THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 52, pp. 44569–44575, December 30, 2011

Comparison of Presenilin 1 and Presenilin 2 γ-Secretase Activities Using a Yeast Reconstitution System*[S]

Received for publication, June 8, 2011, and in revised form, October 25, 2011 Published, JBC Papers in Press, November 10, 2011, DOI 10.1074/jbc.M111.270108

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γ-Secretase is composed of at least four proteins, presenilin (PS), nicastrin (NCT), Aph1, and Pen2. PS is the catalytic subunit of the γ-secretase complex, having aspartic protease activity. PS has two homologs, namely, PS1 and PS2. To compare the activity of these complexes containing different PSs, we reconstituted them in yeast, which lacks γ-secretase homologs. Yeast cells were transformed with PS1 or PS2, NCT, Pen2, Aph1, and artificial substrate C55-Gal4p. After substrate cleavage, Gal4p translocates to the nucleus and activates transcription of the reporter genes *ADE2*, *HIS3*, and *lacZ*. γ-Secretase activity was measured based on yeast growth on selective media and β-galactosidase activity. PS1 γ-secretase was ~24-fold more active than PS2 γ-secretase in the β-galactosidase assay. Using yeast microsomes containing γ-secretase and C55, we compared the concentration of Aβ generated by PS1 or PS2 γ-secretase. PS1 γ-secretase produced ~24-fold more Aβ than PS2 γ-secretase. We found the optimal pH of Aβ production by PS2 to be 7.0, as for PS1, and that the PS2 complex included immature NCT, unlike the PS1 complex, which included mature NCT. In this study, we compared the activity of PS1 or PS2 per one γ-secretase complex. Co-immunoprecipitation experiments using yeast microsomes showed that PS1 concentrations in the γ-secretase complex were ~28 times higher than that of PS2. Our data suggest that the PS1 complex is only marginally less active than the PS2 complex in Aβ production.

γ-Secretase consists of at least four subunits, presenilin (PS),[3] nicastrin (NCT), anterior pharynx defective 1 (Aph1), and presenilin enhancer 2 (Pen2) (1). PS is the catalytic subunit of γ-secretase with aspartic protease activity (2, 3). Amyloid-β (Aβ) peptide, which plays a causative role in Alzheimer disease (AD), is produced after sequential cleavage of amyloid-β precursor protein (APP) by β-secretase and γ-secretase. The Aβ mainly consists of Aβ40 and Aβ42 containing 40 and 42 amino acids, respectively. Aβ42 is more prone to aggregation (4) and more toxic to neuronal cells. Many studies have reported that familial AD (FAD) mutations in PS and APP result in increased ratios of Aβ42 to Aβ40. The high Aβ42 ratio is believed to lead to AD.

PS has two homologs, namely, PS1 and PS2 (67% identical at the amino acid level). Aph1 also has two homologs: Aph1a (with alternative splicing variants Aph1a-S and a-L) and Aph1b. Sato *et al.* (5) reported that γ-secretase contained only one of each subunit, and as such, six distinct γ-secretases exist. Indeed, both PS1 and PS2 form a γ-secretase complex with the other subunits, producing Aβ (6). γ-Secretase cleaves many type I transmembrane proteins including APP and Notch, but the mechanism by which the different γ-secretases select their substrates is unclear. These different γ-secretases may have different functions and substrate selectivity.

Ubiquitous expression of PS1 and PS2 mRNAs in many human and mouse tissues has been reported, with varying expression levels across their tissues and during brain development (7). For example, in human young adult and aged brains, PS1 and PS2 mRNAs expression was similar. The subcellular distribution of PSs are known to be predominantly in the endoplasmic reticulum and the Golgi compartment (8). Levitan *et al.* (9) showed that human PS1 and PS2 substituted for *Caenorhabditis elegans* sel-12, suggesting that PS1 and PS2 are functionally redundant.

Different phenotypes of PS1- and PS2-deficient mice have been reported. PS1 knock-out mice exhibit severe developmental defects and perinatal lethality (10, 11), whereas PS2 knock-out mice show only mild phenotypes (12). Over 160 FAD mutations in PS1, but only 10 in PS2, have been found. These findings suggest that PS1 and PS2 play distinct roles *in vivo*.

