently processed within its transmembrane region by γ-secretase (reviewed in refs *2* and *3*). γ-Secretase cleavage is a critical step that can generate either Aβ40 or the longer and more aggregating Aβ42, which seeds amyloid deposition (*4*), and therefore, γ-secretase is considered to be a potential drug target. Besides cleavage at the Aβ C-terminus, γ-secretase is also involved in the release of the APP intracellular domain (AICD) by cleaving the transmembrane domain at the ϵ-site, near the membrane/cytosol interface (*5*−*8*). Similarly, it can process the transmembrane domains of a subset of type I integral proteins such as the Notch family of receptors to release their cytosolic domain and activate gene transcription (reviewed in refs *9* and *10*). Four distinct gene products, presenilin (PS), nicastrin (NCT), APH-1, and PEN-2 are required for γ-secretase activity (*11*, *12*). Coexpression of these genes results in the association of the corresponding proteins as an enzymatically active complex (*13*−*16*). γ-Secretase is inhibited by aspartyl protease transition-state analogues (*17*−*20*), and presenilin has been proposed to contain the catalytic site of a novel membrane, aspartyl protease, based on the following evidence: presenilin gene knockout abrogates γ-secretase activity (*21*), mutation of PS1 transmembrane aspartate residues 257 and/or 385 mimics the effect of PS1 gene knockout (*22*), reactive derivatives of γ-secretase inhibitors can be cross-linked to presenilin fragments (*23*−*25*), and biotin derivatives of pepstatin associate directly with presenilins (*26*). A similar membrane topology and conserved motifs with signal peptide peptidases further support this hypothesis (*27*).

The combined molecular masses of PS1, nicastrin, APH-1, and PEN-2 would total 200−250 kDa. However, PS1

† This work is supported by the NHMRC (Program Grant 208978) and the NIH-NIA (Ruth L. Kirschstein NRSA Fellowship AG05887 to D.E.H.).

* Corresponding author. E-mail: gmevin@unimelb.edu.au. Tel: +613-8344-4205. Fax: +613-8344-4004.

[1] Abbreviations: Aβ, amyloid-β protein; AD, Alzheimer's disease; AICD, APP intracellular domain; APP, amyloid-β protein precursor; BACE, β-APP-cleaving enzyme; BN-PAGE, blue native polyacrylamide gel electrophoresis; CTF, C-terminal fragment; DDM, *n*-dodecyl β-D-maltoside; DMSO, dimethyl sulfoxide; DSP, dithiobis(succinimidyl propionate); ER, endoplasmic reticulum; HRP, horseradish peroxidase; NCT, nicastrin; NTF, N-terminal fragment; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; PC, phosphatidylcholine; PE, 3-*sn*-phosphatidylethanolamine; PEN-2, presenilin enhancer 2; PL, phospholipids; PS, presenilin; SDS, sodium dodecyl sulfate.

10.1021/bi0481702 CCC: $30.25
Published on Web 02/18/2005

complexes ranging from 100 kDa to 2 MDa have been described, and the stoichiometry of the active γ-secretase complex remains puzzling. Glycerol gradient centrifugations of membranes from PS1-transfected HEK-293 cells extracted with CHAPS (*28*) or digitonin (*29*) separate PS1 complexes of 100−150 kDa. However, membrane extraction with CHAPSO produces a complex of 2 MDa, as determined by size-exclusion chromatography (*18*). The extraction methods that were shown to preserve γ-secretase activity include 1% and 2% CHAPSO (*18*, *30*), 1% CHAPS, and 1% BigCHAPS, whereas more denaturing conditions such as 1% Triton X-100, 1% Nonidet P-40, or extreme pH result in a loss of enzymatic activity (*30*). Brij-35 does not support γ-secretase activity except for cells pretreated with a low-affinity γ-secretase inhibitor (*31*), but γ-secretase can be restored from Brij extracts after exchange with CHAPSO (*30*, *31*). To characterize further the enzymatically active form(s) of the PS1/γ-secretase complex, we used biotinylated derivatives of γ-secretase and aspartyl protease transition-state analogues to probe membrane extracts of human neuroblastoma, SH-SY5Y cells.

EXPERIMENTAL PROCEDURES

Reagents. Buffer chemicals and DMSO (tissue culture grade), Nonidet P-40, sodium deoxycholate, CHAPS, Brij-35, dodecyl maltoside, and Coomassie Brilliant Blue G were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Dithiobis(succinimidyl propionate) (DSP) was from Pierce (Rockford, IL) and CHAPSO from Calbiochem (La Jolla, CA). L-685,458, L-852,505, L-852,646 (*23*), and L-852,-631 [compound Merck F (*32*)] were obtained from Merck Sharp and Dohme Medicinal Chemistry Laboratory, Terlings Park, U.K. Pepstatin biotin derivative Pep-Bt has been described (*26*). 1-Bt and 2-Bt (*24*) were generously provided by Dr. M. S. Wolfe (Brigham and Women's Hospital, Harvard Medical School, Boston, MA).

Cell Culture and Membrane Preparations. SH-SY5Y cells, wild type or stably transfected with PS1, were cultured as described before (*26*). Cells were harvested in PBS, suspended in ice-cold Hepes buffer (20 mM, pH 7.3) plus a protease inhibitor cocktail (P2714 from Sigma), and disrupted manually either by using a Dounce homogenizer or by passing the cell suspension 10 times through a 25 gauge needle. Centrifugation for 15 min at 1000*g* yielded a nuclear pellet that was discarded. The supernatant was centrifuged for 1 h at 100000*g*, and the resulting pellet was resuspended in Hepes buffer plus 5% glycerol and stored at −80 °C. For detergent extraction, membrane aliquots were mixed with detergent-containing buffer and incubated overnight, at 4 °C with end-to-end shaking, followed by centrifugation at 16000*g* for 30 min.

