The First Proline of PALP Motif at the C Terminus of Presenilins Is Obligatory for Stabilization, Complex Formation, and γ-Secretase Activities of Presenilins*

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Mutations in presenilin (PS) genes cause early-onset familial Alzheimer's disease by increasing production of the amyloidogenic form of amyloid β peptides ending at residue 42 (Aβ42). PS is an evolutionarily conserved multispans transmembrane protein, and all known PS proteins contain a proline-alanine-leucine-proline (PALP) motif starting at proline (P) 414 (amino acid numbering based on human PS2) at the C terminus. Furthermore, missense mutations that replace the first proline of PALP with leucine (P414L) lead to a loss-of-function of PS in Drosophila melanogaster and Caenorhabditis elegans. To elucidate the roles of the PALP motif in PS structure and function, we analyzed neuro2a as well as PS1/2 null fibroblast cell lines transfected with human PS harboring mutations at the PALP motif. P414L mutation in PS2 (and its equivalent in PS1) abrogated stabilization, high molecular weight complex formation, and entry to Golgi/trans-Golgi network of PS proteins, resulting in failure of Aβ42 overproduction on familial Alzheimer's disease mutant basis as well as of site-3 cleavage of Notch. These data suggest that the first proline of the PALP motif plays a crucial role in the stabilization and formation of the high molecular weight complex of PS, the latter being the active form with intramembrane proteolytic activities.

Alzheimer's disease (AD) is a progressive dementing neurodegenerative disorder in the elderly characterized pathologically by the presence of senile plaques and neurofibrillary changes in the brains of affected individuals (reviewed in Ref. 1, and references therein). Senile plaques are composed of amyloid β peptides (Aβ) comprising ~40 amino acids that are proteolytically produced from β-amyloid precursor protein (βAPP). βAPP is initially cleaved by β-secretase to generate a 99-residue C-terminal fragment (C99) that then is cleaved by γ-secretase to generate Aβ. A subset of AD is inherited as an autosomal dominant trait (familial AD: FAD). Genetic mutations in βAPP genes that cosegregate with the clinical manifestations of FAD increase production of the amyloidogenic Aβ42 species ending at Ala 42 (2). Aβ42, which normally comprises only ~10% of total secreted Aβ, aggregates much faster than the predominant Aβ40 species (3), and Aβ42 is the initial and predominantly deposited Aβ species in AD brains (4, 5). These data implicated a seminal role of Aβ42 in the pathogenesis of AD.

Mutations in presenilin (PS) 1 and PS2 genes are linked to the majority of early onset FAD. FAD-linked PS mutations affect γ-cleavage of βAPP leading to an increased production of Aβ42 (1). In contrast, ablation of PS1 and PS2 genes in mice completely inhibited production of both Aβ40 and Aβ42, accompanied by accumulation of the βAPP C-terminal stubs (i.e. C99 and C83) that are the direct substrates for γ-secretase (6–8). Furthermore, studies in Caenorhabditis elegans and Drosophila melanogaster, as well as in knockout mice, suggested that PS facilitates Notch signaling by activating the ligand-induced intramembranous proteolysis of Notch receptors at site-3 to release their cytoplasmic domains (NICD) (7–11). This cleavage appears to be very similar to γ-cleavage of βAPP because it occurs close to or within the membrane, inhibited by inactivation of PS genes, and can be blocked by peptidomimetic γ-secretase inhibitors (reviewed in Ref. 12). The physiological role(s) of PS in intramembrane proteolysis has been unclear, but it has been shown that two aspartases within the sixth and seventh transmembrane (TM) domains are required for its γ-secretase activities (13, 14), and the amino acid sequences near these aspartases are homologous to those of the active sites of the polytopic aspartyl proteases of bacterial origin (15). Recently, transition state analogue γ-secretase inhibitors that inhibit aspartic protease(s) have been shown to directly bind fragment forms of PS, strongly suggesting that PS harbors the catalytic center of γ-secretase (16–18).

PS1 and PS2 are polytopic integral membrane proteins that span membrane six to eight times and undergo endoproteolysis to generate N- and C-terminal fragments (NTF and CTF, respectively) (19, 20). These fragments form heterodimers and...
are incorporated into the high molecular weight (HMW) protein complexes that are highly stabilized and acquire long half-life (t1/2 = ~2 h) whereas holoproteins are rapidly degraded (t1/2 = ~2 h) (21–24). Mutagenesis studies showed that the endoproteolysis of PS is not required for its stabilization, complex formation, or function (25–27). In contrast, recent findings indicate that the formation of stabilized HMW complex of PS fragments is tightly related to its function. CHAPSO-solubilized membrane fragments containing PS1 fragments exhibit γ-secretase activity (28); nicarbazin, a novel single-pass transmembrane protein, has been identified as one of the components of the HMW PS complex that may regulate γ-secretase activity (29). Moreover, the facts that the amount of stabilized NTF and CTF of PS are tightly regulated and that overexpression of exogenous PS replaces endogenous PS suggest that some cellular factor(s) of limited amount are required for the stabilization and complex formation of PS (19, 30).