Lai *et al.* (13) indicated that Ps1 (Ps, mouse presenilin) γ-secretase produced 169 times more Aβ than Ps2 γ-secretase, using membrane fractions from Ps1-(+/−), Ps2-(−/−), and Ps1-(−/−), Ps2-(+/+) blastocyst-derived cells from knock-out mice. In their study, γ-secretase activity was calculated as follows: level of produced Aβ/total Ps. They did not use the calculation: level of produced Aβ/Ps in γ-secretase complex and thus did not evaluate the active γ-secretase content.

Yagishita *et al.* (14) developed a novel γ-secretase assay using yeast microsomes. Yeast lacks endogenous γ-secretase and

* This work was supported in part by grants from Human Frontier Science Program and The Ministry of Health, Labor and Welfare, Japan.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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[3] The abbreviations used are: PS, presenilin; APP, amyloid precursor protein; Aβ, amyloid β peptide; Aph1, anterior pharynx 1; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; CTF, carboxyl-terminal fragment; NCT, nicastrin; NTF, amino-terminal fragment; PC, phosphatidyl choline; Pen2, presenilin enhancer 2; FAD, familial Alzheimer disease; TM, transmembrane domain.

APP homologs, and one can reconstitute pure human γ-secretase in yeast and estimate the activity. Using this system, we compared the activity of PS1 and PS2 in γ-secretase complexes. Our data suggested that PS1-containing microsomes had much higher activity than PS2-containing microsomes. However, detailed analysis regarding the "active" γ-secretase complex revealed that the PS1 and PS2 complex produced similar levels of Aβ.

MATERIALS AND METHODS

Construction of γ-Secretase and Substrates—To reconstitute γ-secretase in yeast, human PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and substrates were cloned into the following vectors, as described previously (15). Briefly, PS1 or PS2 and NCT were ligated into KpnI and XbaI sites of the pBEVY-T vector (16). Aph1a-L-HA and FLAG-Pen2 were ligated into the XbaI and KpnI sites of pBEVY-L (16). C55-Gal4p, NotchTM-Gal4p, and C99 were fused to the *SUC2* signal sequence, facilitating translocation to the endoplasmic reticulum, and ligated into the BamHI and EcoRI sites of p426ADH (17). C55, C99, and NotchTM indicate amino acids 672–726 of the human APP770 isoform, 672–770 of the human APP770, 1703–1754 of the mouse Notch-1, respectively.

Myc-tagged PS1 and PS2 were PCR amplified and ligated into the KpnI site of pBEVY-T, using the following two pair of primers, respectively: mycPS1S, 5′-GGGGTACCAAAAATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGACAGAGTTACCTGCACCGTTG-3′ and PS1AS, 5′-GATCCGCTTATTTAGAAGTGTCGAATTCGACCTCGGTACCATGCTAGATATAAAATTGATGGAATGC-3′; mycPS2S, 5′-GGGGTACCAAAAATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGCTCACATTCATGGCCTCTGAC-3′ and PS2AS, 5′-GGGGTACCTCAGATGTAGAGCTGATGGGAGG-3′.

Yeast Transformation—Three plasmids were transformed into *Saccharomyces cerevisiae* strain PJ69–4A (*MATa*, *trp1–901*, *leu2–3*, *112*, *ura3–52*, *his3–200*, *gal4*△, *gal80*△, *LYS2::GAL1-HIS3*, *GAL2-ADE2*, *met2::GAL7-lacZ*) (18). The transformants were selected on SD media plate lacking Leu, Trp, and Ura (SD-LWU). In microsome assays, we used the yeast strain PJ69–4Apep4△(*MATa*, *trp1–901*, *leu2–3*, *112*, *ura3–52*, *his3–200*, *gal4*△, *gal80*△, *LYS2::-GAL1-HIS3*, *GAL2-ADE2*, *met2::GAL7-lacZ, pep4::kanMX*) (14) to avoid endogenous protease activity.

Reporter Gene Expression—Expression of *HIS3* (His) and *ADE2* (Ade) was estimated by transformant growth on SD-LWHUAde. β-Galactosidase assays were performed as described previously (15). Transformants were cultured in SD-LWU media until they reached an A_{600} of ~0.8. Cells were collected after centrifugation and suspended in lysis buffer (20 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol) including protease inhibitor mixture (Sigma), and lysed by glass beads. Protein concentration and β-galactosidase activity of the cell lysates were determined.

