The Mechanism of γ-Secretase Activities through High Molecular Weight Complex Formation of Presenilins Is Conserved in Drosophila melanogaster and Mammals*

Received for publication, May 30, 2002, and in revised form, October 8, 2002
Published, JBC Papers in Press, October 17, 2002, DOI 10.1074/jbc.M205352200

Nobumasa Takasugi, Yasuko Takahashi, Yuichi Morohashi, Taisuke Tomita‡, and Takeshi Iwatsubo‡
From the Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113-0033

Mutations in presenilin 1 (PS1) and PS2 genes contribute to the pathogenesis of early onset familial Alzheimer’s disease by increasing secretion of the pathologically relevant Aβ42 polypeptides. PS genes are also implicated in Notch signaling through proteolytic processing of the Notch receptor in Caenorhabditis elegans, Drosophila melanogaster, and mammals. Here we show that Drosophila PS (Psn) protein undergoes endoproteolytic cleavage and forms a stable high molecular weight (HMW) complex in Drosophila S2 or mouse neuro2a (N2a) cells in a similar manner to mammalian PS. The loss-of-function recessive point mutations located in the C-terminal region of Psn, that cause an early pupal-lethal phenotype resembling Notch, disrupt the HMW complex formation, and abolish γ-secretase activities in cultured cells. The overexpression of Psn in mouse embryonic fibroblasts lacking PS1 and PS2 genes rescued the Notch processing. Moreover, disruption of the expression of Psn by double-stranded RNA-mediated interference completely abolished the γ-secretase activity in S2 cells. Surprisingly, γ-secretase activity dependent on wild-type Psn was associated with a drastic overproduction of Aβ1–42 from human βAPP in N2a cells, but not in S2 cells. Our data suggest that the mechanism of γ-secretase activities through formation of HMW PS complex, as well as its abolition by loss-of-function mutations located in the C termi

Mutations in presenilin (PS1)1 or PS2 genes account for the majority of early-onset familial Alzheimer’s disease (FAD), and these mutations cause an increase in the ratio or levels of production of amyloid β peptides ending at position 42 (Aβ42), that most readily form amyloid deposits (1). Presenilins are polytopic integral membrane proteins that span the membrane eight times and undergo endoproteolysis (2). The endoproteolytic fragments of PS are incorporated into a high molecular weight (HMW) complex (3, 4) and are highly stabilized (t1/2 = ~20 h), whereas holoprotein is rapidly degraded (t1/2 = ~2 h) (5).

This paper is available on line at http://www.jbc.org

* The abbreviations used are: PS1, presenilin 1; AD, Alzheimer’s disease; Aβ, amyloid β peptide; γAPP, β-amyloid precursor protein; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio-2-hydroxy-1-propanesulfonate; CHX, cycloheximide; CTF, C-terminal fragment; dsRNA, double-stranded RNA; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; FAD, familial Alzheimer’s disease; FL, full-length; N2a, mouse neuro2a neuroblastoma; NICD, Notch intracellular domain; NTF, N-terminal fragment; RNAi, double-stranded RNA-mediated interference; TGN, trans-Golgi network; TMD, transmembrane domain; HMW, high molecular weight; LMW, low molecular weight.

‡ To whom correspondence may be addressed: Dept. of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: 81-3-5841-4877; Fax: 81-3-5841-4708; E-mail: taisuke@mol.f.u-tokyo.ac.jp or iwatsubo@mol.f.u-tokyo.ac.jp.

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
Cell Culture and Transfection—Mouse neuro2a (N2a) neuroblastoma cells and 5V4-embryonic mouse embryonic fibroblasts (MEF) derived from PS1<sup>−/−</sup> PS2<sup>−/−</sup> littermates (provided by Dr. B. De Strooper) were maintained as described (15). Generation of stable N2a cell lines co-expressing βAPP<sub>swt</sub> and Notch<sub>α</sub> (NLN) were described previously (25). Stable N2a NLN cell lines expressing Pan or PS2 derivatives were generated by transfecting cDNAs using LipofectAMINE and selected in Dulbecco’s modified Eagle’s medium containing both hygromycin (Wako) at 250 µg/ml and G418 (Calbiochem, San Diego, CA) at 500 µg/ml. Transient transfection of cDNAs into MEF cells were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h of transfection, 10% butyric acid was added for 24 h to drive protein expression.

Drosophila Schneider (S2) cells were maintained in Schneider’s insect medium (Sigma) supplemented with 10% fetal bovine serum, 5% peptone, and penicillin/streptomycin (Sigma medium) at 24 °C (26). Transient transfection of cDNAs into S2 cells was performed using Cellfectin (Invitrogen) according to the manufacturer’s instructions, and samples were collected after 48 h of transfection. Stable S2 cell lines were generated by transfection of cDNAs in pAc5.1/V5-His A vector together with those in pCoHygro (Invitrogen) vector (ratio of transfected cDNAs; 2:1 µg) using Cellfectin and selection in S2 medium containing hygromycin at 250 µg/ml.

