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In vitro reconstitution of γ-secretase activity using yeast microsomes ☆

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ABSTRACT

γ-Secretase is composed of at least four transmembrane proteins, presenilin (PS) 1/2, nicastrin, anterior pharynx-1 (Aph-1) and presenilin enhancer-2 (Pen-2), and cleaves amyloid precursor protein (APP) to produce amyloid β peptides (Aβ) that is deposited in the brains of Alzheimer disease. However, the mechanism of γ-secretase-mediated cleavage remains unclear. To examine the enzymatic properties of γ-secretase, we established an *in vitro* assay system using *Saccharomyces cerevisiae*, which does not possess homologs of human PS1/2, nicastrin, Aph-1, or Pen-2. We transformed these subunits and the substrate in *pep4Δ* cells with vacuole proteases inactivated, and microsome was isolated for *in vitro* assay. In the assay, Aβ40, Aβ42, and Aβ43 were produced with an optimal pH of ~7.0. We also detected Aβ-production by yeast endogenous protease(s), which was abolished by the addition of phosphatidyl choline. This novel system will facilitate the analysis of substrate recognition by γ-secretase.

γ-Secretase is an aspartic protease that catalyzes the final step in amyloid β peptides (Aβ) production. Aβ is produced from amyloid precursor protein (APP) through sequential cleavages by two membrane proteases referred as β- and γ-secretase [1]. β-Secretase, identified as β-site APP-cleaving enzyme 1 or BACE1 [2], cleaves APP, generating a 99-residue fragment called β C-terminal fragment (βCTF). Next, γ-secretase cleaves βCTF in its transmembrane region, producing Aβ and APP intracellular domain (AICD). γ-Secretase is composed of four proteins, presenilin(PS) 1/2, nicastrin (NCT), anterior pharynx-1 (Aph-1), and presenilin enhancer-2 (Pen-2), of which PS is thought to possess aspartyl protease activity [3–5]. Because the substitution of two conserved aspartic acid residues in PS1/2 transmembrane domains 6 and 7 led to a profound loss of γ-secretase activity, these two aspartic acid residues (D257 and D385 in human PS1, D263, and D366 in human PS2) are thought to form the catalytic site(s) [6,7].

The major Aβ species produced by γ-secretase are Aβ40 and Aβ42, named for the number of residues they possess. Aβ40 is the most predominant species (~90%), whereas Aβ42, two amino acids longer species than Aβ40, composes only ~10% of total Aβ species, but is more important in terms of pathology. Due to its self-associating property, Aβ42 accumulates in the brain and triggers the onset of Alzheimer disease [8]. Disease-causing mutations in *APP*, *PS1*, and *PS2* increase the production of Aβ42 [1].

Recent reports indicate that γ-secretase cleaves APP at multiple sites. In addition to the site of Aβ40/Aβ42 production (γ-cleavage sites), various Aβ species such as Aβ43, Aβ45, Aβ46, Aβ48, and Aβ49 were found [9–12]. In contrast, only two C-terminal fragments, AICD49-99 and AICD50-99, have been identified, whereas longer AICDs, the counterpart of Aβ40 or Aβ42 (AICD41-99 or AICD43-99), have not been detected [13–16]. The cleavage sites of AICD were termed as ε-cleavage sites. The function of these multiple γ-secretase cleavage sites and how each subunit contributes to the selection of cleavage sites remains to be elucidated.

To analyze the γ-secretase activity in detail, *in vitro* assay systems have been established using mammalian cells. In these assays, membrane fractions were incubated to analyze Aβ production. Because γ-secretase catalyzes intra-membrane proteolysis, it is desirable to perform *in vitro* reactions with the isolated membrane fraction containing both the protease complex and the substrate.

