The Journal of Neuroscience, December 15, 1999, *19*(24):10627–10634

C Terminus of Presenilin Is Required for Overproduction of Amyloidogenic Aβ42 through Stabilization and Endoproteolysis of Presenilin

Taisuke Tomita,[1] Rie Takikawa,[1] Akihiko Koyama,[1] Yuichi Morohashi,[1] Nobumasa Takasugi,[1] Takaomi C. Saido,[2] Kei Maruyama,[3] and Takeshi Iwatsubo[1]

[1]Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo Bunkyoku Tokyo 113–0033, Japan, [2]Laboratory for Proteolytic Neuroscience, Brain Science Institute, RIKEN, Wako, Saitama 351-0198, Japan, and [3]Laboratory of Molecular Biology, Tokyo Institute of Psychiatry, Kamikitazawa, Setagayaku, Tokyo 156-8585, Japan

Mutations in presenilin (PS) genes cause early onset familial Alzheimer's disease (FAD) by increasing production of the amyloidogenic form of amyloid β peptides ending at residue 42 (Aβ42). To identify a PS subdomain responsible for overproduction of Aβ42, we analyzed neuro2a cell lines expressing modified forms of PS2 that harbor an N141I FAD mutation. Deletion or addition of amino acids at the C terminus and Ile448 substitution in PS2 with the N141I FAD mutation abrogated the increase in Aβ42 secretion, and Aβ42 overproduction was dependent on the stabilization and endoproteolysis of PS2. The same C-terminal modifications in PS1 produced similar effects. Hence, we suggest that the C terminus of PS plays a crucial role in the overproduction of Aβ42 through stabilization of endoproteolytic PS derivatives and that these derivatives may be the pathologically active species of PS that cause FAD.

Key words: presenilin 2; presenilin 1; C terminus; amyloid β peptide; Aβ42; endoproteolysis; stabilization; familial Alzheimer's disease

Alzheimer's disease (AD) is characterized pathologically by a massive deposition of amyloid β peptides (Aβ), which are proteolytically produced from β-amyloid precursor proteins (βAPP) through two sequential cleavages by as yet unidentified proteases termed the β- and γ-secretases (Selkoe, 1991, 1994). Two major forms of Aβ have distinct C termini ending at the 40th and 42nd residues (Aβ40 and Aβ42, respectively), which are differentially cleaved by γ-secretase(s) (Suzuki et al., 1994). Aβ42 aggregates much faster than Aβ40 *in vitro* (Jarrett and Lansbury, 1993), and Aβ42 is the initially and predominantly deposited Aβ species in the brains of patients with AD and Down's syndrome (Iwatsubo et al., 1994, 1995). Moreover, missense mutations in βAPP genes, a rare cause of familial AD (FAD), lead to increased production of Aβ42, strongly implicating Aβ42 in the pathogenesis of AD (Suzuki et al., 1994).

Presenilin (PS) 1 (Sherrington et al., 1995) and PS2 (Levy-Lahad et al., 1995) genes were identified as the major causative genes for early onset FAD that encode homologous polytopic membrane proteins spanning the membrane eight times (Doan et al., 1996; Li and Greenwald, 1998). Although a major proportion of nascent PS is rapidly degraded (Kim et al., 1997), a small fraction of PS is stabilized and undergoes endoproteolysis, resulting in a heterodimeric complex of N- and C-terminal derivatives (NTF and CTF, respectively) (Thinakaran et al., 1996; Capell et al., 1998) with an unusually long half-life (Thinakaran et al., 1996, 1997; Ratovitski et al., 1997). Overexpression of exogenous PS results in the replacement of endogenous PS fragments, suggesting that stabilization of PS is a saturable process competing for a limiting cellular factor (Thinakaran et al., 1996, 1997).

The finding that ablation of PS1 in mice dramatically decreased γ-cleavage of βAPP indicated that PS1 physiologically serves as a coactivator of γ-secretase (De Strooper et al., 1998). Moreover, data from studies in *Caenorhabditis elegans* (Levitan and Greenwald, 1995; Baumeister et al., 1997), PS1 knock-out mice (Shen et al., 1997; Wong et al., 1997; De Strooper et al., 1999), and *Drosophila melanogaster* (Song et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999) suggest that PS1 facilitates *Notch* signaling by activating a γ-secretase-like protease to release Notch intracellular domain (NICD), which activates transcription in nucleus.