Inhibitor Incubations, Precipitations, and Western Blotting. Solubilized membrane protein (20 μg) was incubated in the presence of 4−200 μM inhibitor dissolved in DMSO (2% final concentration). Cross-linking experiments were carried out as described by Li et al. (*23*) with the difference that irradiation was done at 254 nm for 15 min. For inhibitor pull-down experiments, the incubations were diluted to 200 μL with either RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) or 2% CHAPSO in Hepes prior to addition of 10 μL of streptavidin−Dynabeads (Dynal Pty Ltd., Carlton South, VIC, Australia). After gentle end-to-end shaking of the suspension at ambient temperature for 1 h, the beads were trapped with a magnet and washed three times with the incubation dilution buffer, and the proteins were eluted by adding SDS Laemmli sample buffer. The samples were heated at 55 °C for 15 min and separated on 4−20% Tris−Tricine gels (InVitrogen, Carlsbad, CA), the proteins were transferred to nitrocellulose (Transblot membrane; Bio-Rad Laboratories, Regents Park, NSW, Australia), and western blotting was effected as reported before (*26*). For western detection of biotinylated inhibitors, the blots were probed with streptavidin−horseradish peroxidase (Pierce, Rockford, Il) diluted 1000-fold in casein blocking buffer. Chemiluminescence signals were detected either with Hyperfilm (Amersham Biosciences, Castle Hill, NSW, Australia) or using a GeneGnome instrument (Syngene, Cambridge, U.K.). Rabbit polyclonal antibodies 98/1 (raised to PS1 1−20 N-terminal residues) and 00/2 (raised to PS1 loop peptide 301−317) have been described before (refs *33* and *26*, respectively). Anti-nicastrin antibody N-1660 and monoclonal anti-β-COP were purchased from Sigma. Monoclonal antibodies to protein disulfide isomerase (PDI 1D3) and to KDEL (10C3) were from StressGen (Victoria, Canada). Rabbit antibody 02/43 was produced in-house against the sequence 88−110 of human APH-1a synthesized by Auspep (Parkville, Victoria, Australia) and conjugated to keyhole limpet hemocyanin (Pierce) through its N-terminal lysine using glutaraldehyde. This antibody was characterized by western blotting and shown to detect specifically a 25 kDa band in lysates of COS-7 cells overexpressing APH-1a. Rabbit antibody 02/45 (*34*) was produced against sequence 1−15 of human PEN-2 synthesized and conjugated to diphtheria toxoid through its C-terminal cysteine side chain by Mimotopes (Clayton, Victoria, Australia). This antibody detects specifically a 10 kDa signal on western blots of lysates of COS-7 cells transfected with PEN-2.

Blue Native Gel Electrophoresis. Sample buffer (20 μL) containing 0.5% Brilliant Blue G-250 in 50 mM caproic acid and 10 mM Bis-Tris, pH 7.0, plus or minus 0.5% DDM, was added to 10 μg aliquots of membrane protein, and the samples were loaded onto 3−8% Nu-PAGE acetate gels (InVitrogen). Electrophoresis was carried out at 75 V for 30 min and then at 150 V for 2 h using a gel buffer system as described by Schagger and von Jagow (*35*). The proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA) for 30 min at 250 mA using NuPAGE transfer buffer (InVitrogen) containing 10% methanol. The blots were rapidly washed with methanol to remove the blue dye, and western blotting was effected as described above.

Superose-6 Chromatography. SH-SY5Y cell membranes prepared as described above were suspended in 1% CHAPSO in Hepes buffer (20 mM, pH 7.3) supplemented with protease inhibitor cocktail and incubated in the presence of 2% DMSO with or without 2 μM L-685,458. Incubation was allowed to proceed overnight, at 4 °C, with rotary shaking. After brief vortex mixing, the residual membranes were sedimented by centrifugation at 16000*g* for 30 min. Samples of 180 μL (∼100 μg of protein) were diluted twice with Hepes buffer to achieve a final 0.5% CHAPSO concentration. Glycerol was added to a final content of 10%. The samples were chromatographed on a Superose-6 HR column (30 × 1 cm)

equilibrated in elution buffer (20 mM Hepes, 150 mM NaCl, pH 7.0, containing 0.5% CHAPSO). The column was developed at a flow rate of 0.1 mL/min, and 0.75 mL fractions were collected. Samples (20 μL) of each fraction were used for western blot analysis. A fluorogenic assay was used to estimate relative γ-secretase activity in the fractions. Quenched fluorescence peptide MCA-Ile-Thr-Leu-Val-Met-Leu-Lys(DNP)-NH2, which spans the APP ϵ-cleavage site, was synthesized by Auspep and dissolved in DMSO at a stock concentration of 10 mg/mL. Superose-6 fractions (50 μL) were incubated for 18 h with 1 μL of fluorogenic substrate in the presence of protease inhibitor cocktail with no pepstatin. Fluorescence was measured at an excitation/emission wavelength of 355/510 nm, using a Wallac Victor2, 1420 multilabel counter.

Subcellular Fractionation. SH-SY5Y cells were cultured and collected as above. All further operations were done on ice and centrifugations carried out at 4 °C. The cells were resuspended and gently homogenized with a Dounce homogenizer in 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 1 mM magnesium chloride, and complete protease inhibitor cocktail. Nuclei and cellular debris were sedimented by centrifugation at 1000*g* for 10 min and discarded. The supernatant was adjusted to 1.4 M sucrose, and a sucrose gradient was prepared according to Xu and Shields (*36*). All sucrose dilutions were prepared in 10 mM Tris-HCl, pH 7.4. The following solutions were carefully layered in a 13 mL centrifugation tube: 2 mL of 2 M sucrose, 2.25 mL of cell homogenate supernatant, 3.75 mL of 1.2 M sucrose, and 5 mL of 0.8 M sucrose. The gradients were centrifuged for 3 h at 100000*g* in a Beckman L8-80M ultracentrifuge equipped with a SW40 rotor. Membrane vesicles were collected at the gradient interfaces and the suspensions used for western blotting. For BN-PAGE analysis, the vesicles were sedimented by centrifugation for 1 h at 100000*g*, and the pellet was solubilized with 2% CHAPSO in 20 mM Hepes, pH 7.0. Enzymatic activity was assayed using the exogenous substrate C100Flag according to Beher et al. (*37*). Briefly, 10 μg of CHAPSO extract of carbonate-washed microsomal membranes or 10 μL of the above crude membrane vesicle suspensions was incubated for 18 h at 37 °C with 1.8 μM C100Flag in a final 20 μL volume of 50 mM Tris-HCl, buffer, pH 7.4, containing 2 mM EDTA, 150 mM NaCl, and 0.5% CHAPSO. The substrate was heated for 5 min at 65 °C prior to addition to the incubations. Control experiments included incubations at 4 °C or in the presence of L-685,-458 (100 nM). The reactions were terminated by adding SDS sample buffer and the samples analyzed by SDS−PAGE on 15% Tris−Tricine gels, followed by western blotting with anti-Flag M2 monoclonal antibody (Sigma) to detect the ϵCTF or with mouse monoclonal WO2 (*38*) to detect Aβ.