Double-stranded RNA-mediated Interference (RNAi)—For the production of the double-stranded RNA (dsRNA), transcription templates that contained T7 RNA promoter sequences on each end were generated by PCR using cDNAs encoding the T7 RNA polymerase binding site as primer pairs: 5'-TTAATACTGACTATAGGGAGAGAATGGCTGCTGTCAAT-3' for PS1, 5'-TTAATACGACTCACTATAGGGAGATACTTGAGCTGATCATCTTCT-3' for EGFP as sense primers, 5'-TTAATACGACTCATATAGGGAGAGACATCATTCCGACC-3' for Pan, 5'-TTAATACGACTCATATAGGGAGATCGTATTCC-3' for EGFP as reverse primers, respectively. dsRNAs were prepared from transcription templates by using MEGAscript T7 KIT (Ambion, Austin, TX) and transfected into S2 cells using Cellfectin. Cell lysates and conditioned media were harvested after incubation for indicated times.

Antibodies, Immunoblot Analysis, and Fractionation Studies—The following rabbit polyclonal antibodies were used: anti-βAPP against mouse APP (25). BA27 and BC05 that specifically recognize the C terminus of βAPP or βA4 were used as described. Anti-GDN1 against glutathione S-transferase (GST) fused to amino acids 2–52 of Pan, anti-GDL1 against GST fused to amino acids 358–426 of Pan; anti-G2L against GST fused to amino acids 301–361 of human PS2, anti-G2N4 against GST fused to amino acids 2–59 of human PS2, anti-G1N2 against GST fused to amino acids 2–70 of human PS1, and anti-G1L3 against GST fused to amino acids 297–379 of human PS1 have been previously described (15, 16, 20, 22, 27). The rabbit polyclonal antibody C4 against the cytoplasmic C terminus of human βAPP was kindly provided by Dr. Y. Ihara (University of Tokyo). The mouse monoclonal antibodies were purchased from Stressgen (anti-KDEL), Transcription Laboratory (anti-Adaptin-γ), and Roche Diagnostics (anti-c-Myc (9E10)), respectively. Preparation of cell lysates, immunoblot analysis, cycloheximide treatment, glycerol velocity centrifugation, and subcellular fractionation using Iodixanol gradient centrifugation were performed as previously described (15, 22, 23).

Quantification of βA by Two Site ELISAs—Two site ELISAs that specifically detect the C terminus of βA were used as described. BAN50 is an monoclonal antibody raised against a synthetic peptide of human βA1–16; it preferentially reacts with the N-terminal portion of human βA starting at Asp-1, but does not cross-react with N-terminally truncated βA nor with rodent-type βA (20, 28). BA27 and BC05 that specifically recognize the C terminus of βA40 and βA42, respectively, were used with horseradish peroxidase- and Alkaline phosphatase-conjugated secondary antibodies. Culture media were collected after an appropriate incubation period and subjected to BAN50/BA27 or BAN50/BC05 ELISAs as described (20, 29).

**RESULTS**

**Expression and Metabolism of Pan in Drosophila S2 or Mouse N2a Cell Lines—**Drosophila presenilin (Pan) gene encodes 508–541 amino acid proteins with ~50% identity to its vertebrate counterparts (21). The occurrence of endoproteolytic cleavage of Pan protein in vivo and in the Drosophila S2 cell line has also been documented, although a detailed analysis on the metabolism of Pan polypeptides is yet to be performed (18, 30). To examine the expression and metabolism of endogenous and transfected Pan proteins in S2 or mouse N2a cell lines, we stably transfected these cells with Pan and analyzed by immu-

**MATERIALS AND METHODS**

**Construction of Expression Plasmids—**Full-length (FL) cDNAs encoding wild type, FAD mutant N141I human PS2 in pcdNA3 (Invitrogen, Carlsbad, CA) were obtained as described (20). A full-length cDNA encoding 508 amino acid residues of full-length Pan in pC2 vector was provided by Dr. G. L. Boulianne (21). A cDNA coding for the Pan open reading frame was generated by PCR using PhTuru (Strategene, La Jolla, CA), and the following oligonucleotides were used as a PCR primer: 5'-GAATTCATGCGGCGCATTCATCTTCTCAG-3' as a forward primer and 5'-GGCTCAGTTAATATAAACACTCTGTT-3' as a reverse primer. The amplified cDNA was subcloned into pCDNA3 or pAc5.1/V5-His A vector (Invitrogen). A cDNA encoding enhanced green fluorescent protein (EGFP) was digested from pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) and subcloned into pAc5.1/V5-His A vector. cDNAs encoding Pan/D461A, Pan/P507L, Pan/G156E, amino acid numbering based on Pan<sub>541</sub> or human PS2/P423E were generated by the long-PCR protocol (15, 22) using cDNAs encoding wild type, or mutant Pan or PS2 in pcdNA3 vector as the templates, using the following primer pairs: 5'-GGCTCCTGGCGCATTACCTCTTCTGATATTGGCGG-3' for Pan/D461A, 5'-GGGCGAGGCTGCTACAGCGCCTGGCCAAATGGC-3' for Pan/P507L, 5'-GAGGAGCAAGAATATGCTACGAAGGCTGATG-3' for Pan/G156E, 5'-GTTAAAGATGCACAGTCGAGGCTGAGGACG-3' for PS2/P423E as forward primers, 5'-GGCCATATGACAAGCTGAAGATGGTCTGGCAGGCG-3' for Pan/D461A, 5'-GGGCGAGGCTGCTACAGCGCCTGGCCAAATGGC-3' for Pan/P507L, 5'-GAGGAGCAAGAATATGCTACGAAGGCTGATG-3' for Pan/G156E, 5'-GTTAAAGATGCACAGTCGAGGCTGAGGACG-3' for PS2/P423E as reverse primers, respectively. Schematic depictions of modifications of PS used in this study are shown at the left of each sequence. Open and filled triangles show the location of amino acid substitutions that are linked to FAD (i.e. N141I in Pan2, P267S in Pan1) and the loss-of-function (Notch) phenotype in Pan (i.e. Pan507L, Pan156E), respectively. Filled and open arrows indicate an alanine mutation at the 7th TMD of Pan (D461A) and an artificial mutation in Pan at an equivalent position to G156E of Pan, respectively. Open squares show the TMD of Pan. Locations of epitopes of antibodies used in this study are marked by dotted lines, and the names of antibodies are show above the lines.

