Specific combinations of presenilins and Aph1s affect the substrate specificity and activity of γ-secretase

Yoji Yonemura a, d, Eugene Futai b, Sosuke Yagishita c, Christoph Kaether d, Shoichi Ishiura a, *

a Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Tokyo 153-8902, Japan
b Department of Molecular and Cell Biology, Graduate School of Agricultural Science Tohoku University, Miyagi 981-8555, Japan
c Department of Pharmacology, Faculty of Medicine, Saitama Medical University, 38 Moro-hongo, Iruma-gun, Saitama, 350-0495, Japan
d Leibniz Institute on Age Research, Fritz-Lipmann-Institute, Beutenbergstr. 11, Jena, 07745, Germany

1. Introduction

The aspartyl-protease γ-secretase produces amyloid β peptide (Aβ), a causative protein in Alzheimer’s disease (AD). Aβ is produced after sequential cleavage of the amyloid β precursor protein (APP) by β-secretase (BACE1) and γ-secretase. Initially, APP is cleaved by BACE1 to produce a C99 fragment, which is further cleaved by γ-secretase [1]. γ-Secretase cleaves the C99 fragment at several sites, including the ε site and the subsequent γ site [2]. Cleavage at the ε site produces Aβ49 or Aβ48, depending on which side of the site cleavage occurs. Aβ49 is processed to Aβ40 via the release of three tripeptides (ITL, VIV, and IAT), while Aβ48 is processed to Aβ42 via the release of two tripeptides (VIT and TVI) [3], indicating tripeptidylcarboxypeptidase activity of γ-secretase. Aβ in the human brain consists mainly of Aβ40 and Aβ42 at a ratio of approximately 9:1. Aβ42 is more prone to aggregate [4], and an increase in the Aβ42-to-Aβ40 ratio (Aβ42/Aβ40), observed in familial AD patients, is believed to lead to AD pathology [5]. Furthermore, Aβ43 is another neurotoxic Aβ species and PS with familial AD mutations causes higher Aβ43/Aβ40 ratios [6]. Because the Aβ42/Aβ40 ratio is determined by γ-secretase, investigation of γ-secretase may help elucidate the pathogenesis of AD.

The γ-secretase complex comprises four core subunits: presenilin (PS), nicastrin (NCT), anterior pharynx-defective 1 (Aphe1), and presenilin enhancer 2 (Pen2). PS has two homologues, PS1 and PS2. Aph1 has two isoforms, Aph1a and Aph1b, with the former existing as two splice variants Aph1aL and Aph1aS. Each complex consists of one subunit each, resulting in six different γ-secretases. To better understand the functional differences among the γ-secretases, we reconstituted them using a yeast system and compared Notch1-cleavage and amyloid precursor protein (APP)-cleavage activities. Intriguingly, PS2/Aph1b had a clear increase in the Aβ42/Aβ40 ratio over the other γ-secretases. In addition, PS2/Aph1aS γ-secretase produced less Notch intracellular domain (NICD) than the other 5 γ-secretases. Considering that the Aβ42/Aβ40 ratio is relevant in the pathogenesis of Alzheimer’s disease (AD), and that inhibition of Notch cleavage causes severe side effect, these results suggest that the PS2/Aph1aS γ-secretase complex is a potential therapeutic target in AD.

© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
In this study, we examined some of the differences in function and substrate specificity of the γ-secretase complexes, using yeast as a model system [10–12] and human HEK293 cells. Because yeast lacks endogenous γ-secretase, we reconstituted purified human γ-secretases in yeast and compared their respective activities. Our data suggest that different combinations of PSs and Aph1s result in different γ-secretase functions.

2. Materials and methods

2.1. Constructs

To express six different γ-secretases and Gal4 fusion substrates in yeast, PS (PS1 or PS2), NCT, Aph1-ΔHA (Aph1αL-ΔHA or Aph1αS-ΔHA or Aph1b-ΔHA), Flag-Pen2 and C55-Gal4 or Notch-Gal4 were cloned into the pBEVY, as described previously [11]. An Aph1b clone (GNP Clone IRAL037F15) was provided by the RIKEN Bio Resource Center, which participates with the National Bio Resource Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan [13–16].