γ-Secretase Assay and Immunoblotting—Using yeast microsomes, we detected Aβ using an in vitro γ-secretase assay. *In vitro* γ-secretase assays were performed as described previously, with minor modifications (14). Microsomes (80 μg) were solubilized with γ-buffer (50 mM MES (pH 5.5) or 50 mM PIPES (pH 6.0, 6.5, 7.0, 7.5), or 50 mM HEPES (pH 8.0), 250 mM sucrose, 1 mM EGTA) containing 1% CHAPSO on ice for 60 min. Inhibitor mixture, thiorphan, *O*-phenanthroline, CHAPSO, and γ-buffer were added to the solubilized microsomes, as described previously (14). The mixture was incubated at 37 °C for 0 or 24 h. After incubation, the sample was extracted with chloroform/methanol (2:1) followed by addition of sample buffer, and boiled at 100 °C for 5 min. Aβ production was analyzed by Western blotting using the specific antibody, 82E1. Band signal was quantified using an LAS-3000 luminescent image analyzer (FujiFilm, Tokyo, Japan).

Immunoprecipitation of γ-Secretase—Microsomes (400 μg) were solubilized with IP buffer containing 1% CHAPSO and protease inhibitor mixture, on ice, for 60 min. Solubilized membranes were added to 40 μl of anti-FLAG affinity gel (50% slurry) (Sigma) and rotated at 4 °C for 2 h. Beads were washed with IP buffer and suspended in sample buffer containing 8 M urea to prepare the "IP sample" from 400 μg of microsomes. The "input sample" was prepared as follows: 100 μl of sample buffer containing 8 M urea was added to 80 μg of microsomes and incubated at 65 °C for 10 min. Microsomes (8 μg, 10–11 μl) were loaded as input.

Antibodies—The following antibodies were used for immunoblotting: monoclonal antibodies against Aβ, 82E1 (IBL, Fujioka, Japan), HA (12CA5; Sigma), FLAG (M2; Sigma), and polyclonal antibodies against NCT (AB5890; Chemicon, Temecula, CA), Myc, 2272 (Cell Signaling Technology, Beverly, MA), the PS1 loop region (G1L3) (19), and the PS2 loop region (G2L) (20).

RESULTS

PS2 Was Less Active than PS1 in Growth and β-Galactosidase Assays—We constructed recombinant plasmids for γ-secretase and APP-based (C55-Gal4p) or Notch-based substrates (NotchTM-Gal4p) (15). We introduced the vectors into yeast strain PJ69, which expresses *HIS3*, *ADE2*, and *lacZ* under Gal4p control, and generated yeast transformants expressing the γ-secretase subunits (PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2) and an artificial substrate (C55-Gal4p or NotchTM-Gal4p). Gal4p released from C55-Gal4p or NotchTM-Gal4p by reconstituted γ-secretase activates *HIS3* and *ADE2* genes transcription. Therefore, γ-secretase activity was assessed by growth on media lacking histidine and adenine. As a result, yeast expressing PS1 γ-secretase and C55-Gal4p could replicate on the selection media. Yeast expressing PS2 γ-secretase could also grow, but was much slower than that of PS1-expressing yeast (Fig. 1*A*). PS1 L166P, G384A, and PS2 N141I are familial Alzheimer disease (FAD) mutations. Yeast carrying these mutations were unable to grow on media lacking histidine and adenine. After isolating these yeast cell lysates, we measured β-galactosidase activity to estimate γ-secretase activity. PS1 had ~24 times more β-galactosidase activity than PS2 (Fig. 1*B*). The results of the β-galactosidase assay were well correlated with the growth assay results (Fig. 1, *A* and *B*).

Next, we used NotchTM-Gal4p as a substrate instead of C55-Gal4p. The results were similar to those obtained when using