Here, we describe a novel *in vitro* assay system using yeast microsomes to assess γ-secretase activity. We transformed yeast cells with γ-secretase subunits and the substrate βCTF1-55 (C55) or βCTF1-99 (C99), isolated the microsome, and subjected this fraction to *in vitro* assay according to the previous reports [12,17]. This

Abbreviations: Aβ, amyloid β peptides; APP, amyloid precursor protein; AICD, APP intracellular domain; Aph-1, anterior pharynx-1; CTF, carboxyl-terminal fragment; PS, presenilin; NCT, nicastrin; Pen-2, presenilin enhancer-2; wt, wildtype; PC, phosphatidyl choline; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

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reconstituted system may prove to be a useful tool in the analysis of γ-secretase activity.

Materials and methods

γ-Secretase and APP constructs. The expression constructs for γ-secretase were prepared as previously described [5]. The APP-based substrates C55 (amino acids 672–726 from human APP770) and C99 (amino acids 672–770 from human APP770) were prepared by PCR using primers encoding a 19 amino acid signal peptide sequence from yeast invertase (*SUC2*). These fragments were cloned into p426ADH [18]. The Suc2p signal peptide was fused to the N-termini of C55 and C99 to ensure insertion into the endoplasmic reticulum. These expression constructs are referred to as C55 and C99. Plasmids containing mutant PS1(D257A) or PS1(D385A) were generated by site-directed mutagenesis, and were cloned into the vector pBEVY-T [19].

Yeast strain. For biochemical analysis, we generated PJ69-4A strain [20] with PEP4 knocked-out (genotype: *MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ, pep4::kanMX)* by PCR-mediated gene disruption [21]. *PEP4* encodes a vacuolar aspartic protease required for the posttranslational precursor maturation of various vacuolar proteases [22,23]. The generated PJ69-4A*pep4Δ* cells were transformed with the following recombinant plasmids: PS1 and NCT in pBEVY-T, Aph-1-HA, and Flag-Pen-2 in pBEVY-L, and C55 or C99 in p426ADH. The transformants were selected in SD agar medium lacking Leu, Trp, and Ura.

Isolation of microsome from yeasts. Yeast cells were harvested from 2 L culture at an OD600 of ~1.0, and washed with Tris/DTT (100 mM Tris–HCl [pH9.4], 10 mM dithiothreitol [DTT]), followed by centrifugation at 6000*g* for 5 min. The pellets were suspended with lyticase buffer (75% YP, 0.7 M sorbitol, 0.5% glucose, 10 mM Tris–HCl [pH 7.6], 1 mM DTT) containing lyticases, and incubated for 60 min at 30 °C. The mixtures were centrifuged, and the pellets (spheroplasts) were washed twice with 2× JR buffer (40 mM HEPES [pH 7.4], 0.4 M sorbitol, 100 mM KOAc, 4 mM EDTA). The spheroplasts were diluted twofold and homogenized using a Potter-Elvehjem homogenizer. After centrifuging the homogenates at 1000*g* for 5 min to remove nuclei and unbroken cells, the supernatant was centrifuged at 27,000*g* for 10 min. The pellets (crude microsome) were suspended into B88 buffer (20 mM HEPES [pH 6.8], 250 mM sorbitol, 150 mM KOAc, 5 mM $Mg(OAc)_2$) and loaded onto a step gradient, containing 0, 1.2, and 1.5 M sucrose in B88 buffer. The gradient was centrifuged at 200,000*g* for 3 h at 4 °C (P40ST, Hitachi, Tokyo, Japan). The interface between 0 and 1.2 M sucrose was carefully removed to avoid vacuole contamination. Microsomes were collected from the interface between 1.2 and 1.5 M sucrose, were diluted fivefold in gamma buffer (50 mM PIPES [pH 7.0], 250 mM sucrose, 1 mM EGTA) and centrifuged at 27,000*g* for 5 min; the resulting pellet was suspended in gamma buffer and stored at −80 °C until use. Microsome protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA).