2.2. Yeast transformation and reporter assays

To reconstitute each γ-secretase complex, three plasmids were transformed into Saccharomyces cerevisiae strain PJ69-4A (MATa, trp1-901, leu2-3,112, his3-200, gal4d, gal80d, LYS2:GAL1-HIS3, GAL2-ADE2, met2:GAL7-lacZ) [17]. The transformants were selected on SD-LWU plates.

The expression of Gal reporters HIS3 (His), ADE2 (Ade), and lacZ were estimated via growth assays on SD-LWUH Ade plates and β-galactosidase activities. The β-galactosidase assays were performed as described previously [11,12].

2.3. Cell lines and transfection

Human embryonic kidney (HEK293) cell lines stably expressing Swedish mutant APP and γ-secretase [18] were kindly provided by Harald Steiner. Briefly, PS1 or PS2, respectively, was stably transfected into A47–1 double knockdown HEK293 cells already stably expressing Aph1-ΔL, -S or -Δb and Swedish mutant APP. Cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal bovine serum and G418 (200 μg/ml), Blasticidin (10 μg/ml), Zeocin (400 μg/ml), Puromycin (0.5 μg/ml), 1% Penicillin-Streptomycin.

2.4. ELISA

To detect levels of Aβ40, Aβ42 and Aβ43 secreted in the culture media, human Aβ40, Aβ42 and Aβ43 assay kits (IBL international Res59781, Res59791 and Res59711, respectively) were used. ELISA was performed according to the instruction manual. Culture media were diluted 250-fold, 20-fold and 5-fold for the Aβ40, Aβ42 and Aβ43 ELISA, respectively. Three independent samples from each cell line were tested for each Aβ.

2.5. Antibodies

The following antibodies were used for immunoblotting: polyclonal antibodies against NCT (N1660), Pen2 N-terminus (1638), PS1 loop region (3027) [20], Aph1α L/S loop (O2F1, PRB-S51P), Aph1b C-terminus (435) [21], Aβ (2D8), APP C-terminus (6687), β-actin (Abcam, ab8227), and monoclonal antibodies against PS2 (BLHFS5c) [22], GFP (Clontech, 632381).

3. Results

3.1. PS2/Aph1b γ-secretase cleaves APP- but not Notch-derived substrates in a yeast reconstitution system

To compare the APP- and Notch-cleavage activities of the six different γ-secretases, we introduced PS (PS1 or PS2), NCT, Aph1 (Aph1αL-ΔHA or Aph1αS-ΔHA, or Aph1b-ΔHA), and Flag-Pen2 into the yeast strain PJ69-4A [17]. This strain expresses HIS3, ADE2, and lacZ under Gal4 control. As a substrate for γ-secretase, C55-Gal4 (‘C55’ indicates amino acids 672–726 of human APP770 isoform) or Notch-Gal4 (‘Notch’ indicates amino acids 1703–1754 of mouse Notch1) was co-expressed. Transformants expressing different γ-secretases (PS1/Aph1αL, PS1/Aph1αS, PS1/Aph1b, PS2/Aph1αL, PS2/Aph1αS or PS2/Aph1b) were streaked on SD media plates lacking Leu, Trp, and Ura (SD-LWU) and SD media plates lacking Leu, Trp, His, Ura, and Ade (SD-LWHUAde). Yeast growth was observed 3 days after streaking. Growth of yeast cells only occurs upon cleavage of Gal4 containing substrate.

