Pen2 and Presenilin-1 Modulate the Dynamic Equilibrium of Presenilin-1 and Presenilin-2 γ-Secretase Complexes

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γ-Secretase is known to play a pivotal role in the pathogenesis of Alzheimer disease through production of amyloidogenic Aβ42 peptides. Early onset familial Alzheimer disease mutations in presenilin (PS), the catalytic core of γ-secretase, invariably increase the Aβ42:Aβ40 ratio. However, the mechanism by which these mutations affect γ-secretase complex formation and cleavage specificity is poorly understood. We show that our in vitro assay system recapitulates the effect of PS1 mutations on the Aβ42:Aβ40 ratio observed in cell and animal models. We have developed a series of small molecule affinity probes that allow us to characterize active γ-secretase complexes. Furthermore we reveal that the equilibrium of PS1- and PS2-containing active complexes is dynamic and altered by overexpression of Pen2 or PS1 mutants and that formation of PS2 complexes is positively correlated with increased Aβ40 ratios. These data suggest that perturbations to γ-secretase complex equilibrium can have a profound effect on enzyme activity and that increased PS2 complexes along with mutated PS1 complexes contribute to an increased Aβ42:Aβ40 ratio.

β-Amyloid (Aβ)5 peptides are believed to play a causative role in Alzheimer disease (AD). Aβ peptides are generated from the processing of the amyloid precursor protein (APP) by two proteases, β-secretase and γ-secretase. Although γ-secretase generates heterogenous Aβ peptides ranging from 37 to 46 amino acids in length, significant work has focused mainly on the Aβ40 and Aβ42 peptides that are the major constituents of amyloid plaques. γ-Secretase is a multisubunit membrane aspartyl protease comprised of at least four known subunits: presenilin (PS), nicastrin (Nct), anterior pharynx-defective (Aph), and presenilin enhancer 2 (Pen2). Presenilin is thought to contain the catalytic core of the complex (1–4), whereas Aph and Nct play critical roles in the assembly, trafficking, and stability of γ-secretase as well as substrate recognition (5, 6). Lastly Pen2 facilitates the endoproteolysis of PS into its N-terminal (NTF) and C-terminal (CTF) fragments thereby yielding a catalytically competent enzyme (5, 7–10). All four proteins (PS, Nct, Aph1, and Pen2) are obligatory for γ-secretase activity in cell and animal models (11, 12). There are two homologs of PS, PS1 and PS2, and three isoforms of Aph1, Aph1aS, Aph1aL, and Aph1b. At least six active γ-secretase complexes have been reported (two presenilins × three Aph1s) (13, 14). The sum of apparent molecular masses of the four proteins (PS1-NTF/CTF = 53 kDa, Nct = 120 kDa, Aph1 = 30 kDa, and Pen2 = 10kDa) is ~200 kDa. However, active γ-secretase complexes of varying sizes, ranging from 250 to 2000 kDa, have been reported (15–19). Recently a study suggested that the γ-secretase complex contains only one of each subunit (20). Collectively these studies suggest that a four-protein complex around 200–250 kDa may be the minimal functional γ-secretase unit with additional cofactors and/or varying stoichiometry of subunits existing in the high molecular weight γ-secretase complexes. CD147 and TMP21 have been found to be associated with the γ-secretase complex (21, 22); however, their role in the regulation of γ-secretase has been controversial (23, 24).

Mutations of PS1 or PS2 are associated with familial early onset AD (FAD), although it is debatable whether these familial PS mutations act as “gain or loss of function” alterations in regard to γ-secretase activity (25–27). Regardless the overall outcome of these mutations is an increased ratio of Aβ42:Aβ40. Clearly these mutations differentially affect γ-secretase activity for the production of Aβ40 and Aβ42. Despite intensive studies of Aβ peptides and γ-secretase, the molecular mechanism con-

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5 The abbreviations used are: Aβ, β-amyloid; AD, Alzheimer disease; APP, amyloid precursor protein; CTF, C-terminal fragment; FAD, familial early onset AD; NTF, N-terminal fragment; PS, presenilin; Nct, nicastrin; Aph, anterior pharynx-defective; Pen2, presenilin enhancer 2; WT, wild type; MES, 4-morpholineethanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid; PIPES, 1,4-piperazineethanesulfonic acid; L458, L-685,458; L646, L-852,646.

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controlling the specificity of γ-secretase activity for Aβ40 and Aβ42 production has not been resolved. It has been found that PS1 mutations affect the formation of γ-secretase complexes (28). However, the precise mechanism by which individual subunits alter the dynamics of γ-secretase complex formation and activity is largely unresolved. A better mechanistic understanding of γ-secretase activity associated with FAD mutations has been hindered by the lack of suitable assays and probes that are necessary to recapitulate the effect of these mutations seen in cell models and to characterize the active γ-secretase complex.

In our present studies, we have determined the overall effect of Pen2 and PS1 expression on the dynamics of PS1- and PS2-containing complexes and their association with γ-secretase activity. Using newly developed biotinylated small molecular probes and activity assays, we revealed that expression of Pen2 or PS1 FAD mutants markedly shifts the equilibrium of PS1-containing active complexes to that of PS2-containing complexes and results in an overall increase in the Aβ42:Aβ40 ratio in both stable cell lines and animal models. Our studies indicate that perturbations to the equilibrium of active γ-secretase complexes by an individual subunit can greatly affect the activity of the enzyme. Moreover they serve as further evidence that there are multiple and distinct γ-secretase complexes that can exist within the same cell and that their equilibrium is dynamic. Additionally the affinity probes developed here will facilitate further study of the expression and composition of endogenous active γ-secretase from a variety of model systems.