RESULTS

Photo-Cross-Linking of L-685,458 Derivatives to Presenilin Fragments. Two derivatives of the potent γ-secretase inhibitor L-685,458 were used for this experiment. L-852,-505 comprises a benzophenone photoreactive group at its C-terminus (*23*) whereas L-852,631 incorporates two benzophenone groups, one at its N-terminus and one at its C-terminus (*32*). These compounds have been shown to retain γ-secretase inhibitor potency. Both inhibitor derivatives have a C-terminal extension with a spacer arm and a biotin moiety to allow retrieval or detection with avidin reagents. The two compounds were used in cross-linking experiments using membranes from PS1-transfected SH-SY5Y cells. The membranes were solubilized with 2% CHAPSO, and the extract was incubated with the inhibitor derivatives, followed by irradiation to activate the photoreactive group and induce chemical cross-linking. After addition of RIPA buffer to dissociate the complex, the inhibitor-bound fragments were captured with streptavidin beads and analyzed by western blotting. The results shown in Figure 1 demonstrate that L-852,505, the derivative with a C-terminal photoreactive group, had cross-linked to the PS1 C-terminal fragment preferentially to the PS1 N-terminal fragment (lane 2) and that binding of this biotinylated probe was displaced by preincubation with an excess of the parent compound L-685,-458 (lane 3). These data are consistent with the report by Li et al. (*23*) although these authors showed only binding of the L-852,505 probe to PS1 CTF. The derivative L-852,-631, which contains the two photoreactive groups, was found to bind only to the N-terminal fragment (lane 4) and was also displaced by its parent compound (lane 5). Schroeter et al. (*32*) were able to show that this compound binds to both PS1 N- and C-terminal fragments. Different methods may be the cause for this discrepancy, particularly the use of different irradiation conditions and different precipitation and detection reagents. Taken together, these results indicate that the transition-state analogue L-685,458 interacts closely with PS1 fragments, supporting further the corollary that PS1 contains the active site of an aspartyl protease.

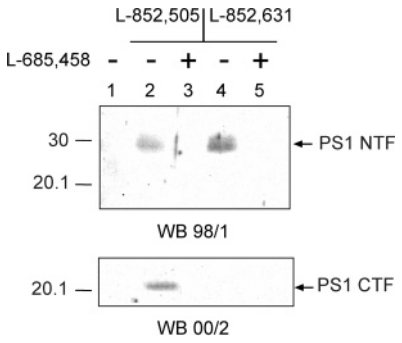

FIGURE 1: Cross-linking of L-685,458 derivatives to PS1 fragments. Microsomal membranes prepared from SH-SY5Y cells stably transfected with PS1 were solubilized with 2% CHAPSO, and the extract was incubated for 1 h in the presence of inhibitors, followed by irradiation for 15 min at 254 nm. RIPA buffer was added to dissociate the complex prior to capture with streptavidin−Dynabeads. Western blot analysis of the precipitates with PS1 antibodies 98/1 (directed to PS1 N-terminus) and 00/2 (raised to PS1 large loop region) shows cross-linking of L-852,631 to PS1 NTF and cross-linking of L-852,505 to PS1 CTF.

γ-Secretase Inhibitors Associate with the PS1 C-Terminal Fragment. To study comparatively the binding of alternative classes of inhibitors to the γ-secretase complex, we used also Pep-Bt, a derivative of pepstatin A with a biotin moiety conjugated to its C-terminus through a long spacer arm (*26*), and 1-Bt, a difluoro alcohol transition-state analogue based on the APP sequence, which is also derivatized with biotin at its C-terminus (*24*). 2-Bt, the biotin derivative of the corresponding substrate analogue with a scissile bond, served as a control (*24*). CHAPSO extracts from PS1-SH-SY5Y membranes were incubated with the biotinylated compounds followed by immunoprecipitation with PS1 N-terminal antibody, 98/1. The precipitates were analyzed by western

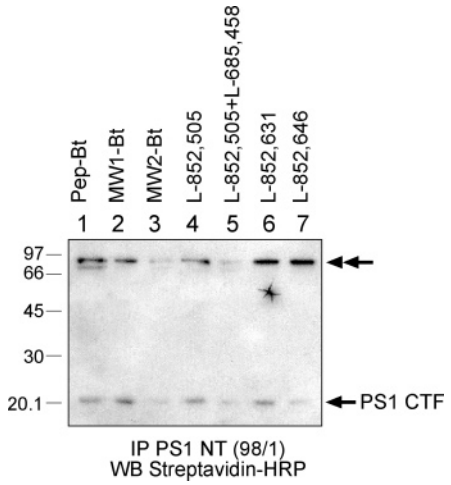

FIGURE 2: Transition-state analogues strongly associate with the PS1 C-terminal fragment. CHAPSO membrane extract from PS1-transfected SH-SY5Y was incubated overnight with 4 μM biotin derivatives of pepstatin A (Pep-Bt), the difluoro alcohol 1-Bt, or the corresponding substrate analogue 2-Bt or with the L-685,458 derivatives. Binding competition was done using a 20-fold excess of L-685,458. The incubations were immunoprecipitated with PS1 N-terminal antibody in the presence of 1% CHAPSO to preserve the integrity of the γ-secretase complex. The precipitates were electrophoresed on a native Tris−Tricine gel. Western blotting with streptavidin−HRP detected inhibitor-bound species of ~20 kDa, corresponding to the apparent molecular mass of PS1 CTF, and 80 kDa (indicated with the double arrow) that will require further characterization. These were barely detectable in samples incubated with 2-Bt or in the presence of an excess of nonbiotinylated inhibitor L-685,458.

blotting with streptavidin−horseradish peroxidase to detect inhibitor-bound proteins. With all of the inhibitors tested, an ~20 kDa species was recovered (Figure 2, lanes 1−7) that has a size consistent with that of PS1 CTF. This was not detected in the absence of inhibitor (not shown). 1-Bt gave a robust signal (Figure 2, lane 2) whereas, as expected, the signal obtained with 2-Bt was hardly detectable (lane 3). An ~80 kDa signal was also detected that will require further characterization as it may represent association of the inhibitors with PS1 fragments and/or with other partners of the complex (Figure 2, lanes 1−7). Preincubation with a 20-fold excess of the parent compound L-685,458 displaced binding of the L-852,505 biotinylated probe to both the 20 and 80 kDa species (lane 5), indicating the specificity of the signals detected. A signal corresponding to the PS1 N-terminal fragment was not detected. These results suggest that in the absence of covalent binding through cross-linking, a sufficient amount of the γ-secretase inhibitors remained associated with the PS1 C-terminal fragment under electrophoresis conditions to allow detection of a signal. Such a tight association suggests interaction of the inhibitors with the active site of γ-secretase.