We first tested cleavage efficiency of APP-derived substrate, C55-Gal4, by six different γ-secretases. Yeast expressing PS1 γ-secretase with any of the Aph1 subunits grew well on SD-LWHUAde plates, whereas yeast expressing PS2 γ-secretase grew poorly, with the exception of PS2/Aph1b, indicating that PS2/Aph1αL or S isoforms do not efficiently process C55-Gal4 (Fig. 1a). The β-galactosidase activity of the yeast lysates confirmed that all PS1 containing γ-secretases but only PS2/Aph1b γ-secretase efficiently cleaved APP (Fig. 1c).

Next, we evaluated whether cleavage of the Notch-Gal4 was affected by different PSs and Aph1s. Yeast expressing PS1 γ-secretase grew normally on the SD-LWHUAde plates, whereas yeast expressing any of the PS2 γ-secretases grew poorly, implying that PS1 γ-secretase cleaves Notch-Gal4 more efficiently than PS2 γ-secretases (Fig. 1b). The β-galactosidase activities of the yeast lysates confirmed the growth assay. A high level of β-galactosidase activity was observed in the lysates from transformants expressing PS1 γ-secretase, with the PS1/Aph1αS variant showing the highest activity. In contrast, low activity was observed in the lysates from transformants expressing PS2 γ-secretase (Fig. 1c). The Aph1 isoform appeared to have no significant effect on the activities of the PS2 γ-secretases (Fig. 1c). These two results from yeast Gal4 reporter assays suggest that combinations of PSs and Aph1s contribute to substrate specificity.

3.2. PS2 γ-secretases produce Aβ with higher Aβ42/43/Aβ40 ratio in HEK293 cells stably expressing 6 different γ-secretases

Different substrate specificities for Notch and APP in yeast prompted us to check the substrate specificities of distinct γ-secretases in mammalian cells. To this end, we analysed Aβ production by each γ-secretase using HEK293/Swe cells stably expressing each combination of PSs and Aph1s [23]. HEK293/Swe are human kidney cells stably expressing APPswe, a mutated APP that produces high amounts of Aβ. Aβ in the culture media of each line was collected for 6 h, and cells were harvested. Expression levels of different combinations of PSs and Aph1s were confirmed by Western blot analysis, showing that each cell line expresses almost single γ-secretase (Fig. 2). Long exposure blots of Fig. 2 showed that there are almost no residual expressions of Aph1, whereas there are slight residual expressions of PSs (Supplementary Fig. S1). Importantly, each cell line also expresses similar amount of CS9, the substrate of γ-secretase to produce Aβ (Fig. 2). The amounts of Aβ released into the culture media were analysed by Enzyme-Linked Immuno-sorbent Assay (ELISA) for Aβ40, Aβ42 and Aβ43. Cells expressing PS1/
Aph1aS and PS2/Aph1aS γ-secretases secreted the highest amounts of Aβ40, Aβ42 and Aβ43, respectively, suggesting that γ-secretase with Aph1aS produces more Aβ than the other complexes. Only in the case of Aβ43, PS1/Aph1b secretes slightly more Aβ than PS2/ Aph1aS (Fig. 3a–c). Consequently, the total amount of Aβ production (Aβ40, Aβ42 and Aβ43) in HEK293/Swe cells expressing Aph1aS γ-secretases was higher than those in cells expressing Aph1aL and Aph1b (Fig. 3d). In contrast, γ-secretases containing Aph1aL produced the lowest amounts of Aβ40, 42, 43, respectively (Fig. 3a–c), reflected also in lowest total production in Fig. 3d. These

Fig. 1. Gal4 reporter assays in a yeast reconstitution system. a and b, yeast cells were transformed with PS (PS1 or PS2), NCT, FLAG-Pen2, Aph1 (Aph1aL-HA, Aph1aS-HA, or Aph1b-HA), and C55-Gal4 (a), or Notch-Gal4 (b). Three independent clones were streaked and cultured on SD-LWU or SD-LWHUAde media at 30°C for 3 days. c, β-galactosidase activity was measured using each yeast lysate. Lysates were prepared from yeast cells expressing C55-Gal4 or Notch-Gal4 using glass beads. One unit of β-galactosidase activity corresponds to 1 nmol O-nitrophenyl β-D-galactopyranoside hydrolysed min⁻¹, and activity was expressed as units min⁻¹ (mg of protein in lysate)⁻¹. Statistical analyses were performed by one-way analysis of variance followed by Tukey’s multiple comparison test. The data are presented as means ± SD (n = 3), *p < 0.05, ***p < 0.001, ****p < 0.0001.
results were confirmed by Western blot analysis (Supplementary Fig. S2).