**Pan**

![Schematic depiction of modified PS used in this study.](Image)

The names of the Drosophila Pan and human PS2 or PS1 cDNAs are shown at the left of each sequence. Open and filled triangles show the location of amino acid substitutions that are linked to FAD (i.e. N141I in Pan2, P267S in Pan1) and the loss-of-function (Notch) phenotype in Pan (i.e. Pan507L, Pan156E), respectively. Filled and open arrows indicate an alanine mutation at the 7th TMD of Pan (D461A) and an artificial mutation in Pan at an equivalent position to G156E of Pan, respectively. Open squares show the TMD of Pan. Locations of epitopes of antibodies used in this study are marked by dotted lines, and the names of antibodies are show above the lines.

![Schematic depiction of modified PS used in this study.](Image)
nblotting with antibodies against the N terminus or hydrophilic 6th loop of Psn (i.e. anti-GDN1 and anti-GDL1, respectively). Immunoblot analysis of lysates of untransfected S2 cells revealed a ~27-kDa N-terminal fragment (NTF) as well as a ~32-kDa C-terminal fragment (CTF) (Fig. 2A). These bands disappeared when the blots were probed by antibodies preadsorbed with immunogen proteins (data not shown). A faint band of ~55–60 kDa, corresponding to the full-length Psn protein, also was detectable. These results confirmed the previous reports on the endoproteolysis of Psn as well as the predominance of fragment forms as endogenous Psn, which was similar to those seen with mammalian PS (18, 30).

We next analyzed the lysates of S2 cells stably transfected with Psn (Fig. 2A). A ~55-kDa band corresponding to a FL Psn polypeptide was detected by the N- and C-terminal antibodies, whereas the levels of NTF and CTF did not increase, suggesting that the levels of Psn fragments also are regulated by a “limiting co-factor” in a similar manner to mammalian PS (31). To further characterize the metabolism and function of Psn, we stably transfected the Psn cDNA into a mouse N2a cell line stably expressing both bAPP_NL and Notc3E (N2a NL/N cell line) (Fig. 2A). Immunoblot analysis revealed that Psn polypeptides expressed in N2a NL/N cells underwent endoproteolysis to give rise to NTF and CTF of the same molecular weights as the endogenous ones in S2 cells. Moreover, the overexpression of Psn in N2a NL/N cells compromised the accumulation of endogenous murine PS fragments, suggesting that Psn retains the capacity to replace the endogenous PS by competing for limiting cofactor(s) in a similar fashion to that observed with mammalian PS (Fig. 2B).

Fragments of mammalian PS are highly stabilized and incorporated into HMW protein complexes of ~200–600 kDa that are distributed in the ER as well as in Golgi/TGN, whereas holoproteins are rapidly degraded, fractionated in the low molecular weight (LMW) range of ~100–200 kDa, and exclusively distributed in ER (15). To examine the stability of Psn protein, we treated S2 or N2a NL/N cells stably expressing Psn with cycloheximide (CHX) (Fig. 3A). The levels of endoproteolytic fragments of Psn did not decrease during CHX treatment of 10–12 h, whereas the Psn holoproteins were rapidly degraded similarly to mammalian PS holoproteins. To examine the capacity of Psn proteins to form HMW complexes, we solubilized the membrane fractions of S2 or N2a NL/N cells in 1% CHAPSO, and separated the extracted proteins on a linear glycerol gradient (Fig. 3B). Endoproteolytic fragments derived from Psn were predominantly distributed in the HMW range of 232–443 kDa, whereas Psn holoproteins were fraction-
ated in the LMW range of 140–232 kDa. Moreover, subcellular fractionation studies using discontinuous Iodixanol gradients showed that endoproteolytic Psn fragments were recovered in fractions containing ER vesicles as well as Golgi membranes, whereas holoproteins were detected in ER fractions in N2a NL/N cells (Fig. 3C and data not shown). These data suggest that Psn proteins are metabolized in Drosophila S2 cells by a similar cellular machinery to that working in mammalian cells, and appropriately metabolized by a mammalian PS-metabolic pathway (i.e. properly folded, assembled with binding partners, stabilized, and forming HMW complex) in mouse N2a cells.