Alternative Detergents Solubilize PS1 Complexes Sized from 350 to 900 kDa by Blue Native Gel Electrophoresis. To obtain more information on the γ-secretase complex, SH-SY5Y membranes were solubilized with various detergents, and PS1 complexes were analyzed by blue native gel electrophoresis (BN-PAGE), followed by western blotting. SH-SY5Y membranes were extracted with either 1% CHAPSO, 1% CHAPS, 1% Brij-35, or 1% dodecyl maltoside (DDM). The glycoside DDM solubilized a PS1 complex of ~350 kDa (Figure 3A). This result is consistent with the previous report where we showed that 350 kDa complexes can be extracted with DDM from mouse and human brains and that these are reactive with antibodies to PS1 N- and C-terminal regions, nicastrin, PEN-2, and APH-1b (*34*). SH-SY5Y CHAPS extract smeared over the gel, due to the poor compatibility of the detergent with this chromatographic technique, but this smear covered a broad range of molecular masses, from 900 to 400 kDa (Figure 3A). CHAPSO produced a more defined pattern with major species detected at 400, 500, and 900 kDa (Figure 3A). As phospholipids were shown to improve γ-secretase activity in a cell-free system (*39*), the effect of preincubating extracted membranes with phospholipids (PL) was tested. PL did not alter the migration of the PS1 complexes but perhaps enhanced their detection, suggesting increased stability. CHAPSO-extracted membranes were also treated with the cross-linking reagent dithiobis(succinimidyl propionate) (DSP) prior to BN-PAGE analysis. This resulted in the disappearance of the 400 and 500 kDa species and an increase of the 900 kDa and higher molecular mass species, supporting that these higher molecular mass species dissociate upon electrophoresis into 400 and 500 kDa subcomplexes. Probing an identical blot with NCT C-terminal antibody detected the 350−900 kDa complexes. The DDM extract contained an additional 220 kDa NCT subcomplex. Adding 0.5% DDM to the sample buffer resulted in the complete breakdown of the 900 kDa PS1 complex into the 400 and 500 kDa complexes (Figure 3B). Taken together, these results indicate that PS1 associates within a high molecular mass complex of 900 kDa that is kept intact by the detergents CHAPSO and CHAPS but becomes broken down into smaller complexes with DDM.

γ-Secretase Inhibitors Bind and Stabilize the 900 kDa Complex. To identify the minimum complex size required to bind a γ-secretase inhibitor, SH-SY5Y membrane extracts were incubated with biotinylated inhibitor derivatives, sample buffer containing 0.5% DDM was added, and the proteins were separated by BN-PAGE and transferred to PVDF for analysis. Probing the blot with streptavidin−horseradish peroxidase followed by chemiluminescence detection revealed that the three inhibitors bound to the 900 kDa complex, whatever detergent was used to solubilize the cell membranes (Figure 4A). The complex derived from Brij-35 extraction migrated slightly slower, possibly due to association of large detergent micelles with the complex. Reprobing the blot for PS1 highlighted the complexes observed previously (Figure 3B) as well as faint signals corresponding to complexes of higher molecular masses.

To examine further the effect of the inhibitors on the stabilization of the 900 kDa complex, the DDM extract was incubated with the inhibitors (Figure 4B). Streptavidin−horseradish peroxidase detected a 900 kDa signal for all inhibitor incubations (Figure 4B, lanes 3−5). A weak signal at 350 kDa was also observed with L-852,505 and L-852,-646, indicating these inhibitors also bound to the complex of lower molecular mass but that either they preferentially associated with the 900 kDa complex or they became more easily dissociated from the 350 kDa complex. This signal was not detected in the absence of inhibitor (lane 1). In addition, this signal was not observed with 2-Bt (lane 2), which is a substrate analogue and thus a weak inhibitor, so it is likely that it would dissociate from the complex under the electrophoresis conditions.

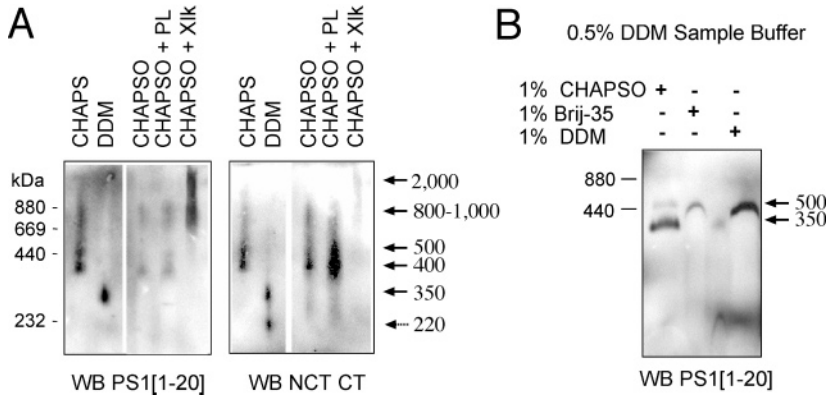

FIGURE 3: Blue native gel analysis of PS1 complexes solubilized with alternative detergents. (A) SH-SY5Y (WT) microsomal membranes were solubilized with either 1% CHAPS, 1% CHAPSO, or 1% dodecyl maltoside (DDM). Samples were also analyzed that had been treated with phospholipids (PL; PE, 5 mg/mL, and PC, 10 mg/mL) or with 2 mM DSP cross-linking agent (Xlk). Ten micrograms of protein was separated by BN-PAGE for western blotting. (B) Separation of CHAPSO and Brij-35 extracts after mixing with sample buffer containing 0.5% DDM. Sharper band resolution was obtained, but only 350 and 500 kDa PS1 complexes were detected, indicating that DDM broke down the higher molecular mass complexes.