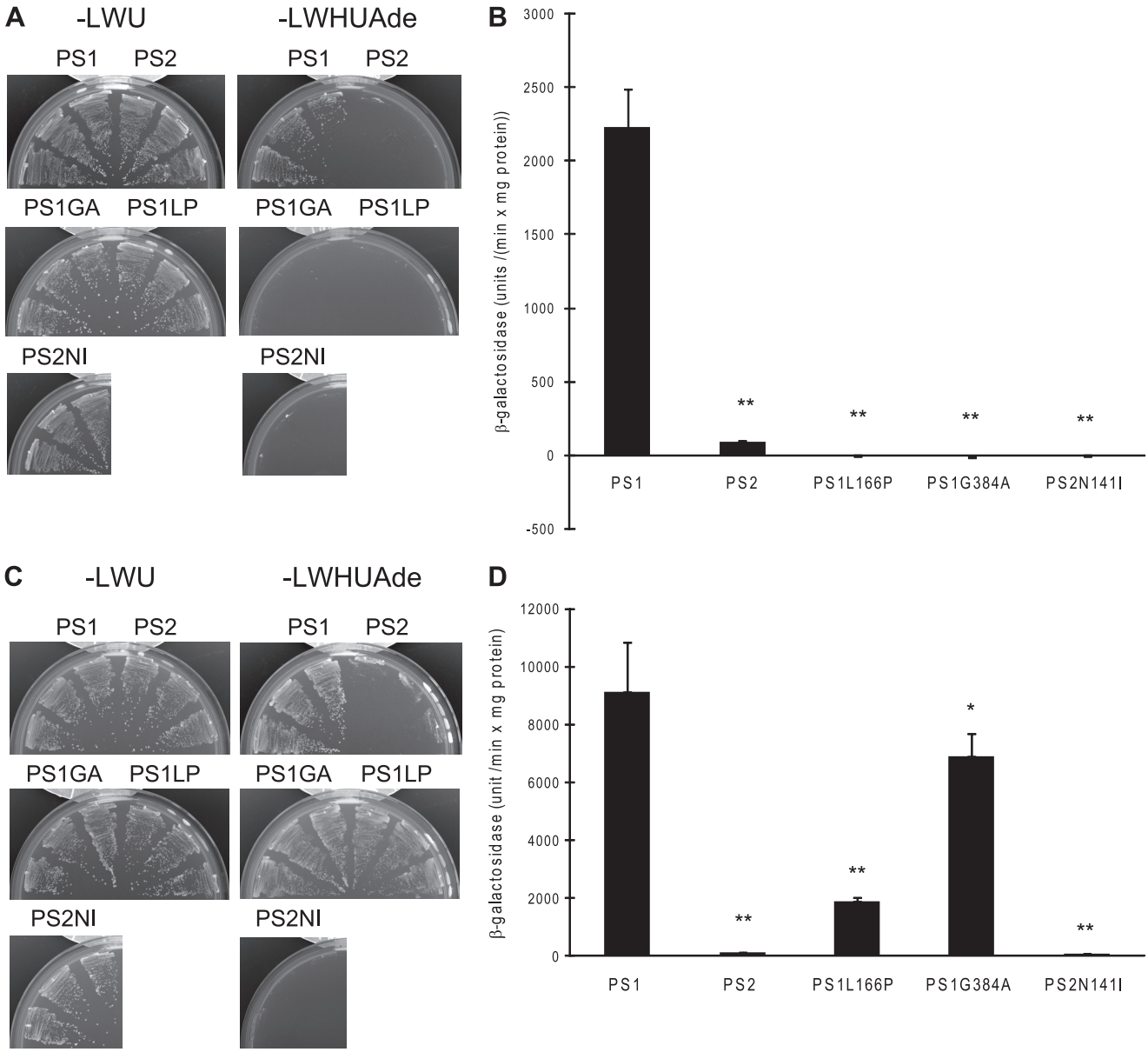

FIGURE 1. **Estimate of reconstituted PS1 or PS2 γ-secretase activity in yeast.** *A* and *C*, yeast cells were transformed with PSs (PS1 or PS2, or PS with FAD mutations), NCT, FLAG-Pen2, Aph1a-L-HA, and C55-gal4p (*A*), or NotchTM-gal4p (*C*). Three independent clones were cultured on non-selection media (SD-LWU) or selection media (SD-LWHUAde) at 30 °C for 3 days. Yeast cells not expressing PS did not grow on SD-LWHUAde. *B* and *D*, β-galactosidase activity was measured for each yeast lysate. Lysates were prepared from yeast cells using glass beads. One unit of β-galactosidase activity corresponds to 1 nmol of *O*-nitrophenyl β-d-galactopyranoside hydrolyzed per min, and activity was calculated as unit/(min × mg of protein in lysate). The activity was normalized by subtracting the activity in the absence of PS, 65 unit/(min × mg protein). Data are presented as mean value ± S.D., $n = 18$ (*A*), $n = 3$ (*C*) *, $p < 0.05$; **, $p < 0.01$ (analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test). Statistical analyses were performed with PRISM software.

the C55-Gal4p, with the following two exceptions. Notch1 was more likely to be cleaved by γ-secretase than C55 (APP) (Fig. 1, *B versus D*) and yeast cells expressing PS1 with FAD mutations (L166P and G384A) were able to grow on SD-LWHUAde, whereas cells expressing PS2 N141I were not (Fig. 1*D*). These results suggested that PS1 with the FAD mutations cannot cleave APP, whereas they can cleave Notch like wild-type γ-secretase.