Mutations of the Highly Conserved Amino Acid Residues at the C Terminus of Psn or Their Equivalents in Human PS2 Affect the Formation of Stable HMW PS Complex—The formation of the stabilized HMW complex of mammalian PS, that requires the integrity of the conserved PS C terminus, is essential to the acquisition of γ-secretase activity, and an aspartate residue within 7th TMD (TMD7) is crucial to the γ-secretase activity in mammalian PS (9, 32). To verify the effects of missense mutations in Psn that cause Notch (i.e. loss-of-function) phenotype in Drosophila in vivo, on the metabolism of Psn polypeptides, we introduced the two types of amino acid substitutions (i.e. P507L or G516E) and stably expressed the mutant Psn in N2a NL/N cells. In addition, we established N2a NL/N cells stably coexpressing Psn carrying D461A mutation that replaces the highly conserved aspartate residue in the C terminus of Psn (Fig. 4B) and data not shown). The overexpression of Psn/D461A resulted in a complete replacement of endogenous murine PS1 fragments, and a portion of Psn/D461A was stabilized as a holoprotein, as previously described in aspartate mutants of mammalian PS (i.e. PS1/D385A, PS2/D366A) (9, 15, 32). We next analyzed the HMW complex formation of Psn and its derivatives (Fig. 4C). The unstable Psn/P507L or Psn/G516E holoproteins were fractionated exclusively in the LMW range. In contrast, Psn/D461A, which was stabilized but not cleaved, was present as holoproteins broadly within LMW and HMW ranges in a similar manner to that of mammalian PS2/D366A (15).

To further elucidate the structural and functional roles of the conserved glycine residue at the C terminus of PS2, we constructed cDNAs encoding wild type or N141I FAD mutant human PS2 harboring a G423E mutation, which is equivalent to G516E mutation of Psn, and stably transfected them in N2a NL/N cells. Western blot analysis revealed that PS2/G423E was expressed as holoproteins but neither underwent endoproteolytic cleavage nor replaced endogenous PS1 CTF (Fig. 5A). FAD-linked N141I mutation did not affect the metabolism of G423E mutant PS2 polypeptides. We next analyzed the half-life and HMW complex formation of PS2/G423E. CHX treatment showed that PS2/G423E holoproteins were unstable (Fig. 5B). Moreover, the PS2/G423E polypeptides were fractionated exclusively in LMW fractions by glycerol velocity centrifugation, in a similar manner to unstable PS2 proteins (e.g. PS2 holoprotein or PS2/P414L) (15), indicating that the G423E mutation abolished the HMW complex formation of PS2 protein (Fig. 5C). These results suggest that the conserved glycine residue in the C terminus of PS plays an important role for the stabilization and formation of HMW complex of PS polypeptides in diverse organisms including Drosophila as well as mammals, as we have previously shown with the conserved proline residue at the PALP motif (15).

Thus, the two loss-of-function mutations of Psn at the conserved amino acid residues at the C terminus abolished the stabilization and HMW Psn protein complex formation, and stabilized Psn proteins participated in the formation of HMW Psn complexes, whereas unstable Psn proteins formed only LMW protein complexes. Taken together, these data strongly suggested that the molecular mechanism of PS metabolism is preserved beyond species from Drosophila to humans.

γ-Secretase Activity of Psn in Mouse N2a Cells—To evaluate the γ-secretase activity of Psn, we analyzed the levels of secreted Aβ1–40 and Aβ1–42 in conditioned media from N2a NL/N cells stably expressing wild type Psn or Psn/D461A by ELISA (Fig. 6A). Surprisingly, overexpression of wild type Psn resulted in a ~5–7 fold increase in Aβ42 secretion as compared with those secreted from cells expressing an empty vector or
that are the direct precursors of p3 and Aβ peptides, lost the Psn/G516E, or PS2/G423E, that failed to undergo stabilization pressing wild type FL PS2. These data suggest that Psn/P507L, FAD mutant PS2/G423E was comparable to those in cells ex-

that the stabilization and formation of HMW complex of PS2 with or without G423E and/or N141I mutations. In panels A and B, bars represent the mean ± S.E. in four independent experiments, and names of transfected Psn (A) or PS2 (B) cDNAs are indicated below the columns. C, PS-null fibroblasts were transiently cotransfected with cDNAs encoding Psn with or without mutations, together with NotchΔE, and Notch processing to give rise to NICD was analyzed by immunoblotting with a monoclonal anti-c-Myc antibody 9E10. Arrow and arrowhead indicate the NotchΔE and its proteolytic derivative NICD, respectively. The names of the transfected cDNAs are shown at the top of each lane.