In vitro assay using the yeasts microsome. An *in vitro* assay for γ-secretase activity was performed according to previous reports [12,17]. Microsomes were solubilized in gamma buffer containing 1% CHAPSO for 1 h, and the mixtures were diluted fourfold in gamma buffer containing various protease inhibitors (50 μM diisopropyl fluorophosphate, 50 μM phenylmethylsulfonyl fluoride, 0.1 μg/mL N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 0.1 μg/mL antipain, 0.1 μg/mL leupeptin, 100 μM EGTA, 1 mM thiorphan, 5 mM phenanthroline) for a final concentration of 0.25% CHAPSO and 400 μg protein/mL. The mixtures were incubated at 37 °C. Where indicated, we added 0.1% phosphatidyl choline (PC) (Catalog No. P3556; Sigma) or 1 μM of the γ-secretase inhibitor {1*S*-benzyl-4*R*-[1*S*-carbamoyl-2-phenylethylcarbamoyl-1*S*-3-methylbutylcarbamoyl]-2*R*-hydroxy-5-phenylpentyl} carbamic acid *tert*-butyl ester (L685, 458) [24]. The reaction was terminated using chloroform/methanol (2:1). After extracting lipids, the protein fractions were analyzed by Western blotting.

To detect total Aβ, the samples were subjected to conventional 16.5% polyacrylamide Tris/Tricine gels. To identify Aβ species, the samples were separated on 10% polyacrylamide separation gels containing 8 M urea (pH 8.45) as previously described [9,10]. Aβ was detected by a monoclonal antibody 82E1 (highly specific for Asp-1 of human Aβ) (IBL, Fujioka, Japan). The blots were developed by an Enhanced Chemiluminescence system, and intensities of the signals were quantified using an LAS-3000 luminescent image analyzer (Fuji Film, Tokyo, Japan).

Results

Characterization of the in vitro assay using yeast microsomes

To establish a novel assay system for γ-secretase, we generated yeast transformants expressing the four subunits of γ-secretase (wtPS1, NCT, Aph-1, and Pen-2) and a substrate C55. We isolated the microsome by sucrose gradient centrifugation, which allowed us to remove vacuoles with high protease activity. To prevent Aβ degradation by yeast proteases, we used a strain in which the *PEP4* gene, which activates various vacuolar proteases [22,23], had been eliminated (PJ69-4A*pep4Δ*). Because some previous reports showed γ-secretase activity was enhanced in the presence of 0.25% CHAPSO or 0.1% PC [12,25], we also examined the effect of CHAPSO or PC on this system. When the microsomes were incubated without CHAPSO or PC, we observed faint Aβ production