We and others have shown that the ectopically expressed NTFs of PS1 or PS2 are not stabilized and do not overproduce Aβ42 on FAD mutant basis (31–33). Furthermore, we have found that the integrity of the C terminus of PS is required for its stabilization and abnormal γ-cleavage of βAPP (i.e, Aβ42 overproduction) (34). PS are evolutionarily conserved proteins that are present in almost all multicellular organisms including vertebrates and invertebrates as well as plants, and the C-terminal region of PS is highly conserved. Notably, we found that all known PS proteins contain an amino acid motif comprised of four consecutive amino acids, proline-alanine-leucine-proline, at the C terminal, including C. elegans SPE-4 that harbors a shorter C-terminal region, which we designated PALP motif (Fig. 1A). In C. elegans and Drosophila, many of the loss-of-function mutants of PS defective in Notch signaling have been reported, and the first proline of PALP motif is replaced with leucine in recessive loss-of-function mutants of C. elegans spe-4 (35) and Drosophila Psnl (36). In C. elegans spe-4 (eb-12) allele, which results in P440L (corresponding to the first proline of PALP) amino acid substitution of SPE-4 protein, morphogenesis of fibrous body-membranous organelle complexes is defective and spermatogenesis arrests at an unusual cellular stage in a similar manner to a null phenotype, despite robust synthesis of mutant proteins (35). Drosophila Psnl (Dpsn) mutant allele causing P507L amino acid substitution also resulted in reduction of Notch signaling similar to that in a Pan null mutant allele (36). These results suggested that the first proline of PALP motif plays an important role in the γ-secretase functions of PS. In contrast, it has been documented that the second proline of PALP motif is replaced with serine (37) or glutamate (38) in some PS1 mutant FAD pedigrees. To elucidate the structural and functional roles PALP motif in the C terminus of PS, we analyzed neuro2a and PS1 null fibroblast cell lines transfected with PALP-mutated PS and examined their effects on the metabolism of PS proteins as well as on γ-cleavage of βAPP and site-3 cleavage of Notch.

**MATERIALS AND METHODS**

**Construction of Expression Plasmids**—A full-length cDNA encoding wild type (wt), N141I (mt), and M239V FAD mutant human PS2 in pcDNA3 (Invitrogen, San Diego, CA) were obtained as described (20, 32). cDNAs encoding PS2/D366A, PS2/P414L, or PS2/P417S were generated by the long-PCR protocol using 5'-TGTCCTGTTGTTGAAGCCTGCAGACAGC-3' for PS2/D366A as a template. All full-length cDNA encoding wt or P267AS FAD mt human PS1 in pcDNA3 has been described (20). cDNAs encoding PS1/D385A or PS1/P433L were generated by the long-PCR protocol using 5'-CTAGCTTCTCAAATCTCCAT-3' for PS1/D385A as a forward primer, and 5'-CAGAAAGTCTTCTGAACTCGCAT-3' for PS1/P433L, 5'-AGTGTACTAGTGGGCAA-3' for PS1/D366A as a reverse primer, respectively. All constructs were sequenced using Thermosequenase (Amersham Pharmacia Biotech) on an automated sequencer (Li-Cor, Lincoln, NE). cDNAs encoding mouse Notch1E in pcDNA3.1/hyg (+) (14) or pCS2+ MT (39) vectors have been described previously.

**Cell Culture and Transfection**—Mouse neuro2a (N2a) neuroblastoma cells were maintained as described previously (20). Generation of SV40-transformed fibroblasts derived from PS1−/− PS2−/− littersmates (40) by B. D. S. will be described elsewhere. Stable N2a cell lines were generated by transfecting cDNAs using LipofectAMINE (Life Technologies, Inc.) and selection in DMEM containing G418 (Life Technologies, Inc., or Calbiochem, San Diego, CA) at 500 μg/ml as described (32). N2a cells stably coexpressing Notch1E and PS2 were generated by transfecting a cDNA encoding mouse Notch1E in pcDNA3.1/hyg (+) (14) and expressing PS2 and selection in DMEM containing hygromycin at 160 μg/ml. Transient transfection was performed by LipofectAMINE or LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer’s instructions, and expression of transgenes was driven by addition of 10 mM butyric acid for 12–24 h.

**Antibodies, Immunoblot Analysis, and Cycloheximide Treatment**—The following rabbit polyclonal antibodies were used: anti-G2N3 against glutathione S-transferase (GST) fused to amino acids 2–74, anti-G2N4 against GST fused to amino acids 2–59, anti-G2L against GST fused to amino acids 301–361 of human PS2 (32), respectively, and anti-G1L3 against GST fused to amino acids 297–379 of human PS1 (34). The rabbit polyclonal antibody C4 against the cytoplasmic C terminus of human βAPP was kindly provided by Dr. Y. Ihara. The mouse monoclonal antibodies were purchased from Stressgen (anti-KDEL), Transduction Laboratory (anti-adaptin-γ and -Vti1b), and Roche Diagnostics (anti-c-Myc (9E10), respectively.)