wild type human PS2. Whereas the percentage of Aβ42 as a fraction of total Aβ (Aβ1–40 + Aβ1–42) (%Aβ42) secreted by untransfected N2a NL/N cells was ~10%, the %Aβ42 secreted from N2a cells expressing wild type Psn was constantly elevated to ~50–75%. Overexpression of Psn/D461A in N2a cells inhibited γ-secretase cleavage of βAPPNL, resulting in a marked decrease in the secretion of both Aβ1–40 and Aβ1–42 accompanied by the accumulation of βAPP C-terminal stubs (i.e., C83 and C99), that are the direct precursors of p3 and Aβ, respectively (data not shown). We therefore analyzed the levels of secreted Aβ from N2a cells expressing Psn/P507L or Psn/G516E (Fig. 6A). In contrast to the expression of wild type Psn, the levels of Aβ or %Aβ42 secreted from cells expressing Psn/P507L or Psn/G516E were comparable to those in cells with wild type FL PS2. These results indicated that the PS2/G423E mutation abrogated the FAD-linked mutant-like Aβ42-promoting effect of Psn in N2a cells. We finally analyzed Aβ secreted from N2a NL/N cells expressing C-terminally modified PS2 (Fig. 6B). Total levels or %Aβ42 of secreted Aβ from N2a cells expressing wild type or FAD mutant PS2/G423E was comparable to those in cells expressing wild type FL PS2. These data suggest that Psn/P507L, Psn/G516E, or PS2/G423E, that failed to undergo stabilization and HMW complex formation, lost the γ-secretase activities, as we have previously observed with PS2/P414L, a PS2 equivalent of P507L mutant of Psn. These data further support our view that the stabilization and formation of HMW complex of PS2 mediated by the integrity of its C terminus is required for the γ-secretase activity (15).

Psn is known to serve as a critical component for Notch signaling in vivo by executing the proteolytic release of Notch intracellular domain (NICD) at site-3 (1, 6). To examine the activity of Psn in γ-cleavage-like site-3 cleavage in mammalian cells, we transiently co-transfected wild type or mutant Psn, together with NotchΔE, in an immortalized PS-null fibroblast cell line derived from PS1/PS2 double-knockout mice (7, 15). Overexpression of wild type Psn restored the proteolytic generation of NICD, suggesting that Psn harbors a site-3 protease activity in mammalian cells. In sharp contrast, Psn/D461A, Psn/P507L, and Psn/G516E did not restore the proteolytic release of NICD in PS-null fibroblasts. We therefore conclude that Psn exhibits γ-secretase activities that partially recapitu-
late those of FAD-mutant PS (i.e. overproduction of Aβ42) in mammalian cells, and that these activities are dependent on the formation of HMW PS complex as well as on the aspartate residue within the TMD7, in a similar manner to mammalian PS.

γ-Secretase Activity to Generate Aβ in Drosophila S2 cells—Psn-dependent γ-secretase activity in Drosophila has been shown to cleave Notch and other transmembrane proteins in vivo (6, 33–35). The amino acid sequence of APPL, a Drosophila homologue of βAPP, is not homologous to that of mammalian βAPP especially within the TMD, and γ-cleavage of APPL has not been documented (36). However, it has been shown that overexpression of the C-terminal 99 amino acid fragment of human βAPP elicits the cleavage to generate Aβ1–40 by a γ-secretase-like activity in Drosophila SL-2 cells, although Drosophila cells lack β-secretase activity (37). To evaluate the γ-secretase-like activity for proteolytic processing of the TMD sequence of human βAPP in Drosophila S2 cells, we transiently transfected a cDNA encoding SC100, that corresponds to the C-terminal fragment of human βAPP starting at the 1st residue of Aβ preceded by a signal peptide, and analyzed the conditioned media by ELISA (20, 29). Aβ secretion was readily detectable in conditioned media of cells expressing SC100; surprisingly, however, %Aβ42 was ~19%, which was in sharp contrast to the robust Aβ1–42 overproduction in mouse N2a cells, that is mediated by the same PS species, i.e. wild type Psn (Fig. 7A). To exclude the possibility that γ-secretase-like activity in S2 cells is incapable of producing excessive amounts of Aβ1–42, we constructed a cDNA encoding SC100 harboring an asparagine to valine substitution at residue 716 of βAPP (SC100/T716F), that has been shown to cause robust increase in Aβ1–42 secretion in COS cells (38). Transfection of SC100/T716F into S2 cells resulted in a dramatic increase in Aβ1–42 secretion and simultaneous decrease in Aβ40 secretion (Fig. 7B), suggesting that the endogenous γ-secretase-like activity mediated by Psn normally cleaves the TMD sequence of human βAPP predominantly at Aβ40 position, but is capable of cleaving predominantly at position 42 under pathogenic conditions (e.g. AβPP mutation) in S2 cells. Thus, Psn-dependent γ-cleavage in S2 cells shows similar characteristics to those in mammalian cells, whereas it may be shifted to position 42 by some unknown mechanism in mouse N2a cells.