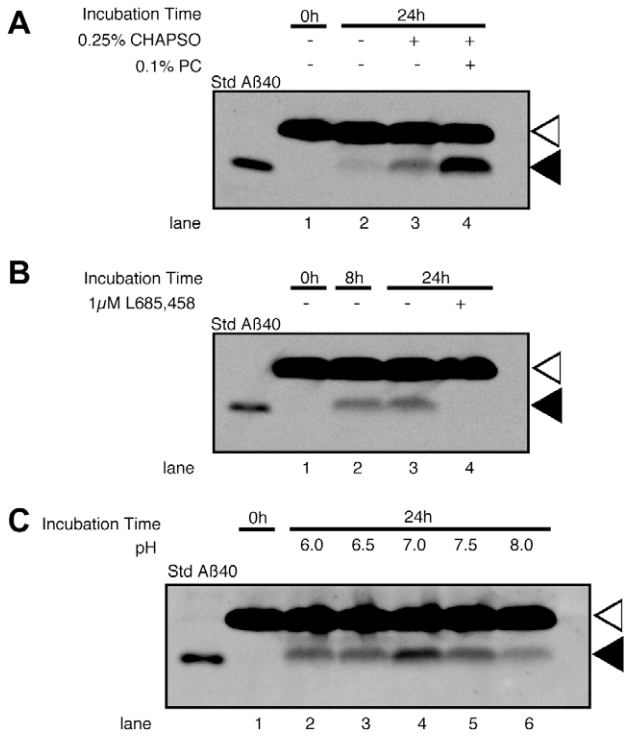

Fig. 1. *In vitro* γ-secretase assay in yeast microsomes using C55 as a substrate. (A) Isolated microsome from yeasts expressing wtPS1 and C55 was subjected to an *in vitro* assay. The produced Aβ increased in the presence of 0.25% CHAPSO or 0.1% PC. (B) The produced Aβ increased according to incubation time, and the Aβ production was inhibited by L685,458. (C) Maximal Aβ production occurred at pH 7.0. Synthetic Aβ40 (30pg) was loaded as a marker. Open and closed arrowheads indicate C55 and Aβ, respectively.

from C55 (Fig. 1A, lane 2). The amount of Aβ increased in the presence of 0.25% CHAPSO or 0.1% PC (Fig. 1A, lanes 3 and 4).

We next investigated the time profile and the effect of L685,458, a transition-state analog γ-secretase inhibitor. As shown in Fig. 1B (lanes 2–4), the level of Aβ increased in a time-dependent manner, whereas Aβ production was blocked by L685,458. To examine the effect of pH on γ-secretase activity, the assay was repeated at pH values of 6.0, 6.5, 7.0, 7.5 and 8.0. We observed maximal Aβ production at pH 7.0. (Fig. 1C, lanes 2–6).

Next, to determine whether this system functions when using the full-length substrate, we generated yeast transformants expressing C99, and isolated the microsomes for the *in vitro* assay. We detected Aβ production from C99, which was enhanced in the presence of 0.25% CHAPSO or 0.1% PC (Fig. 2A, lanes 2–4) and inhibited by L685,458 (Fig. 2B, lanes 3–4). Maximal Aβ production was observed at pH 7.0 (Fig. 2C, lanes 2–6).

C55/C99 processing by γ-secretase harboring a single aspartic acid mutant of PS1

To validate this new assay system, we next investigated the effect of γ-secretase harboring a mutation in one of the two essential aspartate residues in PS1 [6]. We generated yeast strains expressing PS1 mutants, D257A or D385A, and C55. The microsomes were subjected to our *in vitro* assay. Unexpectedly Aβ production was detected in microsomes from both strains, which was inhibited by L685,458 (Fig. 3A, lanes 1–9). To identify the Aβ species, the samples were separated on an 8 M urea/Tris/Tricine gel. While the production of Aβ40, Aβ42, and Aβ43 was identified with wtPS1 (Fig. 3D, lane 2), the production of Aβ43 was identified in both PS1 D257A and PS1 D385A (Fig. 3D, lanes 5 and 7). To test whether the observed Aβ-production activity was dependent upon