Next, the Aβ42(43)/Aβ40 ratios, important for the pathogenesis of Alzheimer’s disease, were calculated. HEK293/Swe cells expressing PS1 γ-secretase with Aph1aL or Aph1aS produced Aβ with lower Aβ42(43)/Aβ40 ratio than did the cells expressing the four other γ-secretase variants (Fig. 3e). These results from ELISA implied that all PS2 γ-secretases produced Aβ in higher Aβ42(43)/Aβ40 ratio than did PS1/Aph1aL γ-secretase (Fig. 3e). The data suggest that PS2 contributes to higher Aβ42(43)/Aβ40 ratios or alternatively that Aph1α contributes to lower ratios in PS1 γ-secretase. Processing of Aβ43 to Aβ40 by each γ-secretase was also tested. PS1/Aph1b γ-secretase showed lower efficiency of conversion of Aβ43 to Aβ40 compared to PS1/Aph1a γ-secretases, although there were no significant differences (Fig. 3f). PS2/Aph1αS and PS2/Aph1b γ-secretases showed higher efficiency of processing Aβ43 to Aβ40 compared to PS1 γ-secretases, though PS2/Aph1αS didn’t show significant difference compared to PS1 γ-secretases (Fig. 3f).

3.3. PS2/Aph1αS γ-secretase cleaves less NICD in HEK293 cells stably expressing 6 different γ-secretase

We next tested the efficiency of Notch-cleavage by distinct γ-secretases using the above cell lines. Cells were transfected with NotchΔE-eGFP [19], and 24 h after transfection, the cells were collected and cell lysates were prepared. To assess the relative activity of each γ-secretase, the ratio of NICD-eGFP to NotchΔE-eGFP was determined by Western blot analysis. In all cell lines NICD-eGFP was produced, but with different efficiencies. Aph1α containing γ-secretase produced NICD-eGFP most efficiently, whereas Aph1αS containing complexes were the least efficient, especially with PS2 (Fig. 4a and b). Although these results slightly differ from those observed in the yeast reporter assay, also in mammalian cells different combinations of PSs and Aph1s affected Notch-cleavage efficiency.

4. Discussion

A number of studies have reported that different γ-secretases have different substrate specificities and/or functional differences [18,24,25]. To date, more than 90 γ-secretase substrates are known, including APP and Notch1 [26]. Thus, general γ-secretase inhibitors, such as DAPT and L-685,458, suppress production of Aβ, NICD and all other substrates. Since NICD is needed for cell differentiation and cell fate decision [27], inhibition of γ-secretase-induced NICD production causes severe side effects in vivo [28–30]. Recently, a phase III trial of a γ-secretase inhibitor, semagacestat, was terminated because of several adverse events, including skin cancers and infections [31]. However, if different γ-secretases have different substrate specificities, it may be possible to develop a drug that inhibits a specific γ-secretase complex that cleaves APP but not Notch1. Therefore, it is important to evaluate the substrate specificities of the six different γ-secretases (PS1 or PS2 with Aph1αS or Aph1αL or Aph1b).