To examine whether Psn plays an essential role in Aβ generation by a γ-secretase-like activity in S2 cells, we generated a S2 cell line stably expressing SC100 (S2-SC100) and suppressed the expression of endogenous Psn gene by double-stranded RNA (dsRNA)-mediated interference (RNAi). After a 48-h transfection of Psn dsRNA, the expression of Psn polypeptide in fragment forms was completely and specifically abolished in S2-SC100 cells, although the expression of other endogenous or exogenous genes (i.e. tubulin and EGFP) was not affected (Fig. 7C and data not shown for EGFP co-transfection).

In this study, we examined the metabolism and function of Psn protein in mammalian and Drosophila cell lines and showed the following: (i) Psn is metabolized in a manner similar to that of human PS. (ii) Loss-of-function mutations of Psn that result in an early pupal-lethal phenotype in Drosophila completely disrupt the stabilization and HMW complex formation of Psn polypeptides. (iii) Overexpression of wild type Psn in N2a cells increases the secretion of Aβ1–42, whereas alanine substitution of the aspartate at position 461, that corresponds to one of the putative catalytic aspartates in mammalian PS, abolishes the γ-secretase activity. (iv) Expression of Psn in PS-null murine fibroblasts restores the γ-like site-3 cleavage Notch, and (v) the disruption of the expression of Psn by RNAi resulted in a complete loss of γ-secretase activity (Fig. 7D). Thus, Psn-dependent γ-secretase activity is required for Aβ generation from a human βAPP derivative (i.e. SC100) in Drosophila S2 cells.

**DISCUSSION**

In this study, we examined the metabolism and function of Psn protein in mammalian and Drosophila cell lines and showed the following: (i) Psn is metabolized in a manner similar to that of human PS. (ii) Loss-of-function mutations of Psn that result in an early pupal-lethal phenotype in Drosophila completely disrupt the stabilization and HMW complex formation of Psn polypeptides. (iii) Overexpression of wild type Psn in N2a cells increases the secretion of Aβ1–42, whereas alanine substitution of the aspartate at position 461, that corresponds to one of the putative catalytic aspartates in mammalian PS, abolishes the γ-secretase activity. (iv) Expression of Psn in PS-null murine fibroblasts restores the γ-like site-3 cleavage Notch, and (v) the disruption of the expression of Psn by double-stranded RNAi completely abolish the γ-secretase activity in S2 cells. These data suggest that the formation of HMW complex containing PS underlying the γ-secretase activities is a highly conserved process that is common to Drosophila and mammals.

Psn polypeptides underwent endoproteolysis to give rise to NTF and CTF in cultured cells as previously documented (18, 30). These fragments were highly stabilized and formed a HMW complex in a similar manner to mammalian PS. Moreover, overexpression of wild type Psn resulted in a complete replacement of endogenous PS in mammalian cells. These results suggest that Drosophila Psn protein is metabolized in a similar manner to mammalian PS and competes for the “limiting cofactor” with mammalian PS (5, 31). We further studied the molecular mechanism of loss-of-function caused by Psn640 and Psn645 alleles (18, 19), and found that these mutations...
corresponding to Pro507 in Psn), play an important role in the cleavage of Notch (Danio rerio) PS (39), whereas the C. elegans PS, i.e., SEL-12, failed to recapitulate these features in mammalian cells (Ref. 40). The amino acid sequences of the C-terminal ~11 residues of PS family proteins are highly homologous among mammals, zebrafish, and Drosophila, whereas they are relatively divergent in C. elegans Sel-12 and Spe-4. Taken together, it is strongly suggested that the integrity of the C terminus, as well as a couple of highly conserved amino acid residues flanking this region including the PALP motif (Ref. 15; the first proline corresponding to Pro Superscript 667 in Psn), play an important role in the common molecular mechanism underlying the γ-secretase-like functions that are conserved from Drosophila to mammals.

We have generated a Drosophila S2 cell line stably expressing the C-terminal stub of human βAPP (SC100), and found that endogenous Psn forms HMW protein complexes in a similar pattern to mammalian PS, and that γ-secretase-like activity cleaves SC100 to secrete Aβ. Moreover, RNAi-based “knockdown” technique confirmed that Aβ-generating protease activities in S2 cells are dependent on Psn expression, as previously shown for the Notch site-3 activities (41). The present experiment also highlights the usefulness of RNAi in the molecular dissection analysis of the PS complex; indeed, Francis et al. (42) have recently identified two additional cofactors of Psn, i.e., APH-1 and PEN-2, using genetic screen in C. elegans, and demonstrated by RNAi that expression of these proteins are essential to the Aβ-generating activities of Drosophila cells transfected with Notch or APP C100, using a cellular system similar to ours.