**EXPERIMENTAL PROCEDURES**

**Affinity Probes and Antibodies**—L-685,458 (L458), L-852,646 (L646), and compounds 2 and 4 were prepared as described previously (1, 29). Synthesis of compounds 1 and 3, newly developed γ-secretase inhibitors, will be published elsewhere (30). The monoclonal 9E10 antibody against Myc was obtained from the Memorial Sloan Kettering Cancer Center core facility. Antibodies against PS1-NTF and Pen2 were kindly provided by Dr. Min-tain Lai (Merck Research Laboratories) and Dr. Jan Näslund (Karolinska Institutet). Antibodies against PS2-CTF (PC235) and PS1-CTF (MAB5232) and Aph1al (38-3600) were purchased from Calbiochem, Chemicon, and Zymed Laboratories Inc., respectively. Antibodies against APP and Nct were generated by our laboratories.

**Cell Lines**—N2A cell lines that stably express human wild type (WT) PS1 and FAD mutants were cultured as described previously (31). The HEK293-APP stable cell line (a gift from Dr. Elizabeth Chen, Merck Research Laboratories) was transfected with Myc-tagged Pen2 expressed from the pcDNA 4.1 vector and selected with Zeocin (Invitrogen) at 0.4–0.8 mg/ml. Cultures were maintained in Dulbecco’s modified Eagle’s medium high glucose containing 10% fetal calf serum and antibiotics.

**Membrane Preparation and in Vitro γ-Secretase Assay**—Membrane fractions were isolated from HeLa-S3 cells (National Cell Culture Center). Briefly 1-liter equivalents were resuspended in Buffer A (50 mM MES, pH 6.0, 150 mM KCl, 5 mM CaCl₂, 5 mM MgCl₂, and protease inhibitors) and lysed by passage through a French press. Nuclear debris were pelleted, and the resulting supernatant was ultracentrifuged 100,000 × g for 1 h. The resulting pellet represented the total membrane fraction. Total membrane fractions were solubilized with 1% CHAPSO in Buffer A for 1 h at 4 °C. Non-solubilized material was removed by ultracentrifugation at 100,000 × g for 1 h, and the resulting supernatant represented the CHAPSO-solubilized fraction. Protein concentration was determined with the DC Protein Assay kit (Bio-Rad) according to the manufacturer’s instructions. γ-Secretase activity was measured by electrochemiluminescence as described previously (18, 33). CHAPSO-solubilized membrane was incubated in Buffer B (50 mM PIPES, pH 7.0, 150 mM KCl, 5 mM CaCl₂, 5 mM MgCl₂, and protease inhibitors) with 0.25% (v/v) CHAPSO, 1 mM substrate, and 0.1% bovine serum albumin (v/v) in the presence or absence of γ-secretase inhibitors for 2.5 h at 37 °C. The reaction mixture was incubated with biotinylated 4G8 and ruthenylated G2-10 or G2-11 in Buffer C (1× phosphate-buffered saline, 0.5% (w/v) bovine serum albumin, and 0.5% (v/v) Tween 20) for 2 h at 25 °C, and immunocomplexes were captured with magnetic streptavidin beads (Dynal). Aβ40 and Aβ42 production was measured by electrochemiluminescence on an Analyzer (Bio-Veris) and expressed as relative light units.

**Affinity Capture of Endogenous γ-Secretase and Western Blotting**—CHAPSO-solubilized membrane was incubated in the presence or absence of 2 μM L458 in Buffer B for 0.5 h at 37 °C. A biotinylated affinity probe at 20 nM was added for an additional 1.5 h at 37 °C. Streptavidin-agarose (Pierce) was added to the reaction and incubated overnight 4 °C. Captured complexes were washed in Tris-buffered saline with 0.1% (v/v) Tween 20 and eluted with 2× Laemmli sample buffer. Samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The following antibodies were used for Western blotting: PS1-NTF (1:1000), PS1-CTF (1:1000), Aph1al (1:250), Nct (1:1000), Pen2 (1:500), and PS2-CTF (1:1000). Anti-mouse or anti-rabbit horseradish peroxidase-conjugated (Amersham Biosciences) secondary antibodies were used in conjunction with standard electrochemiluminescence detection methods. In all cases, blots shown are representative of three or more experiments.

**Photolabeling of γ-Secretase**—Solubilized membrane was incubated in the presence or absence of 2 μM L458 in Buffer B for 0.5 h at 37 °C. 20 nM biotinylated affinity probe was added for an additional 1 h at 37 °C. Samples were then irradiated at 350 nm on ice for 0.75 h. The reaction was either denatured by addition of RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, and 0.5% (w/v) deoxycholic acid) or left in the native condition for 1 h at 25 °C. Streptavidin-agarose was added to the reaction mixture and incubated overnight at 4 °C. Captured complexes were washed with RIPA buffer and eluted with 2× Laemmli sample buffer. Samples were Western blotted as described above. In all cases, blots shown are representative of three or more experiments.