More than 50 missense mutations in PS1, and two in PS2, have been identified in FAD pedigrees (Hardy, 1997). Accumulating data suggest that PS mutations cause AD by promoting the secretion of Aβ42 (Borchelt et al., 1996; Duff et al., 1996; Citron et al., 1997; Tomita et al., 1997), although the mechanism whereby mutant (mt) PS leads to the increased production of Aβ42 remains unknown.

We recently reported that NTF of FAD mt PS2 alone cannot promote secretion of Aβ42 (Tomita et al., 1998), and others showed that NTF of mt PS1 also does not enhance Aβ42 production (Citron et al., 1998; Steiner et al., 1998). These data prompted us to postulate that a subdomain in the PS C terminus mediates Aβ42 overproduction and to undertake molecular dissection studies to identify this subdomain.

Received July 14, 1999; revised Sept. 10, 1999; accepted Sept. 29, 1999.

This work was supported by Grants-in-Aid from the Ministry of Health and Welfare, the Ministry of Education, Science, Culture and Sports, and CREST of Japan Science and Technology Corporation, Japan. We thank Drs. J. Q. Trojanowski for helpful comments on this manuscript, H. R. Kaback for valuable suggestions on the structure and stability of membrane proteins, Hiroshi Iwata for help in immunoprecipitation experiments, and Takeda Chemical Industries for continuous support.

Correspondence should be addressed to Dr. Takeshi Iwatsubo, Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo 7-3-1 Bunkyoku Hongo Tokyo 113-0033, Japan. E-mail: iwatsubo@mol.f.u-tokyo.ac.jp.

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MATERIALS AND METHODS

Construction of expression plasmids. A full-length cDNA encoding wild-type (wt) or N141I FAD mt human PS2 was obtained as described (Tomita et al., 1997, 1998). cDNAs encoding C-terminally modified wt or mt PS2 were generated by PCR using *Pfu* polymerase (Stratagene, La Jolla, CA), and the following oligonucleotides were used as PCR primers: 5′-CCGGGATCCAGACCTCTCTGCGGCCCCAAG-3′ as a sense primer, 5′-CCGGATCCCTACTTCTTGAACACAGC-3′ for PS2/411stop, 5′-CTGCTCGAGCTACAGGGTGTCCATGAA-3′ for PS2/441stop, 5′-CCGGAATTCTACTGATGGGAGGCCAG-3′ for PS2/445stop, 5′-GGGCTCGAGTCAGATGTAGGCCTGATGGGA-3′ for PS2/L446A, 5′-ACACCAGAATTCTCAGATGGCGAGCTGATGGGA-3′ for PS2/Y447A, 5′-ACACCAGAATTCTCAGGCGTAGAGCTGATGGGA-3′ for PS2/I448A, 5′-CCGGAATTCTAGACGTAGAGCTGATGGGA-3′ for PS2/I448V, 5′-CCGGAATTCTAGAAGTAGAGCTGATGGGA-3′ for PS2/I448F, 5′-CCGGAATTCTACCTGTAGAGCTGATGGGA-3′ for PS2/I448R, 5′-CCCGGGAATTCTAATGGTGATGGTGATGATGGATGTAGAGCTGATGGGA for PS2/CHis and 5′-CCCGGGAATTCTATATATATAATTGGTGACTGATGTAGAGCTGATGGGA for PS2/CDup as antisense primers, respectively. cDNAs encoding N-terminally truncated wt or mt PS2 were similarly generated by PCR using the following primers: 5′-GGCACTCGAGTGTAAAACTATACAACTGC as an antisense primer, 5′-CCGGATCCACCATGTCGGCCGAGAGC-3′ for PS2/dAS, and 5′-CCGGGATCCATGGAGGAAGAGCTGA-3′ for PS2/dN as sense primers, respectively. A full-length cDNA encoding wt human PS1 containing VRSQ motif was obtained by PCR from a normal human brain cDNA library, and the P267S PS1 mutation was introduced by the dU-template method as described previously (Tomita et al., 1997). cDNAs encoding C-terminally modified wt or mt PS1 were generated by PCR using the following primers: 5′-CCCAAGCTTGCCACCATGACAGAGTTACCT-3′ as a sense primer, 5′-CCCGGGAATTCTATAATTGGTCCATAAA-3′ for PS1/460stop, and 5′-CCCGGGAATTCTAGCGATAAAATTGATG-3′ for PS1/I467R as antisense primers, respectively. Schematic depictions of truncated and/or mutated PS derivatives are shown in Figure 1. All constructs were sequenced using Thermosequenase (Amersham, Arlington Heights, IL) on an automated sequencer (Li-Cor, Lincoln, NE) as described (Tomita et al., 1997, 1998).