Another intriguing finding in this study was the difference in preponderant γ-cleavage sites by wild type Psn in N2a and S2 cells: In N2a cells, overexpression of wild type Psn caused a robust Aβ1–42 overproduction, which was dependent on the aspartate residue in TMD7. Similar overproduction of Aβ1–42 by transfection of “wild type” PS has also been observed with zebrafish PS1 (39). We compared the deduced amino acid sequence of Psn for variations at positions with known mutations causing FAD in human PS, and found that ~8 amino acid residues in wild-type human PS1 (e.g. Met Superscript 664, Met Superscript 139, Cys Superscript 263) are different from the corresponding codon in Psn (Lys Superscript 106, Leu Superscript 661, Ser Superscript 265), respectively, where FAD-linked mutations have been identified (although the substituted amino acids are not identical). One possibility is that the naturally occurring differences in amino acid sequences, which coincidentally behave like human FAD mutations, caused the overproduction of Aβ1–42 in mammalian cells. In contrast, Psn-dependent γ-secretase activity in Drosophila S2 cells did not cause Aβ1–42 overproduction and the %Aβ42 was at normal level (~15%). The molecular mechanism of overproduction of Aβ1–42 caused by FAD-linked amino acid substitutions in human PS still remains unknown. However, our observation that overexpression of SC100/1716F mutant in S2 cells resulted in an enormous secretion of Aβ1–42 like in mammalian cells indicates that Psn-dependent γ-secretase activity in S2 cells retains the capacity to cleave the TMD sequence of βAPP at Aβ42 position. Another speculative idea is that the differences in the composition or structure of components, as well as in the three-dimensional structures, of PS complexes might have caused the differences in substrate recognition or cleavage sites. Alternatively, the difference in the composition and metabolism of membrane lipids between mammalian and Drosophila cells may underlie the distinct behaviors in γ-secretase activities, related to the unusual enzymatic characteristics of γ-secretase to take place within membranes. In fact, it has been shown that phosphatidylethanolamine is the predominant phospholipid in cellular membranes of Drosophila, whereas the major phospholipid in mammalian cells is phosphatidycholine (43). Genetic, biochemical and proteomic approaches to determine the components of PS complex in mammalian and S2 cells, as well as the extent to which γ-cleavage in vitro, will clarify these problems.

Acknowledgments—We thank Drs. M. Miura, H. Kanuka, and T. Igaki for kind suggestions and help in the culture of S2 cells and RNAi experiments. Drs. B. De Strooper, G. L. Boulianne, R. Kopan, and Y. Ihara for providing mouse embryonic fibroblasts lacking PS1 and PS2, Psn cDNA, NAE cDNA and anti-C4 antibody, respectively. Takeda Chemical Industries for continuous support for our studies, and R. Takikawa, T. Watabiki, and M. Tsuourka for helpful discussions and technical assistance.