**RESULTS**

Pen2 Overexpression Causes an Increase in the Aβ42:Aβ40 Ratio in Cells and in Vitro—Pen2 is essential for γ-secretase activity and is thought to stabilize the complex as well as promote endoproteolysis of PS (9, 10). To further investigate the function of Pen2 on γ-secretase activity and complex forma-
no effect on the expression level of APP in the Pen2 cell line compared with the parental cell line (Fig. 1c).

We next determined the effect of Pen2-Myc expression on γ-secretase activity for the 40 and 42 site cleavage of APP. Total membrane fractions isolated from the Pen2 and APP cell lines were incubated with the APP transmembrane substrate, and Aβ40 and Aβ42 cleavage products were measured (33). Production of Aβ40 was greatly reduced in the Pen2 cell line, but there was no effect on the production of Aβ42 (Fig. 2, left panel). This resulted in an overall increase in the Aβ42:Aβ40 ratio in the Pen2 line (0.25) compared with the APP line (0.15) (Fig. 2, right panel). To verify the in vitro results, we examined the secretion of Aβ40 and Aβ42 peptides from both cell lines (Fig. 2, middle panel). Consistent with the in vitro data, we found that there was a marked reduction in Aβ40 secretion in the Pen2 cell line but little effect on the secretion of Aβ42 compared with the APP cell line. The Aβ42:Aβ40 ratio for the Pen2 cell line was 0.29 and was 0.09 for the APP cell line (Fig. 2, right panel). The very similar results in both the cellular and in vitro assays strongly suggest that overproduction of Pen2 alters γ-secretase activity for Aβ40 and Aβ42 production, which is reminiscent of the effect of PS1 and PS2 FAD mutations.

Capture of the γ-Secretase Complex with Biotinylated Active Site-directed Inhibitors Depends on the Length of the Biotin Linker—To better understand the role of tagged Pen2 in the formation of γ-secretase complexes, we set out to examine the active γ-secretase complexes present in the Pen2 and APP cell lines. It is known that only a small percentage of PS is engaged in catalytically active complexes (35, 36). Therefore, traditional Western blotting and immunoprecipitation methods would be inadequate for our study because they fail to distinguish between catalytically active and inactive complexes. To overcome this, we attempted to develop small molecular affinity probes that would allow us to capture and characterize the active γ-secretase complex under native conditions.

L458 is a potent transition state analog that selectively binds to catalytically active γ-secretase, and its analogs have been used to characterize γ-secretase (17, 36) (Fig. 3a). We first attempted to use L646, a biotinylated γ-secretase inhibitor, to isolate the γ-secretase complex (1). CHAPSO-solubilized

![Figure 1. Overexpressed Pen2 is incorporated into the γ-secretase complex and results in reduced expression of γ-secretase components.](image)

(a) Western blot (WB) showing overexpression of Pen2 as detected with anti-Pen2 and anti-Myc antibodies. (b) Pen2 is associated with Nct. CHAPSO-solubilized membrane fractions were immunoprecipitated (IP) with anti-Nct and Western blotted for Pen2 with anti-Myc. (c) Overexpression of Pen2 causes a reduction in the protein levels of components of γ-secretase but does not affect the expression levels of the APP substrate. 10 μg of CHAPSO-solubilized membrane proteins were resolved by SDS-PAGE and Western blotted for PS1-NTF, PS1-CTF, Nct, PS2-CTF, and APP.

![Figure 2. Pen2 overexpression increases the Aβ42:Aβ40 ratio in both in vitro and cellular assays.](image)

For the in vitro assay (left panel), CHAPSO-solubilized membrane from the APP- or Pen2-expressing cell lines were assayed for Aβ40 and Aβ42 production using an APP transmembrane substrate. Background was defined as activity remaining in the presence of 1 μM L458. Values are the average of background-subtracted activity and are represented as units/min/μg (average ± S.E., n = 3). For the cellular assay (middle panel), APP and Pen2 cells were grown in 96-well plates, and the amount of Aβ40 and Aβ42 secreted into the medium was measured in the electrochemiluminescence (ECL) assay. Background was defined as the amount of Aβ40 and Aβ42 secreted when cells were grown in the presence of 1 μM L458. The concentration of Aβ40 and Aβ42 was determined with peptide standard curves (average ± S.E., n = 3). The Aβ42:Aβ40 ratio for the APP (black bar) and Pen2 (gray bar) cell lines for both the in vitro and cellular (secreted Aβ) assays are shown in the right panel.
membranes were incubated with L646 in the presence or absence of excess L458, and the bound complexes were captured with streptavidin-agarose, resolved by SDS-PAGE, and Western blotted for the core components of 