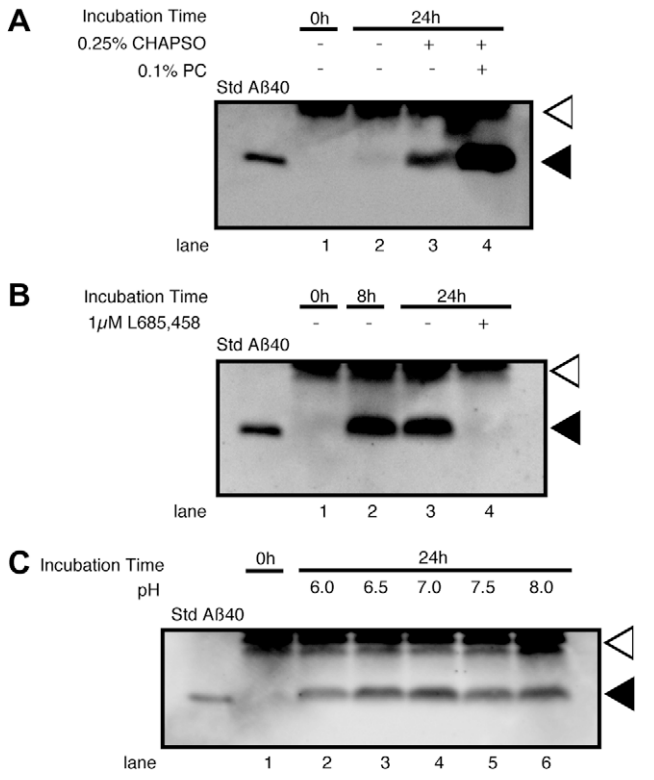

Fig. 2. *In vitro* γ-secretase assay in yeast microsomes using C99 as a substrate. (A) Isolated microsome from yeasts expressing wtPS1 and C99 was subjected to *in vitro* assay. The produced Aβ increased in the presence of 0.25% CHAPSO or 0.1% PC. (B) The produced Aβ increased by incubation, and the Aβ production was inhibited by L685,458. (C) The optimum pH of the Aβ production was pH 7.0. Synthetic Aβ40 (30 pg) was loaded as a marker into the leftmost lanes. Open and closed arrowheads indicate C99 and Aβ, respectively.