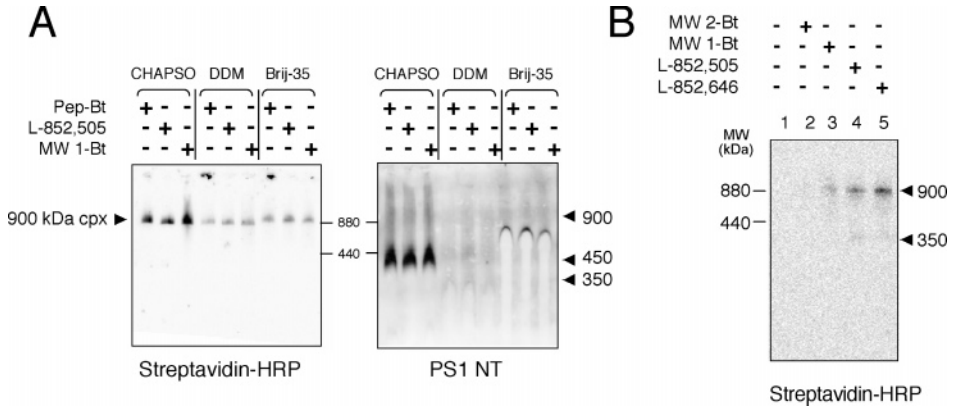

FIGURE 4: γ-Secretase inhibitors bind and stabilize an ~900 kDa complex. (A) Membrane extracts were incubated in the presence of Bt inhibitors and the proteins separated by BN-PAGE and transferred to PVDF. Probing the blot with streptavidin−HRP, followed by chemiluminescence detection, revealed that the three inhibitors bound to an ~900 kDa complex (left panel). Reprobing the blot with 98/1 showed PS1 complexes ranging from 350 to ≥900 kDa (right panel). (B) DDM solubilized membranes were incubated with biotinylated inhibitors, separated by BN-PAGE, and transferred to PVDF, and the blots were probed with streptavidin−HRP. Again the major signal obtained corresponds to labeling of a 900 kDa complex.

Gel Filtration Chromatography Shows Stabilization of the 900 kDa Species after Incubation with L-685,458 Inhibitor. SH-SY5Y membranes, solubilized with 1% CHAPSO, were fractionated on a Superose-6 gel filtration column equilibrated in 0.5% CHAPSO, and the effect of pretreating the membrane extract with the γ-secretase inhibitor, L-685,458 (1 μM), was studied. The fractions were analyzed by western blotting for PS1 and NCT (Figure 5A). Without inhibitor treatment, PS1 and nicastrin show a similar distribution and eluted principally in fractions corresponding to 669−350 kDa. Full-length PS1 and immature NCT were detected in the fractions of lowest molecular mass whereas only mature, highly glycosylated NCT (140 kDa) and PS1 NTF were detected in the 669−440 kDa fractions. Small amounts of PS1 NTF and mature NCT were also detected in a fraction corresponding to ≥900 kDa. After inhibitor treatment, we observed a redistribution of both PS1 NTF and mature NCT to fractions of higher molecular mass. To further analyze these results, the PS1 NTF signal was quantitated by image densitometry (Figure 5B). The data indicate that, in the absence of inhibitor, 13% of PS1 was recovered in the high molecular mass fractions. After inhibitor treatment, there was 49% of PS1 immunoreactivity in the high molecular mass fractions. These data are consistent with those observed in BN-PAGE experiments and confirm that the inhibitor stabilizes the 900 kDa species.

As the previous results suggested that the transition-state analogue inhibitor L-685,458 bound and stabilized the 800−1000 kDa species, we reasoned that this complex corresponds to the active enzyme. To test this hypothesis, we compared γ-secretase activity in the two peaks (800−1000 and 400−500 kDa) of PS1 immunoreactivity of the Superose fractionations of membranes treated with either DMSO or L-685,458, using a quenched fluorescence peptide assay. The activity, normalized to the levels of PS1 immunoreactivity in the samples, was 5-fold higher in the 800−1000 kDa fraction than in the 400−500 kDa fraction (Figure 5C). The absence of activity in the corresponding 800−1000 kDa peak eluted from Superose fractionation of inhibitor-treated membranes was expected and further demonstrates that these fractions contain γ-secretase. Some enzymatic activity detected in the 400−500 kDa fraction was not totally inhibited with L-685,458, indicating the presence of contaminating protease activity also capable of cleaving the synthetic substrate.

Analysis of γ-Secretase Complex Components in Subcellular Fractions of SH-SY5Y Cells. Wild-type SH-SY5Y cells were lysed in isotonic conditions, and the organelles were