For immunoblot analysis of cell lysates, cells were lysed in 2% SDS sample buffer and briefly sonicated. The samples were separated by SDS-PAGE without previous heating, transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA), and probed with each antibody as described (20, 32, 34). The immunoblots were developed using an ECL system (Amersham Pharmacia Biotech) or Immunostar reagents (Wako), and detected on Hyperfilm ECL (Amersham Pharmacia Biotech) or using LAS-1000plus (Fujifilm). Scanned images were quantitated with Image Gauge (Fujifilm) or Scion Image (Scion Corp.) software.

To evaluate the half-lives of transfected PS proteins and fragments thereof, blocking total cellular protein synthesis, cultured cells were treated with cycloheximide (30 μg/ml) for 12 or 24 h and then analyzed by immunoblotting with appropriate PS antibodies.

**Metabolic Labeling and Immunoprecipitation**—N2a cells stably coexpressing PS2 and Notch1E were starved for 2 h in methionine- and serum-free DMEM (Life Technologies, Inc.) containing 10 μM butyric acid. Cells were subsequently metabolically labeled with 15 μl for 6 MBq/mg [35S]methionine (Expre35S35S, PerkinElmer Life Sciences) and chased for 60 min in complete DMEM containing 10% fetal bovine serum with or without addition of 10 μM clasto-lactacystin β-lactone (Sigma) in culture media. Cells were then washed three times in cold Tris-buffered saline and lysed in RIPA buffer (50 mα Tris-HCl, pH 7.5, 150 μM NaCl, 1% Trition X-100, 1% sodium deoxycholate, 0.1% SDS) containing Complete protease inhibitor mixture (Roche Diagnostics). Before precipitating, the extracts were incubated with 10 μl of protein G-agarose beads (Life Technologies, Inc.) and spun down to remove debris and proteins nonspecifically bound to agarose beads. The supernatants were mixed overnight with 50 μl of protein G-agarose beads and a monoclonal antibody anti-c-Myc (9E10) at 4 °C. Beads were washed with RIPA buffer and then twice with PBS (5 μM Tris-HCl, pH 7.5, 150 μM NaCl), and extracted in SDS sample buffer. Immunoprecipitates were fractionated by SDS-PAGE, and autoradiograms were analyzed using BAS-1800II (Fujifilm).

**Glyceral Velocity Gradient Centrifugation**—Glycerol velocity gradient centrifugation was performed as described previously with some modifications (23, 41). N2a cells stably expressing PS2 cDNAs were grown to confluence on a 15-cm dish. All of the following steps were
carrier out at 4 °C. Scraped cell pellets were resuspended in 4 ml of homogenization buffer A (10 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, Complete protease inhibitor mixture). Cells were disrupted by a Polytron homogenizer (Hitachi) at power level 4 for 30 s, and nuclei and large cell debris were pelleted by centrifugation at 1,500 × g for 10 min. The postnuclear supernatants were centrifuged for 1 h at 100,000 × g. The vesicle pellets were extracted with 0.5 ml of homogenization buffer A containing 1% CHAPSO for 1 h. The resulting membrane extracts were cleared by centrifugation for 1 h at 100,000 × g. The supernatants were loaded on a 11-mL linear 15–30% (v/v) glycerol gradient in the gradient buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 30% (v/v) glycerol–Complete protease inhibitor mixture). Molecular mass marker proteins (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; bovine serum albumin, 67 kDa; Amersham Pharmacia Biotech) were fractionated in parallel to allow estimation of the molecular mass of the fractions. Sedimentation was carried out for 16 h at 38,000 rpm in a Beckman SW41 rotor, and the gradients were fractionated from the bottom to give 18–20 fractions of 600 µl. 20 µl of each fraction was separated by SDS-PAGE for immunoblot analysis.

Subcellular Fractionation by Iodixanol Gradients—Subcellular fractionation was performed using iodixanol as medium according to the previously described method with some modifications (42, 43). Cell pellets from a 15-cm dish were resuspended in 4 ml of homogenization buffer B (10 mM HEPES (pH 7.4), 1 mM EDTA, 0.25 M sucrose, Complete protease inhibitor). Cells were disrupted by a Polytron homogenizer and centrifuged at 1,500 × g for 10 min in a Beckman SW41 centrifuge tubes by diluting iodixanol (Optiprep, 60% w/v, Life Technologies, Inc.) with homogenization buffer B (2.5% iodixanol, 1 ml; 5%, 2 ml; 7.5%, 2 ml; 10%, 2 ml; 12.5%, 0.5 ml; 15%, 2 ml; 17.5%, 0.5 ml; 20%, 0.5 ml; 30%, 0.3 ml). The resuspended vesicle fractions were loaded on the top of the gradients and centrifuged in a SW41 rotor at 40,000 × rpm for 2.5 h. Then the resulting gradients were collected in 1-ml fractions. 20 µl of each fraction was separated by SDS-PAGE for immunoblot analysis.