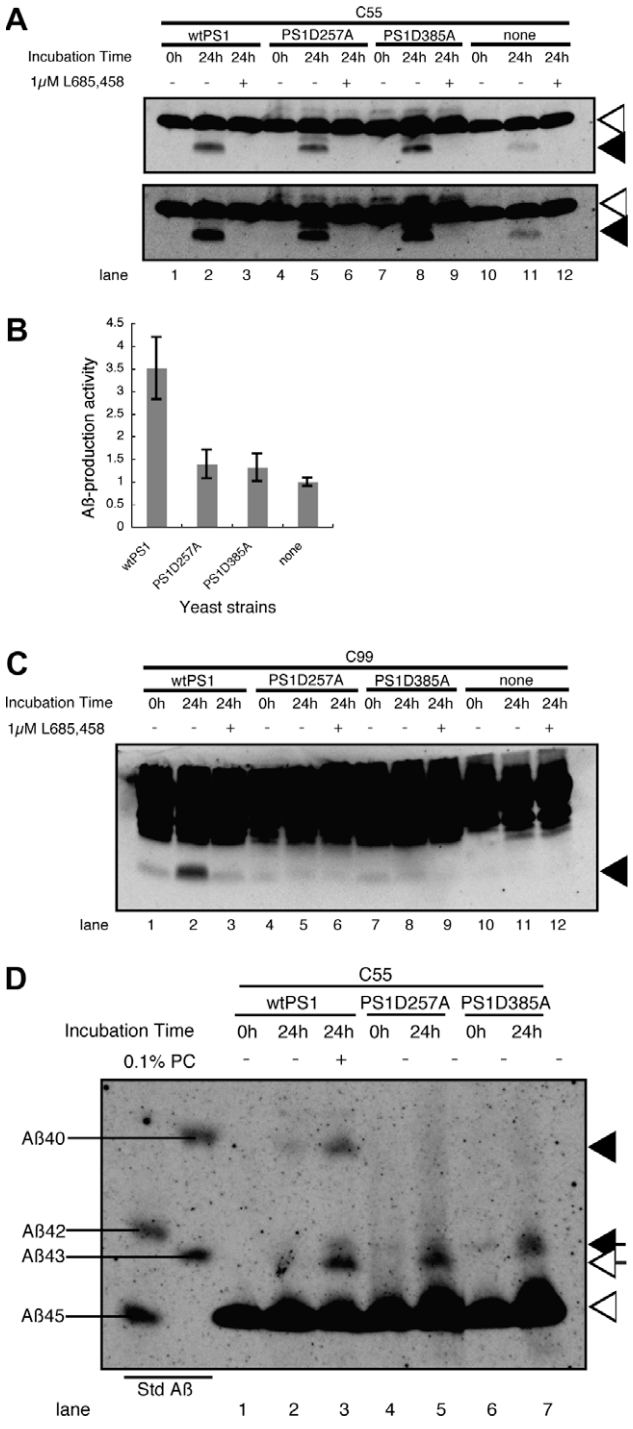

Fig. 3. Effect of single aspartic mutant presenilin1 on γ-secretase activity. (A) Microsomes isolated from the indicated strains were subjected to *in vitro* assays in the presence of 0.25% CHAPSO. All microsomes showed Aβ-producing activity, which was inhibited by L685,458. Open and closed arrowheads indicate C55 and Aβ, respectively. (B) Quantitative Western blotting was performed to determine the ratio of produced Aβ to C55. The column represents mean ± SD of three independent experiments. (C) Microsome isolated from the indicated strains were subjected to *in vitro* assay in the presence of 0.25% CHAPSO. WtPS1 showed γ-secretase activity, while the others do not. The closed arrowhead indicates Aβ. (D) Samples prepared from incubated microsomes were separated on a 10% Tris/Tricine gel containing 8 M urea. Aβ40 (closed arrowhead), Aβ42 (closed arrow) and Aβ43 (open arrow) were detected in wtPS1, whereas only Aβ43 was detected in the remaining strains. The open arrowhead indicates C55. Synthetic Aβ40, Aβ42, Aβ43, and Aβ45 were loaded into the two leftmost lanes.

γ-secretase or endogenous yeast protease(s), we generated a yeast strain expressing C55 alone. Weak but significant Aβ production was detected in microsomes from yeasts expressing C55 alone, which was inhibited by L685,458 (Fig. 3A, lanes 10–12). These results suggest that C55 is cleaved by endogenous yeast protease(s).

Next, we used quantitative Western blotting to estimate the γ-secretase activity based on the ratio of Aβ to C55 (Fig. 3B). Aβ production by wtPS1 was significantly higher compared to the others. However, the production by PS1 D257A or PS1 D385A was not significantly high when compared to the production by yeast protease(s). We concluded that γ-secretase-like activity observed with single aspartic mutant PS1 was due to yeast endogenous protease(s).

We also generated yeast strains expressing PS1 D257A or PS1 D385A and C99. However, the microsome isolated from these strains did not show Aβ production (Fig. 3C, lanes 4–9). We also generated yeasts expressing C99 alone, in which Aβ production was not observed (Fig. 3C, lane 10–12). These results indicate that the yeast proteases could not cleave C99 and the Aβ production from C99 depends on catalytic residues.

Inhibition of the endogenous yeast protease(s) activity by PC

Our previous experiments indicated that endogenous yeast protease(s) was able to process C55, resulting in unwanted Aβ production. To exclude the effect of endogenous protease(s), we searched more appropriate condition for the assay with C55.

We found that PC almost completely inhibited endogenous protease(s) activity, while enhancing exogenous γ-secretase activity (Fig. 4, lanes 2, 4, 6, and 8). When we separated the Aβ products by 8 M urea/Tris/Tricine gel, Aβ40, Aβ42, and Aβ43 was identified (Fig. 3D, lane 3). Thus, the addition of PC allows the reconstitution of γ-secretase activity using C55 as a substrate.

Discussion

To gain insight into the enzymatic properties of γ-secretase, we established a new *in vitro* assay system that excludes the effects of endogenous factors, including endogenous γ-secretase, and ensures that the substrate is properly inserted in the membrane. To reproduce γ-secretase reaction, we thought it is important that both the protease complex and substrate are contained within the membrane. In the assay described here, we transformed human PS1, NCT, Aph-1, Pen-2, and substrates into yeast strains, from which we isolated and assayed the microsome. In addition, a signal peptide for membrane insertion was fused to the substrate sequences. This system is highly advantageous because yeast microsomes do not possess endogenous γ-secretase [5], and the use of a signal peptide ensures the proper localization in the membrane, where it can interact with the protease complex.