According to our Gal4 reporter assays, γ-secretase complex with PS2/Aph1b had a clear substrate specificity. When reconstituted in yeast, which does not express endogenous γ-secretase, all PS1 γ-secretases efficiently cleaved APP, whereas only PS2/Aph1b efficiently cleaved APP among PS2-containing complexes. In contrast, all of the PS1-, but none of the PS2-containing complexes cleaved Notch. Therefore, these results suggest that PS2/Aph1b γ-secretase cleaves APP, but not Notch. If this is the case in mammalian cells, then PS2/Aph1b γ-secretase may be an ideal therapeutic target, since the inhibition of this γ-secretase may suppress Aβ production without the severe side effects caused by inhibition of Notch signalling. However, our results in mammalian cells differed from those observed in the yeast Gal4 reporter assays. Here PS2/Aph1αS showed the lowest Notch-cleavage activity (see below). A possible reason for this discrepancy is that yeast lacks additional proteins which affect the γ-secretase function of Notch processing and Aβ production. Several γ-secretase-associated proteins, including GSAP [32], CD147 [33], and CNTNAP1, COX4I1, CNTN1, and SYP [34], do not exist in yeast. These proteins or other unknown binding proteins might regulate cleavage-efficiencies of γ-secretase substrates, or might regulate localization of enzyme and/or substrate.

Using HEK293 cells stably expressing single γ-secretase [18] (Fig. 2), we analysed Aβ production and Notch-cleavage by distinct γ-secretases. PS1/Aph1b γ-secretase showed a higher Aβ42(43)/Aβ40 ratio compared to PS1/Aph1a γ-secretases, although there were no significant differences (Fig. 3f). PS2/Aph1αS and PS2/Aph1b γ-secretases showed higher efficiency of processing Aβ43 to Aβ40 compared to PS1 γ-secretases, though PS2/Aph1αS didn’t show significant difference compared to PS1 γ-secretases (Fig. 3f).
Fig. 3. Aβ production and Aβ species secreted by HEK293/Swe cells expressing distinct γ-secretases. Aβ levels secreted during 6 h at 37 °C were analysed. Media from HEK293/Swe cells stably expressing distinct γ-secretase subunits were collected after 6 h and subjected to ELISA for Aβ40 (a), Aβ42 (b), and Aβ43 (c). Aβ43 was not detectable in PS2/Aph1aL samples. (d), The total Aβ (Aβ40 and Aβ42 and Aβ43) and (e), Aβ42/Aβ40 ratios were calculated. (f), Aβ40/Aβ43 ratios were calculated. Aβ40/Aβ43 ratios for PS2/Aph1aL γ-secretase couldn’t be calculated because the levels of Aβ43 were undetectable. Statistical analysis was performed by one-way analysis of variance followed by Tukey’s multiple comparison test. The data are presented as means ± SD (n = 3), *p < 0.05.

Fig. 4. NICD production in HEK293 cells expressing distinct γ-secretase. HEK293 cells stably expressing distinct γ-secretase subunits were transfected with NotchΔE-eGFP using Lipofectamine 2000. Cells were collected 24 h after transfection. a, cell lysates were prepared from the collected cells and subjected to Western blot to detect NotchΔE-eGFP and NICD-eGFP using anti GFP antibody. b, NICD-eGFP/NotchΔE-eGFP ratios were calculated from three independent Western blot assays using Multi Gauge software. Statistical analysis was performed by one-way analysis of variance followed by Tukey’s multiple comparison test Error bars indicate SD. n = 3 * p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
for the higher Aβ42(43)/Aβ40 ratio is the location of the enzymes because recent paper showed restricted location of PS2 γ-secretase, which is different from PS1, resulted in higher Aβ42/Aβ40 ratio [37]. We also checked whether conversion of Aβ43 to Aβ40 was affected by different PSs and Aph1s. Previous experiments using cell culture assays showed that PS2 γ-secretases produced a ~1.5–2-fold greater Aβ40/Aβ43 ratio than did PS1 γ-secretases [25], implying that PS2 γ-secretases degrade Aβ43 to Aβ40 more efficiently than do PS1 γ-secretases. This tendency for accelerated processing of Aβ43 to Aβ40 in PS2 γ-secretases was also observed in our cell culture assay (Fig. 3f), at least in the case of PS2/Aph1b and, less pronounced, in PS2/Aph1aS complexes.