Optimal pH for Aβ Production by the PS2 Complex—To study γ-secretase activity *in vitro*, we prepared yeast microsomes from yeast transformants expressing PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and C55 (14). Three previous reports showed that γ-secretase with PS1 maximally produced Aβ at approximately pH 7.0 (14, 21, 22). The optimum pH of Aβ production by γ-secretase with PS2, however, remains unclear. Thus, we investigated the optimal pH of the PS2 complex to produce Aβ. When yeast microsomes prepared from three independent clones were incubated for 24 h at 37 °C with 0.25% CHAPSO and 0.1% PC, we found that the PS2 complex also maximally produced Aβ at approximately pH 7.0 in all three assays (Fig. 2, *A* and *B*), suggesting that the PS1 and PS2 complex have similar pH dependences for Aβ production.

Levels of Aβ Production by PS1 or PS2—We compared the level of Aβ produced by PS1 or PS2 using yeast microsomes. Each microsome was incubated at 37 °C for 24 h in the pres-

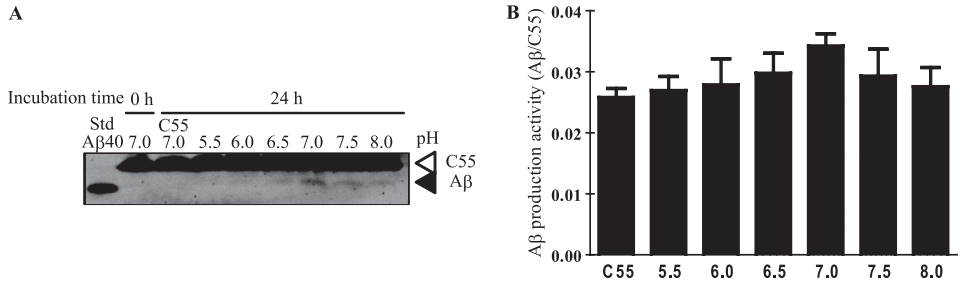

FIGURE 2. **Optimum pH of Aβ production by PS2.** *A*, microsomes (80 μg) prepared from three independent yeast cells transformed with PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and C55, and from yeast expressing C55 were incubated with 0.25% CHAPSO and 0.1% PC at 37 °C for 0 or 24 h. Incubation samples were subjected to immunoblotting to compare Aβ production activity, Aβ/C55. Aβ was detected by 82E1. Synthetic Aβ40 (20 pg) was used as a positive control. Yeast expressing C55 and microsomes incubated for 0 h were loaded as a negative control. *B*, three independent assays were quantified using analyzing software (LAS-3000 luminescent image analyzer, Fuji Film, Tokyo, Japan). The column represents the mean ± S.D. ($n = 3$).

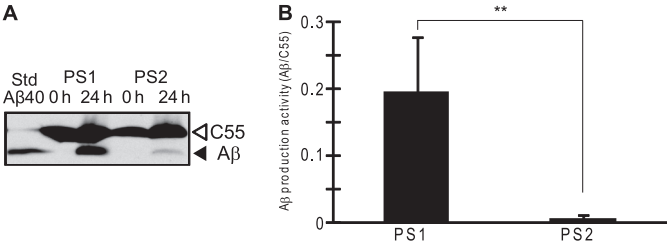

FIGURE 3. **Difference in Aβ production between PS1 and PS2.** *A*, yeast microsomes expressing PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and C55 were subjected to *in vitro* γ-secretase assays at pH 7.0. Aβ produced by PS1 or PS2 γ-secretase was detected. Synthetic Aβ40 (30 pg) was loaded as a marker. *B*, the bands obtained in *A* were quantified to determine the ratio of Aβ to C55 using analyzing software (LAS-3000 luminescent image analyzer, Fuji Film, Tokyo, Japan). The column represents the mean ± S.D. ($n = 5$, **, $p < 0.01$). Data were analyzed by Student's *t* test.

ence of 0.25% CHAPSO and 0.1% PC. We found that the PS1 complex produced significantly more Aβ than PS2 (Fig. 3*A*). By quantifying the Western blotting signals, we calculated that PS1 produced ~24 times more Aβ than PS2 (Fig. 3*B*).