Cell culture and transfection. Mouse neuro2a (N2a) cells were maintained in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin at 37°C in 5% CO_2 atmosphere as described (Tomita et al., 1997, 1998). Stable N2a cell lines were generated by transfecting the cDNAs in pcDNA3 vector using Lipofectamine (Life Technologies, Gaithersburg, MD) and selection in DMEM containing G418 (Life Technologies) at 500 μg/ml. Stable cell lines were analyzed at polyclonal stages unless otherwise stated.

Antibodies, immunoblot analysis, and cycloheximide treatment. The following rabbit polyclonal antibodies were used: anti-G2N2 against glutathione *S*-transferase (GST) fused to amino acids 2–84 of human PS2, anti-G2L against GST fused to amino acids 301–361 of human PS2, (Tomita et al., 1998), anti-PS1N against amino acids 1–22 of human PS1 (Tomita et al., 1997), and anti-G1L3 against GST fused to amino acids 297–379 of human PS1.

Cells were lysed in 2% SDS sample buffer and briefly sonicated. The samples were separated by SDS-PAGE without previous heating, transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with each of the anti-PS antibodies as described (Tomita et al., 1997, 1998). The immunoblots were developed using an ECL system (Amersham).

To evaluate the half-lives of transfected PS proteins and fragments thereof by blocking total cellular protein synthesis, cultured cells were treated with cycloheximide (30 μg/ml) for 4, 10, 12, or 24 hr and then analyzed by immunoblotting with appropriate PS antibodies.

The capacity of transfected PS2 derivatives to replace endogenous PS1 was examined by immunoblotting cell lysates with anti-PS1N or anti-G1L3 antibodies that react with both human and mouse PS1.

Quantitation of Aβ by two-site ELISAs. Two-site ELISAs that specifically detect the C terminus of Aβ were used. 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, but does not react with the 3 kDa fragment (p3) beginning at the Leu-17 residue of Aβ (Fukumoto et al., 1999). BA27 and BC05, monoclonal antibodies that specifically recognize the C termini of Aβ40 and Aβ42, respectively, were conjugated with horseradish peroxidase and used as detector antibodies. The specificity and sensitivity of these ELISAs have been characterized previously (Asami-Odaka et al., 1995). Culture media were collected after an incubation period of 24 hr and subjected to BNT77/BA27 or BC05 ELISAs as described (Tomita et al., 1997, 1998). Four independent measurements in duplicate were performed for each clone.

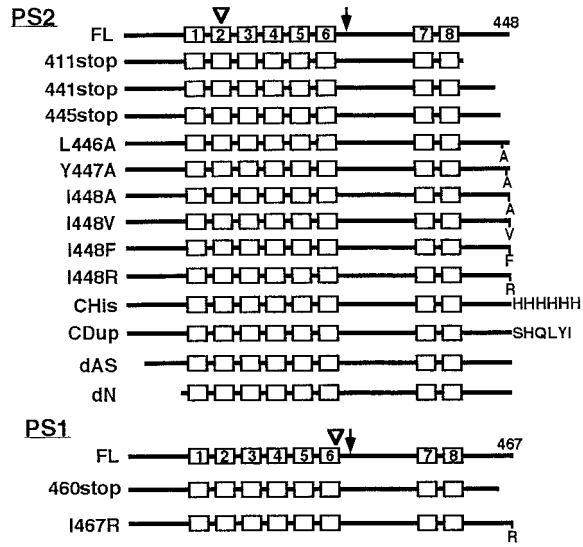

Figure 1. Schematic depictions of modified presenilins. Schematic representations of truncated or modified forms of PS2 (*top*) or PS1 (*bottom*) encoded by the cDNAs used in this study are shown. The names of cDNAs are indicated at the *left* of each *bar*, and *squares* with *numbers* represent putative TM domains. *Open arrowheads* on each *bar* show the location of amino acid substitutions linked to FAD (i.e., N141I in PS2 and P267S in PS1), and *arrows* between the TM 6 and 7 domains represent the sites of endoproteolytic processing. Amino acid substitution or addition at the C terminus of PS2 are shown *below* or at the *right side* of each *bar*, respectively.