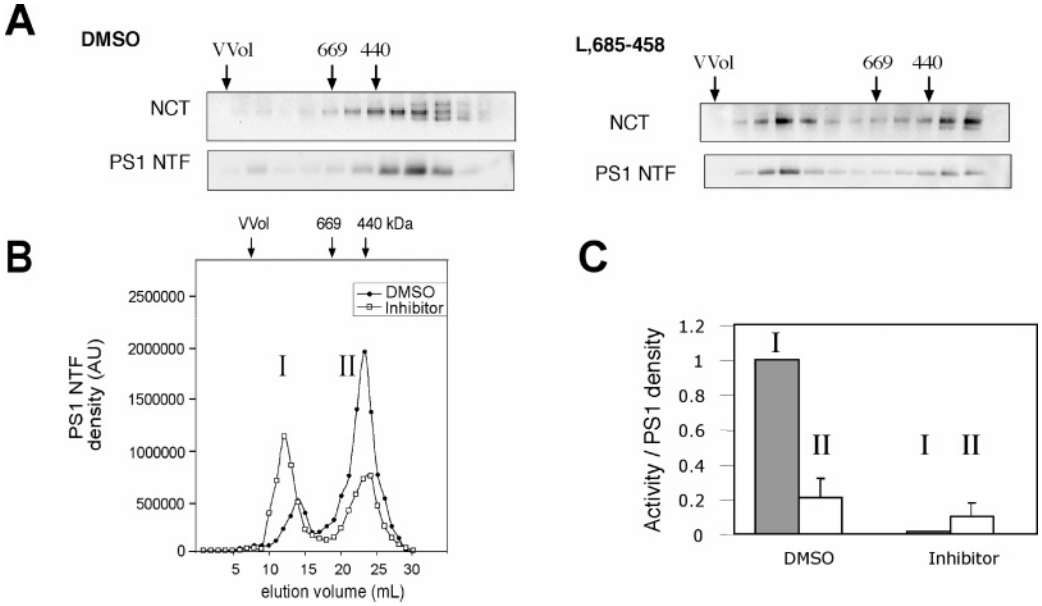

FIGURE 5: Gel filtration of CHAPSO extracts of SH-SY5Y membranes with and without γ-secretase inhibitor treatment. SH-SY5Y membranes solubilized with 1% CHAPSO, in the presence of 2 μM L-685,458 or in the presence of DMSO alone, were chromatographed on a Superose-6 column. (A) Eluted fractions were analyzed by western blotting for PS1 NTF and for nicastrin. (B) The density of PS1 NTF signals captured with a Syngene GeneGnome instrument and quantitated using Gene Tools software was plotted as a function of the volume eluted. In the absence of inhibitor (filled circles), PS1 NTF was recovered as a major peak (II) in fractions ranging from 669 to 350 kDa and as a smaller peak (I) eluting just after the void volume and corresponding to 13% of total PS1 NTF. In the presence of inhibitor (open squares), about 50% of PS1 NTF was eluted in peak I, suggesting that the inhibitor stabilized higher molecular mass complexes. (C) γ-Secretase activity was assayed in pooled fractions of the elution peaks using a quenched fluorescence substrate. The data expressed as the ratio of enzymatic activity toward intensity of the PS1 NTF signal show a 5-fold enrichment of γ-secretase activity in the high molecular mass peak.

fractionated by centrifugation on a discontinuous sucrose density gradient to separate endoplasmic reticulum (ER) and Golgi structures. Three bands of membrane vesicles were collected at the gradient interfaces (labeled B1−B3, from lowest to highest density) and were characterized by western blotting for the organelle-specific markers βCOP, a Golgi coatamer protein, and BiP and protein disulfide isomerase (PDI), two ER lumenal chaperones. The BiP antibody also labels Grp94, which, like BiP, is a soluble glucose-regulated protein that resides in the ER and contains a KDEL motif for retrieval from the Golgi to the ER. Figure 6A shows detection of βCOP in B1 and B2, detection of BiP in all fractions but predominantly in B2, and detection of PDI only in B2 and B3. From these data it can be concluded that B1 contained Golgi, but no ER, vesicles whereas B3 was rich in ER structures and did not contain Golgi vesicles. The same fractions were tested for the presence of PS1, NCT, PEN-2, and APH-1a, the four essential components of the γ-secretase complex (Figure 6B). PS1, full length, was detected only in fraction B3, a finding consistent with PS1 endoproteolysis occurring in the ER. In contrast, PS1 NTF was detected in all fractions, as mature cleaved PS1 is expected to exit the Golgi and traffic through the secretory pathway. Low glycosylation forms of nicastrin corresponding to the immature protein were detected only in fraction B3, but the mature form was present in all fractions. PEN-2 was detectable only in fraction B3, but because the western blot signal was weak, we cannot exclude its presence in the other fractions. APH-1a was present in all three fractions with fragments or immature forms detected in B3. The membrane fractions were then analyzed by BN-PAGE to detect γ-secretase complexes (Figure 6C). Western blotting with NCT antibody showed detection of 250, 400, and 500 kDa complexes in all fractions. An 800−1000 kDa complex was also present in fraction B1, suggesting the formation of higher molecular mass structures as the complex matures through the secretory pathway. A similar pattern was obtained with anti-PS1 antibody, except that the 250 kDa signals were absent. Next, we tested the three membrane fractions for γ-secretase activity using exogenous recombinant C100Flag substrate. Incubation at 37 °C showed detection of robust ϵCTF and Aβ signals obtained from fraction B1 (Figure 6D, lane 6) and that were not detected in 4 °C incubation (lane 5) or in 37 °C incubation in the presence of L-685,458 (lane 7). Only weak signals were detected in the incubations with fractions B2 (lane 9) and B3 (lane12), suggesting that γ-secretase activates as it trafficks through the secretory pathway.

DISCUSSION

Genetic evidence points to only four genes, *presenilin* (or *sel-12*), *nicastrin* (or *aph-2*), *aph-1*, and *pen-2*, as absolutely required for γ-secretase processing of Notch in *Caenorhabditis elegans* and in *Drosophila melanogaster* (*11*, *12*). However, presenilin complexes ranging from 150 kDa to 2 MDa (*28*−*29*, *18*) have been described, and γ-secretase activity has been associated with a 2 MDa complex (*18*). To define the enzymatically active form(s) of the PS1/γ-secretase complex, we used biotinylated derivatives of γ-secretase and aspartyl protease transition-state analogues to probe membrane extracts from human neuroblastoma SH-SY5Ycells.

Using CHAPSO extracts from membranes of SH-SY5Y overexpressing PS1, we showed that derivatives of the hydroxyethylene inhibitor L-685,458 could be cross-linked