We used this assay system to examine γ-secretase activity using C55 or C99. C55 has been used in previous γ-secretase assays, based on the assumption that it is cleaved in the same manner as C99 [5,26]. Consistent with these previous studies, we observed processing of C55 and C99 to Aβ in yeast microsomes containing wtPS1, which was inhibited by L685,458. The amount of Aβ increased in the presence of 0.25% CHAPSO or 0.1% PC, and it reached the maximum at pH 7.0 (Figs. 1 and 2). These results closely reflect the properties of γ-secretase in mammalian cells [27]. We concluded that our yeast microsome assay was successful in reconstituting the γ-secretase reaction *in vitro*.

We next investigated the effect of single aspartic mutant PS1 on γ-secretase activity. A previous study reported that D257 or D385 in the PS1 were essential catalytic residues of γ-secretase activity [6]. Therefore, we generated strains carrying PS1 mutants (PS1D257A or PS1D385A) and analyzed γ-secretase activity using their microsome. As expected, neither mutant produced Aβ peptides from C99, indicating these two aspartate residues are required for catalysis. However, Aβ production was observed in microsomes containing mutant PS1 and C55, and in microsomes containing C55 alone; in all cases, Aβ production was sensitive to L685,458 (Fig. 3A). Quantification of Aβ-production activity showed no significant difference between microsomes containing a catalytic mutant and those containing C55 alone (without PS1). These results indicate that the yeasts possess γ-secretase-like protease activity that is capable of cleaving C55 and is sensitive to L685,458 (Fig. 3B). Note that this yeast endogenous protease(s) produced Aβ43 alone (Fig. 3D).

To exclude endogenous γ-secretase-like activity, we modified our reaction conditions. We found that PC almost completely inhibited endogenous yeast protease(s) activity while enhancing exogenous γ-secretase activity (Figs. 1 and 4). Thus, although both proteases were sensitive to L685,458, they showed very different responses to PC treatment. These results demonstrate that this endogenous yeast protease(s) and γ-secretase possess distinct enzymatic properties.

In this study, we established a new *in vitro* assay system in which exogenous γ-secretase cleaves exogenous βCTF in yeast membranes. Although a previous study reported the reconstitution of human γ-secretase activity in yeast [5], the authors did not report an *in vitro* assay using their system. The *in vitro* assay here includes two key features: *PEP4*-knockout to eliminate most endogenous protease activity, and microsome isolation via sucrose gradient centrifugation to prevent contamination with the vacuole fraction. These changes enabled us to perform biochemical analysis using yeast microsomes, which will facilitate further research into the properties of γ-secretase, such as substrate specificity and the function of each subunit.

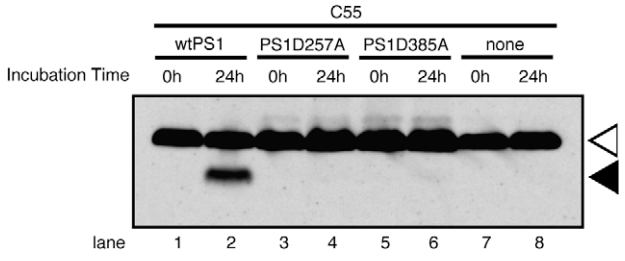

Fig. 4. PC-induced inhibition of endogenous yeast protease(s) activity. Microsomes isolated from the indicated strains were subjected to *in vitro* assays in the presence of 0.25% CHAPSO and 0.1% PC. Endogenous yeast protease(s) activity observed in the absence of PC was almost completely inhibited by PC (lanes 4, 6, and 8), whereas γ-secretase activity was enhanced (lane 2). Open and closed arrowheads indicate C55 and Aβ, respectively.

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