Quantitation of Aβ by immunoprecipitation. Quantitation of Aβ by immunoprecipitation was performed according to the previously described method (Sudoh et al., 1998), with some modifications. Briefly, confluent N2a cells stably expressing PS2 or its derivatives were cultured in DMEM containing fetal bovine serum for 36 hr. Conditioned media (4.5 ml) were incubated with 2 μg of BNT77, 5 μl of goat IgG against mouse IgG, IgA, and IgM (Cappel, West Chester, PA), and 100 μl of 25% protein G agarose (Life Technologies) at 4°C for 12 hr on a rotary shaker. The immunoprecipitates were spun down, boiled in 2% SDS sample buffer, separated by SDS-PAGE on a 16.5% Tris/Tricine gel, and then blotted to a Hybond-ECL membrane filter (Amersham). After boiling in PBS, the membrane was probed with BA27 or BC05 (8 μg/ml, respectively) and then detected by the ECL system as described above.

RESULTS

A small deletion at the C terminus of mt PS2 abrogates increased secretion of Aβ42

We previously showed that C-terminally truncated PS2 harboring the N141I FAD mutation (mt PS2) corresponding to endoproteolytic NTF (terminating at amino acid residue 303; 303stop), or retaining the entire sixth loop but truncated at the putative seventh transmembrane (TM) domain (388stop), lost the capacity to increase secretion of Aβ42 by N2a cells stably overexpressing these proteins (Tomita et al., 1998). To define the minimal PS2 C-terminal region required for the overproduction of Aβ42, we constructed cDNAs encoding mt PS2 (full-length, 448 residues) with the following C-terminal deletions ending at residues 411 (PS2/411stop), 441 (PS2/441stop), or 445 (PS2/445 stop) as shown in Figure 1. Stably transfected N2a cells expressing these constructs were examined to measure the secretion of Aβ42 by Aβ C-terminal-specific ELISAs. The percentage Aβ42 (%Aβ42) as a

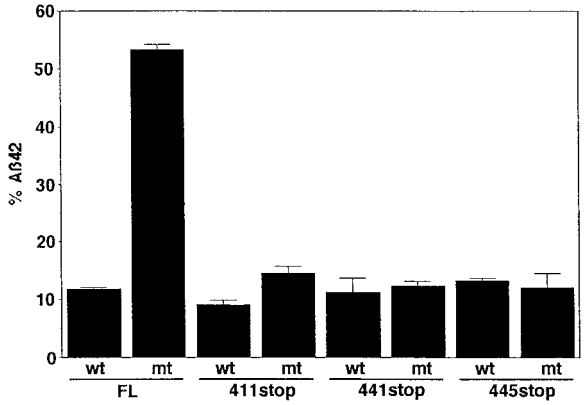

Figure 2. Percentages of Aβ42 secreted from cells expressing C-terminally truncated PS2. Percentages of Aβx-42 as a fraction of total Aβ (= Aβx-40 + Aβx-42) (%Aβ42) secreted from N2a cells stably transfected with full-length (*FL*) or C-terminally truncated (*411stop*, *441stop*, and *445stop*) PS2 genes with (*mt*) or without (*wt*) N141I FAD mutation quantitated by two-site ELISAs. Mean values ± SE in four independent experiments are shown. Transfected PS2 cDNAs are indicated below the *columns*.

fraction of total Aβ (= Aβx-40 + Aβx-42) secreted by cells stably expressing mt PS2/411stop, PS2/441stop or PS2/445stop was ~10%, and this was similar to the %Aβ42 secreted by cells expressing full-length (FL), wt PS2 or wt PS2/411stop, PS2/441stop, or PS2/445stop, whereas the %Aβ42 secreted from cells expressing FL mt PS2 was constantly elevated to ~35–55% as previously documented (Tomita et al., 1997, 1998) (Fig. 2). The total amounts of secreted Aβ from cells transfected with these C-terminally truncated PS2 as determined by ELISA were comparable to those with FL PS2 (data not shown).

Effects of substitution of the C-terminal residues of mt PS2 on Aβ42 production

Because truncation of three amino acid residues at the C terminus of mt PS2 [i.e., Leu (L) 446, Tyr (Y) 447, and Ile (I) 448] completely inhibited the increase in secretion of Aβ42, we next replaced each of these single residues with Ala and examined their effects on Aβ42 secreted by N2a cells, to determine whether one or more of these three residues critically affect the production of Aβ42. mt PS2/L446A and PS2/Y447A increased the %Aβ42 to comparable levels seen in N2a cells with FL mt PS2 (~45–55%), whereas %Aβ42 from cells expressing mt PS2/I448A was ~25%, which was at an intermediate level between those detected in the N2a cells with FL wt and mt PS2 (Fig. 3*A*).