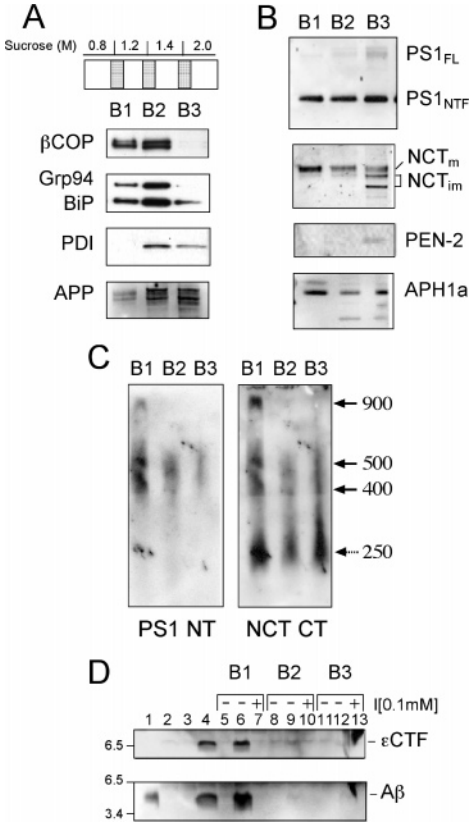

FIGURE 6: Analysis of γ-secretase complex components in subcellular fractions of SH-SY5Y cells. Wild-type SH-SY5Y cells were lysed in isotonic conditions, and organelles were fractionated by centrifugation on a discontinuous sucrose density gradient. Three bands of membrane vesicles were collected at the gradient interfaces (labeled B1−B3, from lowest to highest density). (A) Fractions B1−B3 were characterized by western blotting with organelle-specific marker antibodies, βCOP (Golgi), BiP (which labels both BiP and Grp94, two ER proteins), and PDI (protein disulfide isomerase, another ER marker). B1 was enriched in Golgi vesicles whereas B3 contained mostly ER elements. B2 contained both Golgi and ER vesicles. (B) The fractions were tested for PS1, NCT, PEN-2, and APH-1a. PS1, full length, was detected only in the ER fraction and PS1 NTF in all fractions. Immature forms of nicastrin were detected only in the ER fraction, but the mature form was present in all fractions. PEN-2 was detectable only in the ER fraction. APH-1a was present in the three fractions. (C) The membrane vesicles were solubilized with 1% CHAPSO and analyzed by BN-PAGE, followed by western blotting with NCT antibody. 250, 400, and 500 kDa were detected in all fractions. An 800−1000 kDa complex was also present in fraction B1. (D) γ-Secretase activity assay using exogenous C100Flag substrate (*37*). Membrane vesicle fractions were incubated with C100Flag substrate either at 4 °C (lanes 5, 8, and 11) or at 37 °C, in the absence or in the presence of 0.1 μM L-685,458 (lanes 6, 9, and 12 and lanes 7, 10, and 13, respectively). The incubations were analyzed by western blotting for ϵCTF with anti-Flag M2 antibody or, for Aβ, with WO2 antibody. The data indicate robust γ-secretase activity in fraction B1. A positive control was 37 °C incubation of substrate with SH-SY5Y microsomal membranes solubilized with 0.5% CHAPSO (lane 4). Negative controls were 4 and 37 °C incubations of substrate alone (lanes 2 and 3, respectively). The synthetic Aβ40 peptide standard is shown in lane 1.

to either the PS1 N- or C-terminal fragment, depending on the placement of photoreactive groups relative to the non-scissile bond (Figure 1). This is consistent with previous reports (*22*, *32*) and suggests that both PS1 fragments contain residues in close proximity to the inhibitor binding site. In the absence of covalent binding, we showed that three inhibitor derivatives, L-852,505, pepstatin-Bt, and the difluoro alcohol derivative 1-Bt, interacted with the PS1 C-terminal fragment and this interaction was tight enough to withstand electrophoresis conditions (Figure 2). This result suggests a strong interaction of the PS1 C-terminal fragment with these inhibitors, such as the interaction of a tight-binding inhibitor with a protease active site.

To characterize further the presenilin/γ-secretase complex, we used various detergents to extract SH-SY5Y membrane proteins and analyzed PS1 complexes by BN-PAGE. DDM extracted a complex which migrated as an ~350 kDa species (Figure 3A), a result consistent with our recent report showing solubilization of ~350 kDa presenilin complexes from SH-SY5Y cells and from mouse and human brain. We have shown that presenilin N- and C-terminal fragments, nicastrin, PEN-2, and APH1-b are contained within complexes of this size range (*34*). CHAPSO extract from SH-SY5Y membranes smeared over a broad range of molecular mass (250−1000 kDa), suggesting dissociation of a complex of high molecular mass (Figure 3A, lane 1). Adding DDM to the CHAPSO extract prior to analysis resulted in the resolution of two species of 350 and 450 kDa (Figure 3B, lane 1). The 350−450 kDa complex sizes are consistent with those reported by other groups for PS1 complexes extracted from human brain (*40*) and from embryonic mouse fibroblasts (*41*). Using BN-PAGE, Gu and colleagues have recently reported detection of complexes of 450, 670, and ≥1000 kDa from digitonin-solubilized membranes of PS1-transfected HeLa cells (*42*). Our data, obtained with a wild-type human cell line of neuronal origin, are very consistent with Gu's report, considering the lack of precision in determining molecular mass by BN-PAGE.

To identify the minimal complex size containing γ-secretase activity, we have analyzed by BN-PAGE the various detergent extracts after incubation with γ-secretase inhibitor biotin derivatives (Figure 4). With the three classes of γ-secretase transition-state analogue inhibitors we used, biotin reactivity was recovered in association with the 900 kDa species. The same result was obtained with CHAPSO, DDM, and Brij-35, although extraction with the latest detergents resulted in detection of only 350 and 500 kDa complexes by BN-PAGE. This result indicates that the inhibitors interact with a high molecular mass form and that either they help reassociate the 900 kDa form from the intermediate 350, 400, and 500 kDa species or they stabilize the 900 kDa form and prevent its dissociation during BN-PAGE. In the case of incubations of the DDM extract with L-685,458 derivatives, we have also observed a weak signal at 350 kDa, which suggests an interaction of these inhibitors with this lower molecular mass complex.

Fractionation of 1% CHAPSO membrane extracts on a Superose-6 column showed that PS1 and nicastrin distribute in the same fractions, with only a small percentage (estimated as 13% for PS1) in a ≥900 kDa complex and the rest ranging from 669 to 350 kDa, including immature proteins in the lightest fractions. After preincubating the membrane extract with L-685,458 inhibitor, about half of PS1 immunoreactivity was eluted in ≥900 kDa fractions, again supporting that the inhibitor stabilized the higher molecular mass complex and possibly helped its reconstitution from lower molecular mass species. In addition, we showed that γ-secretase enzymatic activity was 5-fold greater relative to PS1 content in the