Quantitation of Aβ by Two-site ELISAs—Two-site ELISAs that specifically detect the C terminus of Aβ were used as described (20, 32, 34). BNT77, which was raised against human Aβ11–28 and recognizes full-length as well as N-terminally truncated Aβ, was used as a capture antibody; BNT77 binds human as well as rodent-type Aβ, whereas BA27 and BC05 that specifically recognize the C terminus of Aβ react with the 3-kDa fragment (p3) (44). BA27 and BC05 that specifically recognize the C terminus of Aβ react with the 3-kDa fragment (p3) (44). Structures of the PS derivatives used in this study are schematically shown in Fig. 1B. Immunoblot analysis of the cell lysates showed that neither PS2/D366A nor PS2/ΔloopN underwent endoproteolysis to give rise to a 35-kDa NTF and a 23-kDa CTF that are normally produced from full-length (FL) PS2 as reported previously (Fig. 2A, upper panel, arrowhead) (13, 14, 25–27). wt and mt PS2/P414L also were expressed as holoproteins and did not yield endoproteolytic fragments, whereas PS2/P417S were cleaved to form fragments (Fig. 2A, upper panel, arrowheads). Overexpression of exogenous wt FL PS2 or PS2/P414L as well as of PS2/D366A or PS2/ΔloopN resulted in the replacement of endogenous murine PS1 fragments (Fig. 2A, lower panel, arrowhead). These data were consistent with the notion that replacement of endogenous PS is not coupled to endoproteolysis (24, 25, 30). In contrast, replacement of endogenous PS1 did not occur in N2a cells expressing PS2/P414L, suggesting that P414L mutation abolishes the interaction of PS with the cofactor(s) that stabilizes intracellular PS.

To evaluate the half-lives of transfected PS proteins and fragments thereof, we performed a chase experiment upon treatment with cycloheximide that blocks de novo cellular protein synthesis (24, 34). In cycloheximide-treated N2a cells, PS2 holoproteins were rapidly degraded, whereas fragments were stabilized at 24 h of chase. NTF and CTF derived from PS2/P417S also were highly stabilized (Fig. 2B, upper panel, arrowhead). A portion of PS2/D366A or PS2/ΔloopN was stabilized as holoproteins, suggesting that the abolition of endoproteolysis does not affect stabilization of PS (Fig. 2B, lower panel, arrow). In contrast, PS2/P414L holoproteins were degraded in a simi-
FIG. 2. Expression and stability of PS2 derivatives in N2a stable cell lines. A, immunoblot analysis of expression of PS2 and its derivatives. Cell lysates (20 μg of protein) from N2a cells transfected with cDNAs encoding FL PS2 or modified PS2 were separated by SDS-PAGE and analyzed by immunoblotting with anti-G2N4, anti-G2L, and anti-G1L3 antibodies. The names of the transfected cDNA constructs are indicated at the top of each lane. wt and mt indicate wild-type and N141I FAD-linked point mutation, respectively. The holoprotein of wt or modified PS2 is marked by an arrow, NTF, CTF, and endogenous mouse PS1 CTF (mopPS1 CTF) are indicated by arrowheads, respectively. B, analysis of the half-lives of PS2 derivatives. Cells were incubated in culture medium containing cycloheximide (30 μg/ml) for 0, 12, or 24 h. Lysates prepared after each incubation period were analyzed by immunoblotting with anti-G2N4 antibody. Arrows and arrowheads at the top and bottom indicate the mobilities of protein molecular mass markers that are shown in kilodaltons.

FIG. 3. Analysis of modified PS2 proteins by velocity gradient centrifugation. 1% CHAPSO extracted membrane proteins from N2a stable cell lines expressing wt FL PS2 (A), PS2/D366A (B), PS2/ΔloopN (C), PS2/P414L (D), or PS2/P417S (E) were fractionated by centrifugation through 15–30% linear glycerol density gradients. 20 μl of each fraction was analyzed by immunoblotting with anti-G2N4 antibody. Arrows and an arrowhead at the right of each panel indicate holoproteins and NTF of PS2, respectively. Arrowheads at the top and bottom indicate the mobilities of protein molecular mass markers that are shown in kilodaltons.

Formation of High Molecular Weight Complex and Subcellular Distribution of PS2 Proteins—PS proteins are known to form a HMW complex with other components (21–23). To examine the capacity of modified PS2 proteins to form HMW complex, membrane fractions of N2a cells were solubilized in 1% CHAPSO, and extracted proteins were separated on a linear glycerol velocity gradient. Endoproteolytic fragments derived from wt FL PS2 were predominantly present in the 232–443-kDa HMW range, whereas PS2 holoproteins were fractionated in the 140–232-kDa low molecular weight (LMW) range (Fig. 3A). PS2/D366A and PS2/ΔloopN, which were stabilized but not cleaved, were present as holoproteins broadly within LMW and HMW ranges (Fig. 3, B and C). In contrast, unstable PS2/P414L holoproteins were fractionated exclusively in the LMW range (Fig. 3D). Fragments of PS2/P417S, which were stabilized in a similar fashion to FL PS2, were also recovered in HMW fractions, whereas holoproteins were present in the LMW range (Fig. 3E).