We then focused on the role of residue I448, which is unusually hydrophobic for a C-terminal residue oriented at the cytoplasmic side, on Aβ42 secretion and examined the effects that resulted from replacing this residue with amino acids having different properties, i.e., Val (V), which is similarly hydrophobic but has a slightly shorter carbon chain, Phe (F), which also is hydrophobic but harbors an aromatic ring, or Arg (R), which is hydrophilic with positive charges. mt PS2/I448V and PS2/I448F enhanced secretion of Aβ42 from N2a cells at comparable levels to those in N2a cells expressing FL mt PS2. In sharp contrast, %Aβ42 secreted from cells expressing mt PS2/I448R was ~10%, which was similar to levels in cells with wt PS2 (Fig. 3*B*).

We further examined the secretion of Aβ40 and Aβ42 from cells transfected with these C-terminally substituted PS2 by immunoprecipitation with BNT77 and immunoblotting with BA27 and BC05, respectively (Fig. 3*C*). In N2a cells expressing FL wt

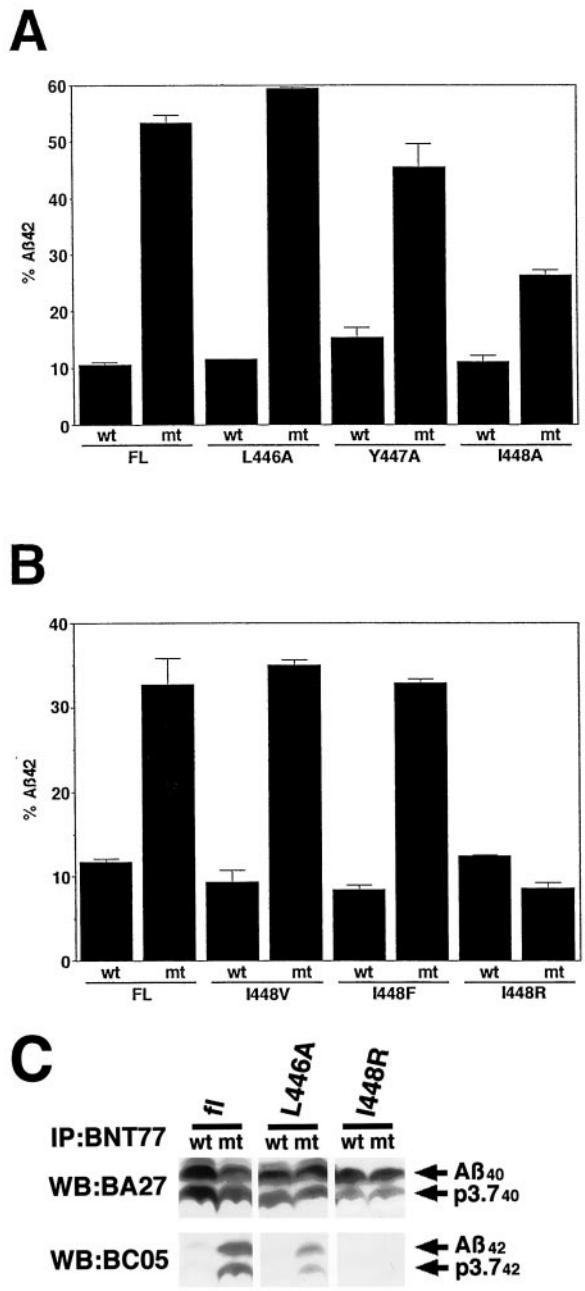

Figure 3. Percentages of Aβ42 secreted from cells expressing PS2 with amino acid substitutions at the C terminus. *A*, *B*, Percentages of Aβx-42 as a fraction of total Aβ (= Aβx-40 + Aβx-42) (%Aβ42) secreted from N2a cells stably transfected with PS2 genes with Ala substitution at Leu446 (*L446A*), Tyr447 (*Y447A*), or Ile448 (*I448A*) (*A*) or substitution at Ile448 to Val (*I448V*), Phe (*I448F*), or Arg (*I448R*) (*B*) with (*mt*) or without (*wt*) N141I FAD mutation quantitated by two-site ELISAs. *FL*, Full-length PS2 without amino acid substitution at the C terminus. Mean values ± SE in four independent experiments are shown. Transfected PS2 cDNAs are indicated *below* the *columns*. *C*, Immunoprecipitation of the 4 kDa $A\beta_{40}$ and $A\beta_{42}$ (*arrows*) as well as the 3.7 kDa $p3.7_{40}$ and $p3.7_{42}$ (*arrows*) from conditioned media of N2a cells stably expressing wt or mt FL PS2 (*fl*), PS2/L446A, or PS2/I448R by BNT77. Immunoprecipitates were visualized by Western blotting with BA27 or BC05.