Equilibrium of γ-Secretase Complexes

FIGURE 3. The capture of active γ-secretase complexes relies on the length of linker. a, chemical structures of compounds used in this study. The photoreactive benzophenone group is marked with a dashed box. The linear distance of the linker between the parental compound backbone and the biotin moiety is measured in Å as determined with 3D ChemDraw. b, PS1 can be captured by biotinylated compounds with linkers of at least 34 Å. Active PS1 was captured from CHAPSO-solubilized HeLa membrane with 20 nm biotinylated affinity probe in the presence (+) or absence (−) of 2 μM L458, isolated with streptavidin-agarose, separated by SDS-PAGE, and Western blotted for PS1-NTF and PS1-CTF as indicated. c, compound 4 is able to capture the core components of γ-secretase in addition to PS1. 20 nm compound 4 was incubated with CHAPSO-solubilized HeLa membrane in the presence (+) or absence (−) of 2 μM L458 and isolated with streptavidin-agarose. The captured complexes were resolved by SDS-PAGE and Western blotted for the indicated γ-secretase subunits. Boc, t-butoxycarbonyl; Ph, phenyl.
Equilibrium of γ-Secretase Complexes

**FIGURE 4. Steric hindrance between streptavidin and the active site of γ-secretase by compounds with short linkers.** a, γ-secretase can be captured in the native conformation when photolabeled with compound 3 but not compound 1. CHAPSO-solubilized HeLa membrane was covalently photolabeled with 20 nM of either compound 1 or 3 in the presence (+) or absence (−) of 2 μM L458 by UV activation of the photoreactive benzophenone group. Photolabeled complexes were captured with streptavidin-agarose under either native (−RIPA) or denatured (+RIPA) conditions, resolved by SDS-PAGE, and Western blotted for PS1-NTF. b, soluble streptavidin (SA) greatly increases the IC₅₀ of compounds with biotin linkers less than 25 Å. CHAPSO-solubilized HeLa membrane was treated with increasing concentrations of the indicated compound and assayed for Aβ40 production using the C100FLAG substrate in the presence (+) or absence (−) of soluble streptavidin. Background is defined as activity remaining in the presence of 1 μM compound. Activity is plotted as percentage of background-subtracted maximal (Max) activity. Curves were generated using a sigmoidal four-parameter logistic fit in SigmaPlot 8.0. (average ± S.D., n = 4). c, schematic illustration of the interaction of the probes with the γ-secretase complex and streptavidin.

only compound 3 was able to capture PS1-NTF under native (−RIPA) conditions (Fig. 4a). In all cases, capture was completely blocked by inclusion of excess L458, indicating specific capture.

These studies suggest that the biotin moiety of the ligand becomes partly occluded by the protein interface of the active site when compounds with short linkers, such as compound 1, bind to γ-secretase under native non-denaturing conditions (Fig. 4a, −RIPA). This partial occlusion prevents streptavidin-agarose from binding to the biotin moiety of the ligand, and therefore labeled γ-secretase is not captured. However, denaturing conditions destroy the γ-secretase complex and thereby eliminate steric hindrance between the protein interface of the active site and streptavidin-agarose allowing isolation of the labeled components (Fig. 4a, +RIPA). To support this conclusion, we determined the inhibitory potency of the biotinylated analogs in the presence or absence of soluble streptavidin (Fig. 4b and supplemental Table S1). Inclusion of soluble streptavidin had no effect on the potency of L458 (Fig. 4b, left panel) but dramatically increased the IC₅₀ values of L646, compound 1, and compound 2 (>220-fold) (Fig. 4c, middle panel, and supplemental Table S1). However, inclusion of soluble streptavidin had much less of an impact on the potency of compound 3 and compound 4 (<20-fold) (Fig. 4c, right panel, and supplemental Table S1). It appears that the effect of streptavidin on the interaction of these inhibitors with γ-secretase is inversely correlated with the length of biotin linker. These data suggest that the active site of γ-secretase is a fairly deep binding pocket, which is consistent with studies that have indicated that the active site of γ-secretase is a hydrophilic pore 20–40 Å in length within the lipid bilayer (37, 38). Furthermore these data also indicate that affinity probes with linkers of at least 34 Å are required to capture γ-secretase under native non-denaturing conditions (Fig. 4c).

**Overexpression of Pen2 Increases the Formation of PS2-containing Active γ-Secretase Complexes**—After establishing that compound 4 was able to fully capture active γ-secretase complexes under native conditions, we characterized the active γ-secretase complexes in the Pen2 and APP cell lines using this probe. First we assessed the affinity of γ-secretase for L458 from both cell lines. We determined that the IC₅₀ values for inhibition of solubilized γ-secretase from the Pen2 and APP cells were 1.5 ± 0.1 and 1.3 ± 0.3 nM, respectively, indicating that overexpression of Pen2 did not affect the affinity of γ-secretase complexes for L458 (supplemental Fig. 1). We then used compound 4, based on the L458 backbone, to analyze the γ-secretase complexes in the APP and Pen2 cell lines. Solubilized membrane fractions from both cell lines were incubated with compound 4 in the presence or absence of excess L458, and streptavidin-agarose was then used to capture the compound 4-γ-secretase complexes. Bound complexes were washed, resolved by SDS-PAGE, and immunoblotted for γ-secretase components: PS1-NTF, Nct, Pen2, and PS2-CTF (Fig. 5a). When we compared the active γ-secretase complexes from the two cell lines, we immediately noticed two remarkable features. First, Pen2 cells possessed significantly more PS2-containing complexes but considerably fewer PS1 complexes than the APP cells even in light of the fact that Pen2 cells express less total PS2 than APP cells (Figs. 5a and 1c). This finding further supports the assertion that the total amount of γ-secretase subunits in cell membrane...
Equilibrium of γ-Secretase Complexes