To examine the subcellular compartmentalization of PS2 proteins, membrane vesicles were separated by fractionation on discontinuous iodixanol gradients (42, 43). The densest fractions, 10–12, exhibited the strongest immunoreactivity for the anti-KDEL antibody, indicating that these fractions contained ER vesicles (Fig. 4F). Fractions enriched in Golgiitrans-Golgi network (TGN) vesicles were identified with antibodies against Adaptin-y or Vti1b (fractions 6–9) (Fig. 4G, data not shown for Vti1b). Fraction 9 contained both ER and Golgi/TGN vesicles. Endoproteolytic fragments derived from FL PS2 were recovered mainly in fractions 7–9 that contained Golgi/TGN vesicles, whereas holoproteins were present in fractions 9–12, which accumulate ER vesicles as described previously (Fig. 4A, arrow and arrowhead) (42, 43). PS2/D366A and PS2/ΔloopN were distributed as holoproteins broadly in fractions 6–12, suggesting that a portion of these mutant holoproteins were transported from ER to Golgi/TGN in a similar manner to endoproteolytic PS fragments (Fig. 4, B and C). In contrast, PS2/P414L holoproteins remained in fractions 10–12 rich in ER vesicles (Fig. 4D). Fragments of PS2/P417S, which were stabilized in a similar fashion to FL PS2, were fractionated mainly in Golgi/TGN fractions 7–9, whereas holoproteins were present in fractions 9–12 (Fig. 4E).

These data suggest that stabilized PS2 proteins (i.e. endoproteolytic fragments and a portion of holoproteins in uncleavable mutants) participate in the formation of HMW PS2 complexes, which are present in Golgi/TGN. In contrast, unstabilized PS2 proteins (i.e. holoproteins of wt FL PS2, PS2/P414L, and nascent form of PS2/D366A and PS2/ΔloopN) form LMW PS2 complexes that remain in ER. These observations support the idea that stabilization and transport to Golgi/TGN of PS proteins require an interaction of PS with unknown cellular cofactor(s), and that the first proline of the PALP motif plays an important role in the formation of PS HMW complex.

Effects of PALP Mutations on Site-3 Cleavage of Notch—PS is known to serve as a critical component of Notch signaling by executing the site-3 cleavage of Notch (7–11). It has been reported that PS2/D366A abrogates the proteolytic cleavage of mouse Notch-1 in mammalian cells and interferes with the Notch signaling in C. elegans in vivo (14). Thus, it is tempting...
to speculate that the loss-of-function mutations in invertebrate PS may suppress Notch signaling by inhibiting the proteolytic release of NICD. To examine whether PS2/P414L or PS2/P417S mutations affect the γ-secretase-like site-3 cleavage activity for Notch, we analyzed the proteolytic release of NICD in N2a cells transiently transfected with a cDNA encoding NotchΔE that lacks the extracellular domain but retains the C terminus, in fractions 9–12, and that Golgi/TGN marker adaptor-γ was distributed mainly in fractions 5–9 (G, arrowhead).

FIG. 4. Subcellular distribution of modified PS2 derivatives in stably transfected N2a cells. Total membrane proteins from N2a stable cell lines expressing wt FL PS2 (A, F, and G), PS2/D366A (B), PS2/ΔloopN (C), PS2/P414L (D), or PS2/P417S (E) were fractionated by 2.5–30% discontinuous iodixanol gradients. 20 μl of each fraction was separated by SDS-PAGE and immunoblotted with anti-G2N3 (A), anti-G2N4 (B–E), anti-KDEL (F), and anti-adaptor-γ (G) antibodies, respectively. Numbers of fractions collected from the top of the iodixanol gradients are indicated at the top and bottom. Arrows and an arrowhead in A–E indicate holoproteins and NTF of PS2, respectively. Note that anti-KDEL antibody probed the ER-resident chaperones GRP78 (BiP) and GRP94 (F, arrowheads), which carry the KDEL sequence at the C terminus, in fractions 9–12.