REFERENCES

1. Selkoe, D. J. (2001) Physiol. Rev. 81, 741–766
2. Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., De Strooper, B., and Wadsworth, J. H. (1996) Nature 384, 509–5094
3. Seeger, M., Nordstedt, C., Petaneska, S., Kovacs, D. M., Gours, G. K., Hahne, S., Fraser, P., Levesque, L., Czemak, A. J., St. Jane, D. G., St. John, P. S., Sidisa, S. S., H9253, S. H., and Zaczek, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 24536–24541
4. Boulianne, G. L., Livne-Bar, I., Humphreys, J. M., Liang, Y., Lin, C., Rogaev, E. I., Shichman, S. H., and Wolfe, M. S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16467–16472
5. Fortini, M. E. (2002) J. Biol. Chem. 277, 673–674
6. Herrerama, A., Serrels, L., Annan, W., Colden, D., Schonhoj, L., and De Strooper, B. (2000) Nature 405, 461–462
7. Lee, Y. M., Xu, M., Lai, M. T., Huang, Q., DiMuzio-Mower, J., Sardana, M. K., Shi, X. P., Yin, K. C., Shaffer, J. A., and Gallard, S. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6138–6143
8. G. L., Boulianne, G., Lai, M. T., Huang, Q., DiMuzio-Mower, J., Levy, M., Cordell, B., Sardana, M. K., Shi, X. P., Yin, K. C., Shaffer, J. A., and Gallard, S. J. (2000) Nature 405, 689–694
9. Lee, Y. M., Xu, M., Lai, M. T., Huang, Q., DiMuzio-Mower, J., Harrison, T., Lelis, C., Shih, R. K., and Wolfe, M. S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 24535–24540
10. Li, Y. M., Lai, M. T., Xu, M., Huang, Q., DiMuzio-Mower, J., Sardana, M. K., Shi, X. P., Yin, K. C., Shaffer, J. A., and Gallard, S. J. (2000) Nature 405, 689–694
11. Li, Y. M., Xu, M., Lai, M. T., Huang, Q., DiMuzio-Mower, J., Harrison, T., Lelis, C., Shih, R. K., and Wolfe, M. S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 24535–24540
12. Efes, W. P., de la, T. S., Moore, C. L., Tsai, J. Y., Rahamti, T., Xia, W., Selkoe, D. J., and Wolfe, M. S. (2000) Nature 405, 6138–6143
13. Seiffert, D., Bradley, J. D., Rominger, C. M., Rominger, D. H., Yang, F., Harding, E. J., Wang, Q., Roach, A. H., Thompson, S. M., Hahne, M., Prakash, J. N., Prakash, A. P., Cathala, A., Hart, P. R., Robertson, D. W., Cordell, B., Stern, A. M., Olson, R. E., and Zaczek, R. (2000) J. Biol. Chem. 275, 34086–34091
14. Efes, W. P., de la, T. S., Moore, C. L., Tsai, J. Y., Rahamti, T., Xia, W., Selkoe, D. J., and Wolfe, M. S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2720–2725
15. Efes, W. P., de la, T. S., Moore, C. L., Tsai, J. Y., Rahamti, T., Xia, W., Selkoe, D. J., and Wolfe, M. S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2720–2725
16. Arduengo, P. M., Appleby, L., Chuang, P., and L’Herault, S. W. (1998) J. Cell Biol. 111, 3645–3654
17. Gus, Y., Levine-Bar, I., Zhou, L., and Boullanne, G. L. (1999) J. Neurosci. 19, 8435–8442
18. Lukinova, N. I., Rossova, V. Y., and Fortini, M. E. (1999) Genetics 153, 1789–1797
19. Tomita, T., Maruyama, K., Saida, T. C., Kame, H., Shinoka, K., Tomikawa, T., Nishizawa, K., Kondo, H., Nakashima, K., and Fujita, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2025–2030
20. Boullanne, G. L., Levine-Bar, I., Humphreys, J. M., Liang, Y., Liu, C., Rogaev, T., Watabiki, T., and Iwatsubo, unpublished observations.
22. Morohashi, Y., Hatano, N., Ohya, S., Takikawa, R., Watabiki, T., Takasugi, N., Imazumi, Y., Tomita, T., and Iwatsubo, T. (2002) J. Biol. Chem. 277, 14965–14975
23. Iwata, T., Tomita, T., Maruyama, K., and Iwatsubo, T. (2001) J. Biol. Chem. 276, 21678–21685
24. Schroeter, E. H., Kisslinger, J. A., and Kopan, R. (1998) Nature 393, 382–386
25. Tomita, T., Catayama, R., Takikawa, R., and Iwatsubo, T. (2002) FEBS Lett. 520, 117–121
26. Hirasaka, S., Kanuka, H., Shoji, S., Yoshikawa, S., Okano, H., and Miura, M. (1998) J. Cell Sci. 111, 667–673
27. Tomita, T., Tokuhro, S., Hashimoto, T., Aiba, K., Saito, T. C., Maruyama, K., and Iwatsubo, T. (1998) J. Biol. Chem. 273, 21153–21160
28. Asami-Odaka, A., Ishibashi, Y., Kikuchi, T., Kitada, C., and Suzuki, N. (1995) Biochemistry 34, 10272–10278
29. Iwatsubo, T., Odaka, A., Suzuki, N., Miazawa, T., Nakina, N., and Ibara, Y. (1994) Neuron 13, 45–53
30. Nowotny, P., Gorski, S. M., Han, S. W., Philips, K., Ray, W. J., Nowotny, V., Jones, C. J., Clark, R. F., Cagan, R. L., and Goate, A. M. (2000) Mol. Cell Neurosci. 15, 88–98
31. Thinakaran, G., Harris, C. L., Ratovitski, T., Davenport, F., Slunt, H. H., Price, D. L., Borchelt, D. R., and Sisodia, S. S. (1997) J. Biol. Chem. 272, 28415–28422
32. Steiner, H., Duff, K., Capell, A., Romig, H., Grim, M. G., Lincoln, S., Hardy, J., Ya, X., Picciano, M., Fechteler, K., Citron, M., Kopan, R., Pesold, B., Keck, S., Baader, M., Tomita, T., Iwatsubo, T., Baumeister, R., and Haass, C. (1999) J. Biol. Chem. 274, 28669–28673
33. Struhl, G., and Greenwald, I. (1999) Nature 398, 522–525
34. Ye, Y., Lukinova, N., and Fortini, M. E. (1999) Nature 398, 525–529
35. Struhl, G., and Adachi, A. (2000) Mol. Cell 6, 625–636
36. Rosen, D. R., Martin-Morris, L., Luo, L. Q., and White, K. (1990) Proc. Natl. Acad. Sci. U. S. A. 86, 4749–4752
37. Foss, A., Bruckner, B., Czech, C., Masters, C. L., Beyreuther, K., and Paro, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13703–13708
38. Lichtenthaler, S. F., Wang, R., Grimm, H., Ulson, S. N., Masters, C. L., and Beyreuther, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3053–3058
39. Leimer, U., Lun, K., Romig, H., Walter, J., Grunberg, J., Brand, M., and Haass, C. (1999) Biochemistry 38, 13622–13629
40. Okochi, M., Eimer, S., Bottcher, A., Baumeister, R., Romig, H., Walter, J., Capell, A., Steiner, H., and Haass, C. (2000) J. Biol. Chem. 275, 40925–40932
41. Ha, Y., Ye, Y., and Fortini, M. E. (2002) Dev. Cell 2, 69–78
42. Francis, R., McGrath, G., Zhang, J., Bddy, D. A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hie, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Hines, C. S., Hiebsch, R., Ruble, C., Nye, J. S., and Curtis, D. (2002) Dev. Cell 3, 85–97
43. Jones, H. E., Harwood, J. L., Bowen, I. D., and Griffiths, G. (1992) Lipids 27, 984–987

E., and St. George-Hyslop, P. (1997) Neurouropt 8, 1025–1029