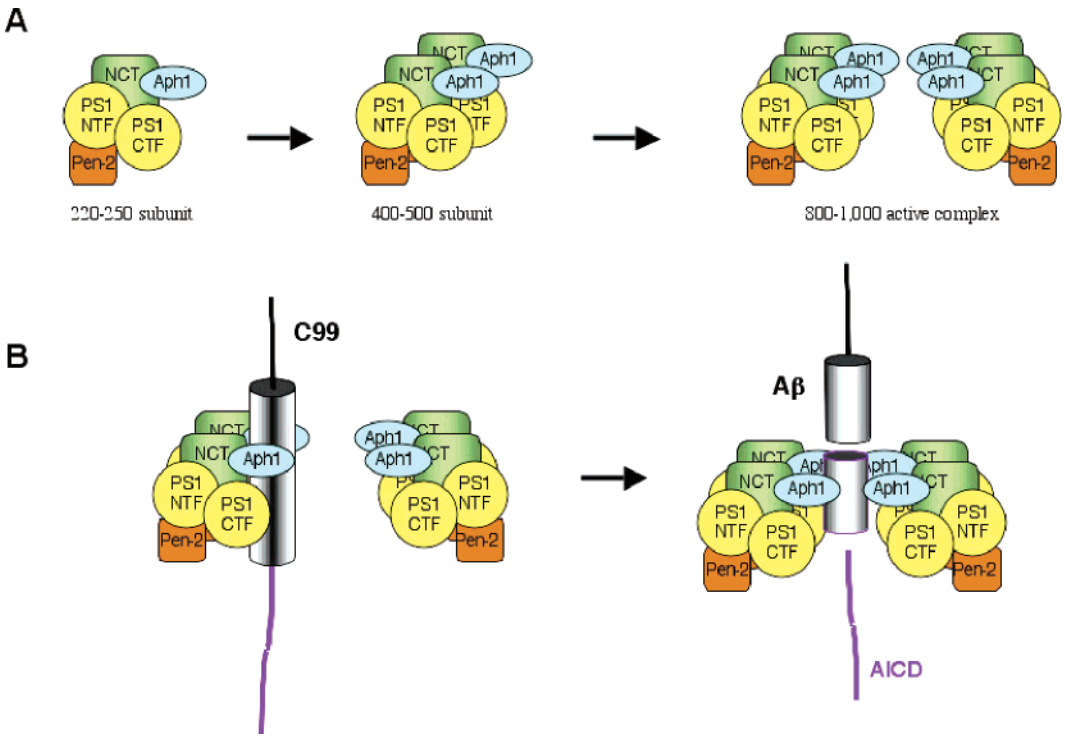

FIGURE 7: Schematic diagram of γ-secretase complex putative assembly. (A) Assembly of PS1 fragments, NCT, APH-1, and PEN-2 would form a complex subunit with a molecular mass of ~200−250 kDa, assuming that each individual component is represented as a single entity. Combination of two of these subunits would form ~400−500 kDa complexes such as those detected by BN-PAGE of CHAPSO membrane extracts. This structure could further dimerize to yield the 800−1000 kDa complexes that represent the most active form of γ-secretase and are stabilized by the transition-state analogue inhibitors. (B) This model contains four putative protease active sites and would be able to process the APP transmembrane domain at sites 40, 42, and 49 to generate Aβ peptides and release AICD. C99 represents the last 99 C-terminal residues of APP and corresponds to the β-secretase C-terminal fragment, which is the direct precursor to Aβ.

≥900 kDa complex than in the 400−500 kDa form. Taken together, these data indicate that the optimally active enzyme is the 900 kDa species. This is consistent with a recent report that γ-secretase activity increases with complex size, as determined by glycerol velocity gradient (*40*). Previous reports (*18*, *38*) have shown recovery of PS1 immunoreactivity and γ-secretase activity in a fraction eluting at the void volume of a Superose-6 column and corresponding to a molecular mass of 2000 kDa. However, size-exclusion chromatography is not a precise technique for determining molecular mass in the ≥1000 kDa range, and variations in detergent concentrations may account for discrepancies in the estimation of complex sizes. Using the same cell line and similar conditions for complex extraction and dilution, it was shown by Beher et al. (*37*) that only about 10% of PS1 can bind to an inhibitor affinity column and that this represents ~70% of total γ-secretase activity present in the cell extract. In concordance with this report, the data presented here indicate that ~10% of PS1 immunoreactivity elutes in a peak of very high molecular mass and that this peak is greatly enriched in γ-secretase activity. The binding of transition-state analogues to this very high molecular mass complex further demonstrates that it represents the active form of the enzyme.

We have also shown that this ≥900 kDa complex is present in Golgi-enriched fractions but absent from ER-enriched fractions, demonstrating that higher molecular mass complexes form as its components mature and traffic through the secretory pathway (Figure 6B,C). Furhermore, we showed γ-secretase activity to be markedly greater in Golgi than in ER fractions (Figure 6D). These data are consistent with a report by Baulac et al. (*43*), which described subcellular fractionation of CHO cells transfected with APP, PS1, APH-1, and PEN-2 and showed the γ-secretase components to interact in Golgi-enriched fractions as well as the presence of enzymatically active γ-secretase in these fractions.

On the basis of our data and recent reports in the literature, we would like to propose a model for γ-secretase where the 900 kDa complex represents a dimer of the 400−500 kDa species that may be itself constituted of two complex subunits (Figure 7A). Indeed, coexpression of PS1, NCT, PEN-2, and APH-1 results in the formation of a 250−350 kDa complex (*44*) that could represent a basic subunit. This could then associate as a dimer, consistent with the size of the complexes of 450−650 kDa extracted by CHAPSO (our data and ref *42*). However, our data demonstrate that the most stable form of the complex that binds to the inhibitors is a ≥900 kDa structure that would be consistent with the assembly of two of the 400−500 kDa subunits, thus forming a complex containing four PS1 complex subunits and consisting of a dimer of dimers. Such a structure has recently been described for an ionotropic glutamate receptor (*44*). In the case of γ-secretase, this type of structure would contain four PS1 CTF reactive aspartates and another four PS1 NTF aspartates that could form multiple catalytic sites to process alternative substrates and carry out various cleavages such as APP cleavages at Aβ residues 40, 42, and 49 (Figure 7B). This structure would allow simultaneous accommodation of a substrate and an inhibitor, as previously indicated (*30*, *37*, *45*). It would also support enzyme kinetic studies showing that γ-secretase contains multiple inhibitor binding sites (*47*). This model is only hypothetical and represents what we

propose to be the catalytic machinery of the γ-secretase activity, without excluding interactions with other components also shown to interact with presenilins, such as β-catenin and other armadillo proteins (*29*, *48*−*50*) or the tyrosine kinase GSK-3β (*51*−*53*), which may regulate the complex association and activity. The precise composition of the γ-secretase complex subunits will have to be thoroughly examined, as these may not be identical and could unravel new clues for designing γ-secretase inhibitors that target APP cleavage at position 42 with lesser effect on cleavages at positions 40 and 49 and which could find a therapeutic application in the treatment of Alzheimer's disease.

ACKNOWLEDGMENT

We thank Dr. Michael S. Wolfe for the generous gift of biotinylated compounds and for helpful comments and Drs. Mark Shearman and Dirk Beher for kindly providing L-685,-458 and derivatives as well as C100Flag substrate. We also thank Dr. Damian Holsinger and Jane Tanner for help with tissue culture and subcellular fractionations.

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BI0481702