FIG. 5. Immunoblot and pulse-chase analysis of site-3 cleavage of Notch in cells expressing modified PS2. A, N2a cells stably expressing wt or modified PS2 were transiently transfected with the cDNA encoding a C-terminally Myc-tagged mouse NotchΔE polypeptide. Lysates of cells (3 μg of protein) were analyzed by immunoblotting with a monoclonal anti-c-myc antibody 9E10. The same membrane was developed upon longer exposure (lower panel). Arrows and arrowheads indicate the NotchΔE and its proteolytic derivative NICD, respectively. The names of the transfected cDNAs are shown at the left of each lane. wt and mt indicate wild-type and N141I FAD-linked point mutation, respectively. B, immunoblot analysis of the steady-state levels of NotchΔE polypeptide and NICD formation in N2a cells stably coexpressing PS2 and NotchΔE. C, N2a cells stably coexpressing PS2 and NotchΔE were pulse-labeled with [35S]methionine for 15 min and chased for 30 and 60 min with or without the presence of 10 μM clasto-lactacystin β-lactone. Lysates were immunoprecipitated with anti-c-Myc antibody 9E10 and analyzed by autoradiography. Arrows and arrowheads indicate NotchΔE and NICD, respectively. Note that NotchΔE polypeptides were rapidly degraded in stably transfected N2a cells, whereas treatment with clasto-lactacystin β-lactone significantly stabilized NotchΔE. D, PS-null fibroblasts were transiently cotransfected with the cDNAs encoding wt or modified PS2 and NotchΔE, and Notch processing to yield NICD were analyzed by immunoblotting as in A (3 μg of protein/lane). Lysate from N2a cells expressing NotchΔE was separated as a control at the left lane.
chase even under treatment with clasto-lactacystin β-lactone, whereas overexpression of PS2/P414L did not affect NICD formation (Fig. 5C, upper right and lower panel, arrowhead). However, very small amounts of NICD were detected at 60 min of chase in cells expressing mt FL PS2 or PS2/P417S, suggesting that these mutations partially abolish the site-3 cleavage activity of PS2.

To overcome the caveat that site-3 cleavage activity associated with endogenous PS masks the activities of transfected PS, we transiently co-transfected PS2 derivatives and NotchΔE in immortalized PS-null fibroblast cell line derived from PS1/PS2 double-knockout mice (Fig. 5D) (7, 40). NICD generation was completely absent in PS-null fibroblasts, which was restored upon transfection with wt FL PS2 whereas the overexpression of mt FL PS2 did not rescue (Fig. 5D, arrowhead). PS2/D366A did not rescue the site-3 cleavage of NotchΔE as documented previously (14). PS2/P414L and PS2/P417S also did not restore the proteolytic release of NICD in PS-null fibroblasts. We therefore conclude that the amino acid substitution in the first proline of PALP motif interferes with the site-3 cleavage activity of PS2 by inhibiting the formation of HMW PS complex.

**Effects of PALP Mutations on Aβ Generation in N2a Stable Cells**—To examine the effects of mutations in PALP motif on γ-cleavage of APP, we analyzed N2a cells stably expressing PS2/D366A, PS2/P414L, or PS2/P417S. Overexpression of PS2/D366A in N2a cells inhibited γ-cleavage of βAPP, resulting in a marked accumulation of βAPP C-terminal stubs (i.e. C83 and C99), which are the direct precursors for p3 and Aβ, respectively (Fig. 6A, arrowheads) (14). In contrast, cells expressing PS2/P414L or PS2/P417S did not accumulate βAPP CTFs, suggesting that mutations in either of the two prolines of PALP motif in PS2 does not have a dominant negative effect on γ-cleavage of βAPP unlike PS2/D366A. To further characterize the effects of PALP mutations of PS2 on Aβ production, the levels of secreted Aβ40 and Aβ42 in conditioned media were measured by Aβ C-terminal specific ELISAs (44). Secretion of Aβ from N2a cells expressing PS2/D366A was significantly inhibited compared with that in cells expressing wt FL PS2 as reported previously (see Fig. 5D) (14). In contrast, the total levels of Aβ secreted from cells expressing PS2/P414L or PS2/P417S were comparable to those in cells with wt FL PS2 (Fig. 6B). The percentage of Aβ42 as a fraction of total Aβ (Aβ40 + Aβ42) (%)Aβ42) secreted by cells stably expressing mt PS2/P414L was ~10%, and this was similar to the %Aβ42 secreted from cells expressing wt FL PS2 or wt PS2/P414L, whereas the %Aβ42 secreted from cells expressing mt FL PS2 was constantly elevated to ~75% as previously documented (Fig. 6C) (20, 32, 34, 43, 44). Moreover, the %Aβ42 in cells expressing PS2/P417S was also elevated to ~30%. These results indicate that the P414L mutation abrogates the overproduction of Aβ42 on a FAD-linked mutant basis, whereas P417S mutation harbors a pathogenic function like FAD mutant PS to increase Aβ42.