**Affinity Capture**

| Compound 4 | APP | Pen2 |
|------------|-----|------|
| -/+L458    | PS1-NTF | -/+L458 |
| Nct        | -/+L458 | -/+L458 |
| Pen2       | -/+L458 | -/+L458 |
| PS2-CTF    | -/+L458 | -/+L458 |

**FIGURE 5.** Pen2 overexpression causes a shift from PS1-containing active γ-secretase complexes to PS2-containing complexes. *a*, the equilibrium of γ-secretase complexes is altered in Pen2 cells compared with APP cells. Active γ-secretase was captured from CHAPSO-solubilized membrane of APP or Pen2 cells with 20 nm compound 4 in the presence (+) or absence (−) of 2 μM L458. Bound complexes were captured with streptavidin-agarose, resolved by SDSPAGE, and Western blotted for the indicated components of γ-secretase. *b*, relative amounts of captured complex subunits indicate that the stoichiometry of the subunits is dynamic and can be modulated by Pen2 overexpression. Quantification of captured components was calculated by measuring the relative band intensities from the representative blot shown in *a* using the gel analysis function in ImageJ.

Prompted us to consider the possibility that overexpression of PS1 FAD mutants would alter the dynamics of PS1 and PS2 complex formation and mimic the effect seen with Pen2 overexpression. We performed analogous experiments as with the Pen2 cells using established cell lines that expressed either WT or mutated PS1. We characterized N2a cell lines stably expressing either PS1 WT or PS1 harboring the FAD mutations M146L or C410Y. Cell membranes isolated from each cell line were assayed for Aβ40 and Aβ42 production in the *in vitro* γ-secretase assay (Fig. 6a, left panel) (31, 39). Both PS1 M146L and C410Y mutations caused an increase in the Aβ42:Aβ40 ratio (0.20 and 0.36, respectively) compared with PS1 WT (0.08) (Fig. 6a, right panel) in our *in vitro* assay; this is consistent with previous cellular studies (31, 39). M146L slightly augmented Aβ40 production (138% of WT levels) and to a much larger magnitude increased Aβ42 production (346% of WT levels), whereas C410Y greatly decreased Aβ40 production (29% of WT levels) without significantly affecting Aβ42 levels (128% of WT levels). Irrespective of the divergent effect on Aβ40 and Aβ42 production, the end result of overexpression of either PS1 FAD mutant was an increase in the Aβ42:Aβ40 ratio.

Because the PS1 FAD mutants affected the Aβ42:Aβ40 ratio in a manner similar to that of Pen2 overexpression, we characterized the active γ-secretase complexes in the PS1 FAD mutants compared with PS1 WT. We first looked at expression levels of the known components of γ-secretase by Western blotting. Compared with the human WT PS1-expressing cells, the M146L-expressing cells exhibited a similar level of PS1, Pen2, and Nct but had significantly higher amounts of PS2 (Fig. 6b). Although overexpression of C410Y also resulted in higher levels of PS2, there was a decrease in expression of PS1, Nct, and Pen2 (Fig. 6b). After we determined that γ-secretase in these cells exhibited the same affinity for L458 (data not shown), we assessed the relative amount of active PS1- versus PS2-containing γ-secretase complexes between the cell lines. We again used compound 4 to capture the active γ-secretase complexes from CHAPSO-solubilized membrane fractions. Clearly both FAD PS1 and PS2 are capable of incorporating into the active γ-secretase complex. Furthermore M146L and C410Y had increased amounts of PS2-containing γ-secretase complexes compared with WT (Fig. 6c). M146L had slightly more PS1, but roughly equal amounts of Nct and Pen2 engaged in active complexes compared with WT, whereas C410Y had much less PS1, Nct, and Pen2. These data suggest that the amount of PS2-containing complexes is positively correlated with the Aβ42:
Aβ40 ratio generated by these cell membranes. Additionally these data, like the Pen2 overexpression data, suggest the possibility of differential stoichiometries of subunits of the various active γ-secretase complexes. For example, M146L overexpression resulted in a gross increase in PS2-containing active complexes but did not affect the levels of active PS1-containing complexes nor did it alter the incorporation of the critical subunits Nct and Pen2 into active complexes. Clearly the stoichiometry of subunits in the active γ-secretase complexes is dynamic.

To verify the assertion that PS1 WT and PS1 FAD mutants differentially affect the dynamics of the PS1 and PS2 γ-secretase complexes, we conducted similar studies using three additional PS1 FAD stable expressing N2a cell lines. The L286V, H163R, and E280A mutations reduced γ-secretase-mediated Aβ40 production to 15, 23, and 11%, respectively, of WT levels (supplemental Fig. 2a, upper panel). These FAD mutations also caused a reduction in the production of Aβ42 (58, 54, and 84% of WT production, respectively) (supplemental Fig. 2a, middle panel). The net effect of these mutations resulted in an increase in the Aβ42:Aβ40 ratio (0.32, 0.19, and 0.64 for L286V, H163R and E280A, respectively, compared with 0.08 for WT) (supplemental Fig. 2a, lower panel). We again used compound 4 to characterize the active PS1 and PS2 γ-secretase complexes in