PS1 Complexes Were More Abundant than PS2 Complexes—To verify whether PS, NCT, Aph1a-L, and Pen2 form the γ-secretase complex, we isolated membrane fractions from yeast introduced with PS, NCT, Aph1a-L-HA, FLAG-Pen2, and C99, and performed co-immunoprecipitation experiments with the anti-FLAG M2 affinity gel. Both PS1 and PS2 were co-immunoprecipitated with FLAG-Pen2 (Fig. 4, *B* and *C*). NCT and Aph1a-L were also co-immunoprecipitated with FLAG-Pen2 (Fig. 4*A*), suggesting that PS1 and PS2 formed a γ-secretase complex. We also found that the PS2 complex predominantly included non-glycosylated immature NCT, whereas the PS1 complex contained highly glycosylated mature NCT (Fig. 4*A*).

Comparison of the PS1 and PS2 contents in γ-secretase is difficult due to the variable affinity of their specific antibodies. To estimate the amount of PS1 or PS2 in γ-secretase complexes, we constructed Myc-tagged PS1 and PS2. We introduced these constructs into yeast and reconstituted the γ-secretases. Preparing these microsomes, we immunoprecipitated γ-secretase complexes with anti-FLAG affinity gel. The immunoprecipitates were next subjected to immunoblotting. Aph1a-L levels in the PS1 or PS2 complex were similar (Fig. 5*A*). The Myc-tagged PS1 complex included mainly mature NCT, while Myc-tagged PS2 complexes contained immature NCT (Fig. 5*A*). The level of PS1 NTF in γ-secretase complexes (associated with FLAG Pen2) was ~28 times higher than that of PS2 NTF (Fig. 5*B*).

When calculating γ-secretase activity per one γ-secretase complex from these data, a significant difference between PS1 and PS2 does not exist. However, the PS1 complex was 24.15 more active in the β-galactosidase assay. *In vitro* Aβ production assays indicated that PS1 was 24.61 more active than PS2. Comparing PS1 and PS2 contents in γ-secretase in a co-immunoprecipitation experiment, we found that the amount of PS1NTF in the γ-secretase complex was 28.14 times higher than that of PS2NTF. These data suggested that the complete PS2 complex was 1.142 or 1.143 times more active than the PS1 complex.

DISCUSSION

γ-Secretase assays measuring released Aβ into conditioned media from cultured cells have been previously performed. These assays found that γ-secretase with PS FAD mutations increased the Aβ 40/42 ratio. However, very few *in vitro* assays have been reported. To accurately study γ-secretase activity, Yagishita *et al.* (14) established an *in vitro* assay system using yeast, which possesses no γ-secretase homologs. This system enabled us to directly compare activities between the PS1 and PS2 complex.

Yeast growth and β-galactosidase assays using C55-Gal4p or Notch-Gal4p as a substrate revealed that PS1 had a significantly higher activity than PS2. We also found that FAD mutations in PS abolished APP processing activity, and that PS1 L166P and G384A cleaved Notch with reduced activity compared with wild-type PS1. The assembly of PS1 FAD mutants (L166P or G384A) into γ-secretase complex was also assessed by immunoprecipitation (supplemental Fig. S1). The assembly of PS1 L166P mutant was similar to PS1 WT. On the other hand, ~36% of PS1 G384A (comparing to the WT) formed the γ-secretase complex. These results showed that PS1 L166P assembled normally with defective protease activity and PS1 G384A was defective both in the assembly and the protease activity, suggesting that loss of function of PS caused lower cleavage activity. These reductions in processing activity obtained in this report support PS loss of function hypothesis, which is believed to cause FAD (23). We evaluated the activity of other PS1 FAD mutations (A79V, M146L, A231V, M233T, and ΔExon9) in Notch cleavage (data not shown). Our Notch

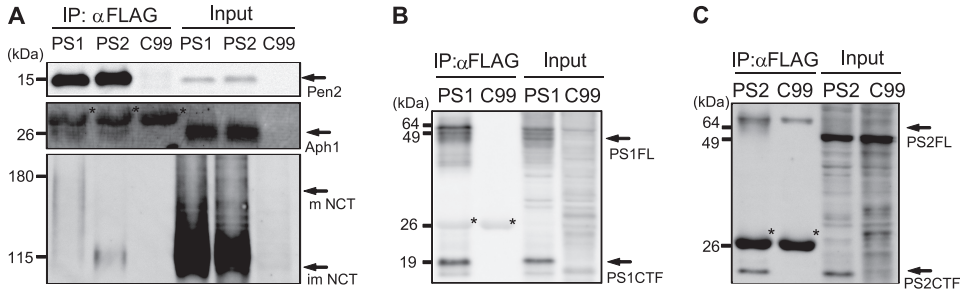

FIGURE 4. **Formation of PS1 and PS2 γ-secretase complexes.** Yeast microsomes expressing PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and C99, and microsomes expressing C99 were solubilized with IP buffer containing 1% CHAPSO and protease inhibitor mixture. γ-Secretase complexes were immunoprecipitated with anti-FLAG affinity gel (Sigma). The immunoprecipitates and input fraction were subjected to immunoblotting. NCT, Aph1, Pen2, and PS were detected by specific antibodies. The *asterisks* indicate nonspecific bands.