**Effects of PS1 Harboring P433L Mutation on Site-3 Cleavage of Notch and Aβ Generation**—To determine whether our results regarding the role of PALP motif of PS2 in site-3 cleavage and Aβ generation are applicable to PS1, we constructed cDNAs encoding wt and P2675S FAD-linked mutant (mt) PS1 with a proline to leucine amino acid substitution of the first proline in PALP motif (P433L). Consistent with the results obtained with PS2, PS1/P433L as well as PS1/D385A did not rescue the site-3 cleavage of NotchΔE in PS-null fibroblasts (Fig. 7A). In stably transfected N2a cells, the total levels of secreted Aβ were not affected by expression of wt or mt PS1/P433L (Fig. 7B). mt FL PS1 increased the %Aβ42 by about 2-fold compared with that of wt FL PS1, whereas %Aβ42 was not elevated in cells expressing mt PS1/P433L (Fig. 7C). These results suggested that the first proline of the PALP motif in PS1 C terminus also is important for its assembly and stabilization that are the prerequisites for γ-secretase activities.

**Effects of P414L Mutation on the Metabolism and the Dominant Negative Function against γ-Secretase Activity of PS2/D366A**—It has been reported that aspartate mutations in PS2 affect not only γ-secretase activity but also HMW complex formation in stably transfected MEF cells (45). However, a portion of PS2/D366A formed a HMW complex of a similar size to that derived from wt PS2, which was stabilized in a similar manner to fragments derived from FL PS2 (Fig. 3). To determine whether the HMW complex formation and stabilization of
PS2/D366A is the prerequisite for its dominant negative function, we stably transfected PS2/D366A harboring P414L mutation (PS2/D366A/P414L) in N2a cells and examined its metabolism as well as its effect on $\gamma$-secretase activity. Western blot analysis revealed that mutated PS2/D366A/P414L was no more capable of replacing endogenous PS1 CTF or accumulating $\beta$APP CTFs (Fig. 8, A and B). These data suggested that P414L mutation affected the metabolism as well as the dominant negative function of PS2/D366A. We next analyzed the half-life and HMW complex formation of PS2/D366A/P414L. Cycloheximide treatment showed that PS2/D366A/P414L protein was unstable (Fig. 8B). Moreover, the fractionation pattern of PS2/D366A/P414L polypeptides in glycerol velocity centrifugation was very similar to that of PS2/P414L, indicating that P414L mutation abolished the HMW complex formation of PS2/D366A protein. Finally, we analyzed the amount of A$\beta$ secreted from N2a cells. Total levels of secreted A$\beta$ from N2a cells expressing PS2/D366A/P414L were comparable to that in cells expressing wt FL PS2, whereas overexpression of PS2/D366A inhibited the secretion of A$\beta$ (Fig. 8D). These data suggest that PS2/D366A/P414L double mutant protein does not undergo stabilization and HMW complex formation, thereby losing its dominant negative effects on $\gamma$-secretase activities.

**DISCUSSION**

Genetic and biochemical studies have shown that PS is not only AD-related pathological proteins, but also critical components of Notch signaling (1, 9). Although the precise molecular function of PS remains elusive, accumulating evidence suggests that PS is involved in intramembranous cleavage of various transmembrane proteins (6–8, 10, 46, 47). In this study, we demonstrated that (i) the first proline of the highly conserved PALP motif at the C terminus of PS plays a critical role in the stabilization, HMW complex formation and transport out of ER to Golgi/TGN of PS, and (ii) lack of assembly and stabilization as a HMW complex of PS caused by the amino acid substitution in this proline deprives PS of the capacities to support intramembranous $\gamma$-cleavage of $\beta$APP and site-3 cleavage of Notch. The results of our studies provide unequivocal evidence that stabilization and HMW complex formation of PS, which require the integrity of the C-terminal domain of PS, are the prerequisites for its physiological and pathological $\gamma$-secretase functions.

The PALP motif is completely conserved in all PS species thus far identified (Fig. 1A). Here we showed that replacement of the first proline of PALP motif for leucine abolished the normal metabolism (i.e. endoproteolysis, stabilization, replacement, and HMW complex formation) of PS in mammalian cells.
Although subcellular localization of site-3 cleavage activity of Notch remains unknown, Golgi vesicle-enriched fractions have been shown to contain γ-secretase activities for βAPP in vitro (42). Thus, the loss-of-function phenotypes of PS2/P414L for cleavages of βAPP and Notch that we have shown in this study may be due to defects in stable HMW complex formation and proper transportation from ER to Golgi/TGN. Similar mechanisms may underlie the recessive PS-null phenotype exhibited by C. elegans spe-4 (eb-12) (35) and Drosophila Pan (Dps) (36) alleles in vivo. Taken together, it is strongly suggested that the first proline of PALP motif plays an important role in the proper metabolism of PS family proteins to confer normal or abnormal functions.