**FIGURE 6.** Overexpression of PS1 FAD mutants causes an increase in the Aβ42:Aβ40 ratio and shifts the equilibrium from PS1-containing active γ-secretase complexes to PS2-containing complexes. a, PS1 FAD mutations increase the Aβ42:Aβ40 ratio. CHAPSO-solubilized membrane from PS1 FAD mutant-overexpressing N2a cells was assayed for in vitro production of Aβ40 and Aβ42 (left panel) using an APP transmembrane substrate. The Aβ42:Aβ40 ratios are shown in the right panel. Background was defined as activity remaining in the presence of 1 μM L458. Activity is graphed as background-subtracted units/min/μg of membrane assayed. b, expression of γ-secretase components is altered by expression of PS1 FAD mutants. CHAPSO-solubilized membrane from PS1 WT and two PS1 FAD mutants (M146L and C410Y) were Western blotted for the indicated components of γ-secretase. c, formation of PS2-containing γ-secretase complexes is increased by expression of PS1 FAD mutants. Active γ-secretase was captured from CHAPSO-solubilized membrane of PS1 WT, PS1 M146L, and PS1 C410Y cells with 20 nM compound 4 in the presence (+) or absence (−) of 2 μM L458. Bound complexes were captured with streptavidin-agarose, resolved by SDS-PAGE, and blotted for the indicated components of γ-secretase. ECL, electrochemiluminescence.
these cell lines. Although similar amounts of PS1 were detected in all four cell lines (supplemental Fig. 1b, upper panel), the amount of PS1 engaged in the active γ-secretase complex is considerably different. WT- and L286V-expressing cells had similar amounts of PS1-containing active γ-secretase, whereas H163R and E280A had much fewer PS1-containing active complexes (supplemental Fig. 2b, lower panel). H163R and E280A contained roughly the same amount of PS2 compared with WT, whereas L286V showed increased PS2 levels compared with WT (supplemental Fig. 2b, upper panel). However, all three FAD mutants contained much more active PS2-containing γ-secretase complexes compared with WT (supplemental Fig. 2b, lower panel). Furthermore using these PS1 FAD mutants and PS1 WT, we determined the relationship among 1) the ratios of Aβ42:Aβ40 and the compound 4-captured PS2:PS1 (referred to (PS2:PS1)captured), 2) the ratios of Aβ42:Aβ40 and the total PS2:PS1 (referred to (PS2:PS1)total), and 3) the ratios of (PS2:PS1)captured and (PS2:PS1)total. We found that 1) the ratio of Aβ42:Aβ40 strongly correlates with the ratio of (PS2:PS1)captured ($r^2 = 0.906$) but not (PS2:PS1)total ($r^2 = 0.018$) and 2) there is little correlation between (PS2:PS1)total and (PS2:PS1)captured ($r^2 = 0.041$) (supplemental Fig. 3). These data demonstrate three critical points. First, the total amount of PS1 and PS2 protein does not always correlate with their incorporation into active γ-secretase complexes. Therefore, isolation of the active γ-secretase complex is necessary for understanding the formation and dynamics of the complexes. Second, PS1 WT and FAD mutants have distinct effects on the equilibrium of PS1 and PS2 γ-secretase complexes, supporting our previous conclusion that γ-secretase complexes are dynamic and that perturbations to complex equilibrium can affect enzyme activity. Third, despite the different amounts of active PS1 and PS2 present in different cell lines, it appears that an increased ratio of captured PS2:PS1 leads to an elevated ratio of Aβ42:Aβ40, which correlates with the amount of characteristic AD plaques in mouse models (40, 41) and with the age of onset of familial Alzheimer disease (42). Therefore, these studies suggest that it is the relative amount of active PS1 and PS2 γ-secretase complexes that plays a critical role in the determination of the ratio of Aβ42:Aβ40.

**FAD Knock-in Mouse Models of AD Show an Increase in PS2-containing γ-Secretase Complexes—**To further validate our conclusion that perturbations to the PS1 and PS2 complex equilibrium results in increased Aβ42:Aβ40 ratios, we determined whether FAD knock-in mouse models also exhibited a biochemical phenotype similar to that seen in the cell models. Knock-in mouse models of AD have been shown to have increased Aβ42:Aβ40 ratios and accelerated AD plaque deposition compared with wild type controls (31, 43, 44). We chose two knock-in models that harbor PS1 mutations, M146V and ΔE10, to validate our findings that PS1 mutations can cause a shift in the equilibrium of PS1- and PS2-containing γ-secretase complexes. In previous studies, we have shown that ΔE10 mice have altered γ-secretase specificity for Aβ40 and Aβ42 production that leads to a 1.7-fold increase in the Aβ42:Aβ40 ratio (40). We determined that M146V mice also show a similar increase in the Aβ42:Aβ40 ratio. Solubilized membrane from WT or PS1 M146V brain was assayed for in vitro Aβ40 and Aβ42 production using the APP transmembrane substrate as done in the above cellular assays. M146V mice showed decreased Aβ40 but increased Aβ42 production compared with WT (Fig. 7a) that led to an increase in the Aβ42:Aβ40 ratio (1.5-fold).