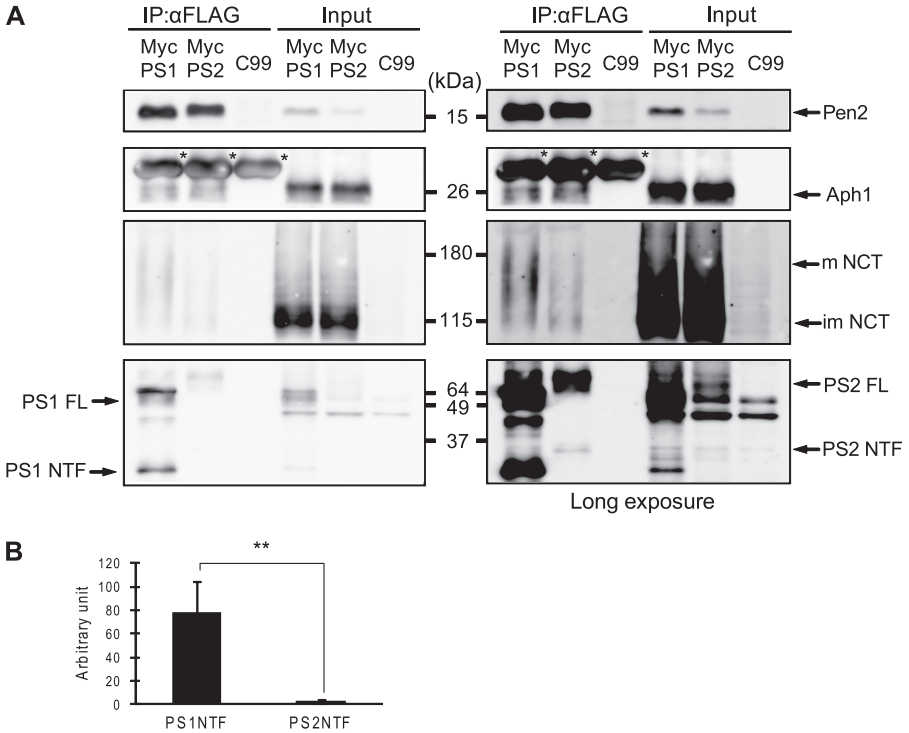

FIGURE 5. **Quantification of PS1 and PS2 in γ-secretase complexes.** *A*, yeast expressing Myc-tagged PS1 or PS2, the other secretase subunits, and C99, were incubated with anti-FLAG affinity gel. The immunoprecipitates were analyzed by immunoblotting. *B*, amount of Myc-tagged PS1NTF and Myc-tagged PS2NTF in the γ-secretase complexes were quantified using LAS-3000 luminescent image analyzer (Fuji Film, Tokyo, Japan). Data were analyzed by Student's *t* test. Error bar shows the mean ± S.D. $n = 4$, **, $p < 0.01$. The *asterisks* indicate nonspecific bands.

cleavage results with PS1 FAD mutations, PS1L166P and G384A, corroborated the findings of earlier studies (24, 25).

Based on the *in vitro* γ-secretase assay using yeast microsomes, we found that γ-secretase with PS2 optimally produced Aβ at approximately pH 7.0. Previous reports have shown that PS1 also maximally produced Aβ at pH 7.0 (14, 21, 22), suggesting that PS1 and PS2 make Aβ using a similar mechanism.

Our co-immunoprecipitation experiments using yeast microsomes containing PS1 or PS2, NCT, Aph1a-L-HA, and FLAG-Pen2 showed that PS2 bound to immature NCT, whereas PS1 bound to the mature NCT. Expression levels of immature or mature NCTs in cells transformed with PS1 or PS2 were similar, but the anti-FLAG affinity gel immunoprecipitates contained different levels of immature and mature NCT. Frånberg *et al.* (26) reported that Ps2 bound to immature NCT in Ps1-deficient (Ps1-(−/−), Ps2-(+/+)) MEF cells and Ps1 bound to mature NCT in Ps2 deficient (Ps1-(+/+), Ps2-(−/−)) MEF cells using affinity capture with an active site-directed γ-secretase inhibitor. This difference in NCT maturation in the complex may affect substrate affinity.