The Notch-cleavage efficiency of Aph1aS containing complexes was lower than that of Aph1aL γ-secretases, especially in combination with PS2, suggesting that Aph1aS is a determinant of low Notch-cleavage specificity, considering that the different subcellular localization of PS1 and PS2 didn’t affect endogenous NICD production [37]. Saito et al. reported that mRNA level of Aph1aS was 1.5–3-fold higher than that of Aph1aL in HEK293 (~3-fold), SH-SYSY (~2.5-fold), Hela (1.5–2-fold) and human adult brain (2.5–3-fold) [38], though it is unknown which combinations of γ-secretase are formed most prominently. It was reported that Aph1b γ-secretases might be a potential target of γ-secretase inhibitors for AD treatment because of their small side effects in knock-out models [24], whereas Aph1a γ-secretases shouldn’t be the target because of their important role in development [39]. Our results, however, revealed that Aph1aS γ-secretase cleaved Notch less efficiently than Aph1aL, suggesting that it is rather Aph1aS that should be targeted. In contrast, according to our data specific inhibition of Aph1b γ-secretases might cause side effect because of equal inhibition of APP and Notch signalling. Previous different conclusion that Aph1b γ-secretases play less important roles in Notch-cleavage compared to Aph1a [24,39] might be explained as follows. Knockdown of Aph1b is reported not to cause reduction of the expression levels of other γ-secretase components, implying that Aph1a γ-secretases still can work properly. In contrast, knockdown of Aph1a caused marked reduction of expression level of the other γ-secretase components [18,40], suggesting that Aph1b γ-secretase formation is also affected.

In this manuscript, we showed that cells expressing PS2/Aph1aS γ-secretase produced Aβ with higher Aβ42(43)/Aβ40 ratio and lower Notch-cleavage efficiency. However, this cell line contains only a slight amount of PS1/Aph1aS γ-secretase because cells expressing PS2 γ-secretase showed residual expression of PS1 and vice versa, but importantly almost no residual Aph1 expression (Supplementary Fig. S1). PS1/Aph1aS γ-secretase showed lower Aβ42(43)/Aβ40 ratio and higher Notch-cleavage efficiency than PS2/ Aph1aS γ-secretase. Therefore, pure PS2/Aph1aS γ-secretase would be expected to show even higher Aβ42(43)/Aβ40 ratio and even lower Notch.

In conclusion, we here show that the six distinct human γ-secretases have different cleavage-efficiencies for Notch and differ in the Aβ species they produce, through it is needed to analyse Notch-cleavage and Aβ production by the distinct γ-secretases in neuronal cells to confirm that our findings are the case with the brain. Recent studies revealed the PS1/Aph1aL γ-secretase structure [41]. Further analysis of different γ-secretase structure might aid in the development of structure-based specific γ-secretase inhibitors. Targeting the PS2/Aph1aS γ-secretase complex would be expected to have the greatest effect of reducing Aβ42(43)/Aβ40 with the fewest side effects caused by inhibition of Notch signalling.

5. Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

The authors thank Dr. Raphael Kopan (Cincinnati Children’s Hospital Medical Center) for the mNotch1 clone, Dr. Christian Haass for Pen2, PS and APP antibodies and Dr. Harald Steiner for HEK cell lines and Aph1b antibody. The authors also thank Dr. Masashi Asai (Nagasaki University) for the helpful discussions and technical suggestions.

Y.Y. was supported by Japan Society for the Promotion of Science. This work was supported in part by an Intramural Research Grant (MHLW; 26-8) for Neurological and Psychiatric Disorders of NCNP, a research grant for Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare (MHLW; H26-Sinkeikinn-ippan-004) and a Grant-in-Aid from the MHLW of Japan (to S.I.).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.09.018.

Transparency document

Transparency document related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.09.018.

References