We then examined the total amount of PS1 and PS2 in the membrane fractions from total brain. We found that there was very little difference in the expression levels of PS1 or PS2 between either of the knock-in mice and their respective wild type controls (Fig. 7b). We next determined the relative ratio of PS1 versus PS2-containing active γ-secretase complexes in brain from the knock-in mice. γ-Secretase was captured from CHAPSO-solubilized membrane fractions of brain with compound 4, and the amount of bound PS1 and PS2 was determined by Western blotting. M146V mice showed an increase in both PS1- and PS2-containing active γ-secretase complexes compared with WT control (Fig. 7c). ΔE10 mice showed no change in PS1-containing complexes but a large increase in PS2-containing complexes (Fig. 7c). In both cases it was evident that the equilibrium between PS1- and PS2-containing γ-secretase complexes was altered in the knock-in mice, and both exhibited an increase in the Aβ42:Aβ40 ratio. These data further support a positive correlation between shifting the equilibrium toward PS2-containing complexes at the expense of PS1-containing complexes and alteration of the Aβ42:Aβ40 ratio.

**DISCUSSION**

Three mutated genes (APP, PS1, and PS2) have been linked to early onset AD. Elucidating the function of these proteins and the effect of their mutations on γ-secretase activity offers a unique opportunity to investigate the reaction mechanism of γ-secretase and the molecular pathogenesis of AD. The small molecule approaches used in our present studies have provided critical insights into the effect of PS1 FAD mutations on γ-secretase activity and the dynamics of γ-secretase complex formation.

PS1 mutations that increase the ratio of Aβ42:Aβ40 in cell and animal models suggest that they affect γ-secretase activity. However, whether these cellular effects are attributed to γ-secretase itself and/or other cellular factors remains to be investigated. In other words, can these cellular observations be biochemically recapitulated in an in vitro system? This study provides biochemical evidence that PS1 mutations directly alter the rate of γ-secretase production of Aβ40 and Aβ42. Furthermore although there was a similar increase in the Aβ42:Aβ40 ratio, the PS1 mutations differentially affected γ-secretase activity for Aβ40 and Aβ42 production. We observed three distinct effects on Aβ40 and Aβ42 production by PS1 FAD mutants: 1) M146L increased both Aβ40 and Aβ42 production, 2) C410Y reduced Aβ40 and increased Aβ42 production, and 3) L286V, H163R, and E280A reduced both Aβ40 and Aβ42 production. Our studies suggest that these mutations mediate an increase in the Aβ42:Aβ40 ratio through distinctive mechanisms. Our data also indicate that expression of C-terminally Myc-tagged Pen2 increases the Aβ42:Aβ40 ratio in a manner similar to that of PS1 FAD mutants and therefore represents yet another distinctive mechanism by which γ-secretase activity can be modulated. Understanding how PS1 mutations and
Pen2 overexpression alters γ-secretase complex formation, which reveals a unifying factor for understanding their diverse effects on γ-secretase activity.

Our current findings reveal that expression of PS1 FAD mutants or C-terminal Myc-tagged Pen2 results in increased formation of active PS2 γ-secretase complexes. These findings clearly suggest that PS1 FAD mutants have a reduced ability to compete for the pool of shared obligatory subunits (Nct, Pen2, and Aph1) with PS2 and therefore lead to increased PS2 γ-secretase complex formation (Fig. 8). The precise mechanism of this reduced ability requires further investigation but potentially results from a reduced affinity for subunit interactions because of conformational changes in PS1. Because of the complexity of γ-secretase as a multisubunit membrane-bound enzyme complex, there has been a lack of both an in vitro reconstruction system and high resolution structural information of the complex. This has made it extremely challenging to obtain kinetic data that would indicate exactly how PS1 FAD mutations affect subunit interactions and complex assembly. Furthermore, the expression of C-terminal Myc-tagged Pen2 also led to an increase in PS2-containing complexes suggesting that PS2 may normally have an increased ability to compete with PS1 for the shared cofactors when the formation and natural dynamic of complex equilibrium are perturbed.

PS1 and PS2 are highly homologous (67% identical) but are engaged in mutually exclusive complexes (13, 35). Both PS1- and PS2-containing complexes are catalytically active; however, PS1-containing γ-secretase complexes display considerably higher specific activity than PS2 complexes (28, 35). Therefore, shifting the equilibrium toward an increase in PS2-containing γ-secretase complex at the expense of PS1-containing complexes should result in a reduction in overall γ-secretase activity; this hypothesis is supported by our current observations. Another critical question is how changing the complex equilibrium alters the ratio of Aβ42:Aβ40. Our preliminary studies indicating that the Aβ42:Aβ40 ratio displayed a strong positive correlation with the relative amount of active PS2:PS1 complexes suggest that PS2 γ-secretase complexes might be capable of processing the APP substrate with a higher Aβ42:Aβ40 ratio (Fig. 8, pathway 1). Concomitantly PS1-mutated γ-secretase also has altered specificity for Aβ42 and Aβ40 production (Fig. 8, pathway 2). Therefore, the alteration of γ-secretase specificity for Aβ40 and Aβ42 production could result from a combination of the increase in PS2 complex formation and altered activity by the mutated PS1 complex. Indeed our previous study showed that the Aβ42:Aβ40 ratio in PS2-expressing cells was 1.4-fold higher than that in PS1-expressing cells was 1.4-fold higher than that in PS1-expressing cells (35). Therefore, it is noteworthy to point out that investigation of the relationship between PS1- and PS2-mediated γ-secretase activity for Aβ40 and Aβ42 production is technically challenging. Utilizing a single form of PS (PS1 or PS2) in the absence of the