In this study, we used Aph1a-L as a γ-secretase subunit, which may facilitate PS2 binding to immature NCT. Also, Aph1a-S expression, or Aph1b as a γ-secretase subunit, may result in alternative binding patterns, such as PS2 binding to mature NCT or PS1 binding with immature NCT. In fact, we observed the PS1 complex with Aph1a-S containing more immature NCT than the PS1 complex with Aph1a-L (data not shown). To date, γ-secretase is known to target many substrates, but how γ-secretase selects its substrates is unclear. These variable γ-secretases may contribute to specific substrate selection.

To compare the γ-secretase activity of PS1 and PS2 precisely, we employed two different approaches. First, we used C55(-Gal4p) or C99 as a substrate instead of C100Flag. NCT

plays a role in binding to the substrate by recognizing N terminus of C99 (27). So, natural N terminus of C99 or C55 is important to assess γ-secretase activity correctly. Using C100Flag as a substrate may result in inaccurate evaluation, because C100Flag possesses one extra amino acid, methionine, on the N terminus. Second, we estimated the amount of PS1 or PS2 in the γ-secretase complex. Lai *et al.* (13) reported Ps1 and Ps2 γ-secretase activity as a function of total protein concentration, but not all PS localizes to the γ-secretase complex. Therefore, γ-secretase activity should be calculated as follows: γ-secretase activity/concentration of PS in γ-secretase complex. γ-Secretase assembly is not a random process, but occurs sequentially. NCT and Aph1 form the NCT-Aph1 subcomplex in the initial step of complex formation. Two hypotheses have been proposed regarding the subsequent steps in γ-secretase complex assembly. One hypothesis is that PS binds to the NCT-Aph1 subcomplex, followed by Pen2, creating a γ-secretase complex (28, 29). Alternatively, the PS-Pen2 intermediate may bind to the preexisting NCT-Aph1 subcomplex to form the γ-secretase complex (30). To evaluate the construction process of the γ-secretase complex, we compared PS1 or PS2 in the γ-secretase complex by co-immunoprecipitating Myc-tagged PS1 or PS2 with anti-FLAG antibody (FLAG tag is on Pen2). Co-immunoprecipitation with other antibodies detecting NCT, Aph1, or PS could lead to inaccurate estimates regarding the amount of Myc-PS in the γ-secretase complex. We found that the concentration of PS2 in the γ-secretase complex was much lower than that of PS1. Because we applied a minimal reconstitution system in yeast, unknown protein(s) may stabilize PS2. This possibility is currently being explored.

In this study, we reconstituted human PS1 and PS2 γ-secretase complexes and compared their Aβ production (per γ-secretase complex). PS1 had 24.65 times and 24.61 times higher activity than PS2 in the β-galactosidase and *in vitro* Aβ production assay, respectively. Based on Co-IP experiments, the amount of PS1 in the γ-secretase complex was 28.14 times higher than that of PS2. Thus, our data suggest that PS1 did not have significantly higher activity than PS2, as has been reported (13). PS1 and PS2 were 67% identical at the amino acid level, suggesting that these two proteins have related functions in the γ-secretase complex. Our results suggest that the difference between PS1 and PS2 is their affinity to the other γ-secretase subunits. The contribution of PS1 on γ-secretase activity is more important than that of PS2 because PS1 knock-out mice exhibit severe phenotypes, whereas PS2 knock-out mice do not. We hypothesize that the differences in PS1 and PS2 knock-out mice phenotypes may result from different amounts of PS1 and PS2 γ-secretases, but not differences in their activity.

Currently, PS1 is believed to have a higher activity than PS2 in γ-secretases, while we showed that they have similar activities. In corroboration of our findings, recent reports have shown that PS2 γ-secretase cleaved more APP than PS1 γ-secretase in microglia cells, regardless of the presence of PS1 (31). Thus, when studying γ-secretase activity, we should consider the concentration of PS in the active γ-secretase complex, which may aid in clarifying the pathogenesis of FAD caused by PS loss-of-function FAD mutations.

Acknowledgments—We thank Dr. Raphael Kopan for the mNotch1 clone and Dr. Philip James for the PJ-69–4A yeast strain. We also thank the members of our laboratory, especially Yusuke Nagara, Natsumi Ohsawa, and Yuri Watanabe for helpful discussions and technical suggestions.

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