In contrast, PS2/P417S underwent endoproteolysis to give rise to stabilized PS complexes and %Aβ42 was increased as observed with other FAD-linked PS mutations. Furthermore, Notch site-3 cleavage was partially suppressed with both N141I and P417S PS2 mutants. It has been documented that the proteolytic production of NICD is impaired by some FAD-linked PS1 mutations (48), although NICD has been shown to be produced normally with other PS1 mutations (49). In this regard, Kulic et al. (50) have recently reported an interesting relationship between Aβ42 overproduction and Notch site-3 cleavage; they have introduced arbitrary mutations at position 286 of PS1 and shown that the strength of Aβ42 promoting effects of PS1 mutations is correlated with the extent of reduction in NICD generation. The precise mechanism underlying these changes in γ- and site-3 cleavage remains unknown. However, one could argue that FAD-related PS2 mutations that are more potent in Aβ42 promoting effects compared with those in PS1 (Ref. 51 and see Figs. 6C and 7C) might have behaved like some of the artificial PS1 mutations, leading to inhibition in Notch site-3 cleavage. It is tempting to speculate that this apparent “loss-of-function” in Notch cleavage caused by some of the FAD-linked PS mutations could be mechanistically related to the shift of γ-cleavage from position 40 to 42 of Aβ. However, the relevance of NICD reduction to the pathophysiology of AD remains unknown; if mutant PS1 allele were inactive for Notch cleavage, sufficient NICD could be produced by the activity of wt PS1 on the normal allele, considering the normal development of heterozygous PS1 KO mice in the absence of PS2 (40, 52), and Notch phenotype has never been reported in FAD patients.

What, then, is the mechanistic role of PALP motif in the stabilization of PS? One possibility would be that this motif serves as the binding site for the cellular factor required for stabilization. Because of its unique side-chain structure, proline is known as the critical amino acid essential to the proper structure of peptide backbone and function of proteins (53). In particular, a number of protein interaction domains harbor proline-rich sequences, that tend to adopt the polyproline II helix, i.e. an extended structure with three residues per turn and thus well placed to interact with the protein. Notably, SH3 domains recognize proline-rich sequences containing the core sequence of PXXP (55). The classification of SH3 ligand is dominated by the location of a positively charged residue that forms a salt bridge with an acidic residue in the SH3 domains, and peptides with a motif +XXPXX or PXPX+ (where + refers to a positively charged residue) correspond to class I and II motifs, respectively. Thus, the PALP motif of PS (i.e. KAL-PALP) fulfills the criteria for a class I SH3 ligand. Indeed, secondary structure prediction using Chou-Fasman method suggested that the C-terminal region of PS flanking the PALP motif forms an α-helical structure, whereas replacement of the first proline disrupted α-helix and converted it to a β-sheet. Although PS-binding proteins carrying polyproline binding domain such as SH3 domain have not been identified, it is tempting to speculate that the α-helical structure around PALP motif serves as the binding site for the limiting cofactor(s).

An alternative possibility would be that the PALP motif is required for maintaining the proper conformation of the C-terminal region of PS. We have shown previously that subtle changes in the C-terminal region of PS disrupt the stability of PS and its γ-secretase functions (34). Similarly, the PALP motif may play a seminal role in the maintenance of proper membrane topology and assembly of the TM domains that are essential to the stabilization and complex formation of PS. It is also possible that the structural integrity of the entire C terminus is required for the association of PS with cofactor(s).

PS2/P414L proteins were fractionated exclusively in LMW ranges, whereas wt PS2 or PS2/P417S were separated in HMW fractions. Moreover, a portion of uncleavable PS2/D366A or PS2/ΔloopN, that were stabilized as holoproteins, were also fractionated in HMW ranges. These data strongly suggest that the stabilization of PS proteins coincides with the formation of HMW complex and that the endoproteolysis of PS proteins is not required for these processes. Thus, the difference in molecular size between unstable and stabilized PS complexes may be due to an association with the cofactor(s) including those required for stabilization (30).

Recently, it was reported that the formation of HMW complex is impaired with aspartate mutants of PS (45). However, a portion of PS2/D366A formed a HMW complex of a similar size to that derived from wt PS2 (Fig. 3). The reason for this discrepancy is unknown, but it may be due to differences in detergents and/or cell types. Nonetheless, the occurrence of stabilization and replacement with PS2/D366A that normally are observed with wt PS2 (Figs. 2A and 9A) suggests that aspartate mutation does not impair the association of PS with the cofactor(s) required for stabilization (Fig. 9B). In addition, introducing P414L mutation in PS2/D366A completely abolished the HMW complex assembly and dominant negative effects on γ-secretase activity (Fig. 8). Aspartate mutant thus acts as a dominant negative molecule because of its ability to replace the active endogenous PS, as well as its inactivity as a γ-secretase.

We have shown previously that PS2/I448R, in which the C-
terminal isoleucine is replaced with arginine, is defective in stabilization and formation of HMW complex (Fig. 9C).

Mutational analysis of the highly conserved PALP motif provided additional evidence that the stabilized HMW complex of PS represents the active form of γ-secretase. Further attempts to define the molecular mechanism of PS stabilization and to identify the components of PS complexes relevant to intramembranous proteolytic activity will facilitate understanding of the pathogenic mechanisms of AD.

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The First Proline of PALP Motif at the C Terminus of Presenilins Is Obligatory for Stabilization, Complex Formation, and γ-Secretase Activities of Presenilins
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