**FIGURE 7.** Two independent knock-in mouse models of FAD have increased Aβ42:Aβ40 ratios and show a shift toward PS2 containing active γ-secretase complexes. *a,* M146V knock-in mice exhibit altered Aβ42:Aβ40 ratios compared with WT mice. CHAPSO-solubilized membrane of total brain from PS1 M146V mice and its respective WT control were assayed for in vitro production of Aβ40 (black bar) or Aβ42 (gray bar) using an APP transmembrane substrate. Background was defined as activity remaining in the presence of 1 μM L458. Activity is graphed as background-subtracted units/min/μg of membrane assayed (average ± S.E., n = 3). The Aβ42:Aβ40 ratios are shown below (average ± S.E., n = 3). b, FAD knock-in mice have comparable basal expression of γ-secretase subunits as compared with WT. CHAPSO-solubilized membrane from total brain of PS1 M146V mice (*left panel*) and PS1 ΔE10 (*right panel*) and their respective WT controls was Western blotted for expression of PS1-NTF and PS2-CTF. c, FAD knock-in mice favor an increase in PS2-containing γ-secretase complex formation compared with WT. Active γ-secretase was captured from CHAPSO-solubilized membrane of total brain from PS1 M146V mice (*left panel*) or PS1 ΔE10 mice (*right panel*) and their respective WT controls with 20 nM compound 4 in the presence (+) or absence (−) of 2 μM L458. Bound complexes were captured with streptavidin-agarose, resolved by SDS-PAGE, and Western blotted for PS1-NTF and PS2-CTF. ECL, electrochemiluminescence.
other does not reflect the endogenous dynamics of PS1- and PS2-containing γ-secretase complexes that compete for the known shared cofactors (Nct, Aph1, and Pen2) when present in the same cells (32). Therefore, the contribution of PS1 mutations to the production of Aβ42 and Aβ40 should be carefully interpreted with consideration to PS2 involvement in the formation of Aβ peptides. Alternatively PS1 γ-secretase complexes may preferentially process Aβ40 peptides, whereas PS2 complexes show no preference for Aβ40 and Aβ42 production (Fig. 8, pathway 3). A loss of PS1 γ-secretase complexes because of an increase in PS2 γ-secretase complexes would selectively reduce Aβ40 peptide production and thereby account for the observed Aβ42:Aβ40 ratio increases we observed. Similarly a loss of Aβ40-generating PS1 complexes because of PS1 FAD mutations would also increase the Aβ42:Aβ40 ratio in the absence of PS2 expression. This is in fact supported by previous studies that showed that expression of PS1 FAD mutations in PS1- and PS2-null cells was capable of elevating the ratio of Aβ42 to Aβ40 (28).

Taken together, these findings reveal a novel mechanism of γ-secretase regulation by dynamic alteration of the equilibrium of PS1- and PS2-containing γ-secretase complexes (Fig. 8). These data provide a possible mechanism for multiple “loss of function” PS mutations distributed within different regions of the protein (25–27); these distinct mutations could exert their effect on Aβ40 production by perturbation of the complex equilibrium. In other words, shifting the equilibrium from PS1 γ-secretase to PS2 γ-secretase alters overall γ-secretase activity and leads to an elevated ratio of Aβ42:Aβ40 (Fig. 8). In addition to favoring an increase in PS2-containing complexes, PS1 FAD mutations also result in formation of a mutated PS1 γ-secretase complex with altered specificity for Aβ40 and Aβ42 production as well as leading to increased Aβ42:Aβ40 ratios (Fig. 8).

Additionally the requirement of affinity probes with longer linkers for capture of native γ-secretase has indicated that that the active site is buried deeply within the hydrophobic membrane. This conclusion is in agreement with other biochemical and structural studies (36–38) that have shown the active site of γ-secretase resides in a hydrophobic pore 20–40 Å in length within the lipid bilayer. Moreover conjugation of L685,458 to biotin by either hydrophobic or hydrophilic linkers had little effect on the inhibitory potency of L-685,458, suggesting that this inhibitor can easily access the active site of γ-secretase regardless of the biophysical properties of linkers. It is likely that L-685,458 accesses the γ-secretase active site through a channel-like mechanism. It is of note to point out that conformation of the γ-secretase complex is dependent on detergent concentration because γ-secretase in CHAPS at concentrations above the critical micelle concentration, in the presence or absence of digitonin, can be captured by a transition state inhibitor immobilized to solid support through a six-atom hydrophilic linker (15, 17). γ-Secretase captured under high detergent conditions such as these may represent a different form of the complex. Therefore we believe our panel of affinity probes with varying linker lengths is a valuable tool for the functional characterization of active and endogenous γ-secretase and allows us to further examine the relationship between γ-secretase specificity and endogenous PS1- and PS2-containing γ-secretase complexes in normal individuals and AD patients. This ability will facilitate elucidation of the pathogenesis of AD and aid in the development of therapeutics.

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