PS2, robust 4 kDa (Fig. 3*C*, $A\beta_{40}$), and 3.7 kDa (Fig. 3*C*, $p3.7_{40}$) bands immunoreactive with BA27 were observed, whereas BC05 detected only a trace amount of 4 and 3.7 kDa Aβ peptides. In N2a cells expressing FL mt PS2, the intensities of the BA27-

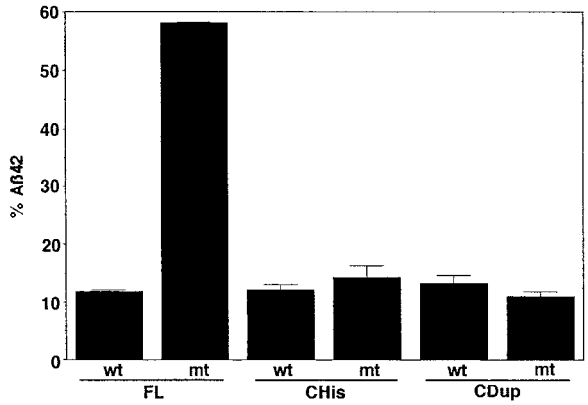

Figure 4. Percentages of Aβ42 secreted from cells expressing PS2 with addition of amino acids at the C terminus. Percentages of Aβx-42 as a fraction of total Aβ (= Aβx-40 + Aβx-42) (%Aβ42) secreted from N2a cells stably transfected with PS2 genes with additional amino acids at the C terminus (*CHis*, six His residues; *CDup*, duplication of the C-terminal six amino acid residues of PS2) with (*mt*) or without (*wt*) N141I FAD mutation quantitated by two-site ELISAs. *FL*, Full-length PS2. Mean values ± SE in four independent experiments are shown. Transfected PS2 cDNAs are indicated *below* the *columns*.

positive bands were weaker compared with those in cells expressing wt PS2, whereas stronger BC05-positive 4 kDa (Fig. 3*C*, $A\beta_{42}$) and 3.7 kDa (Fig. 3*C*, $p3.7_{42}$) bands were observed. The amounts of the BC05-positive bands were similarly increased in N2a cells expressing mt PS2/L446A, whereas no increase in the amount of BC05-positive bands was observed in cells expressing mt PS2/I448R. These data were in agreement with those obtained by ELISA, suggesting that the levels as well as ratios of Aβ40 and Aβ42 as determined by ELISA correctly represent those of bona fide Aβ peptides (the 4 kDa peptides may correspond to full-length Aβ and the 3.7 kDa peptides to N-terminally truncated Aβ, respectively). The total amounts of secreted Aβ from cells transfected with these C-terminally substituted PS2 as determined by ELISA also were at levels similar to those with wt PS2 (data not shown).

Addition of amino acids to the PS2 C terminus abolishes increased Aβ42 secretion

We next examined the effects of the addition of amino acid residues to the C terminus of mt PS2 on Aβ42 secretion. To this end, we used two different types of six amino acid long sequences: one contained six His residues (designated CHis), which is often used as an epitope tag, and the other was Ser-His-Gln-Leu-Tyr-Ile (designated CDup), which was identical to the last six amino acid residues of PS2. The latter was designed to determine whether the effect of mt PS2 on Aβ42 secretion is dependent on the integrity of the C-terminal amino acid sequences, regardless of the length of the C terminus. When stably transfected into N2a cells, neither mt PS2/CHis nor mt PS2/CDup retained the capacity to increase secretion of Aβ42, and the %Aβ42 was ~10%, which was similar to cells with wt PS2 (Fig. 4). The total amounts of secreted Aβ from cells transfected with PS2 with additional residues at the C terminus again were comparable to those with FL PS2 (data not shown).

Relationship between overproduction of Aβ42 and stabilization and endoproteolysis of PS2

It has not been definitively proven whether nascent holoproteins or stable complexes of endoproteolytic fragments (composed of

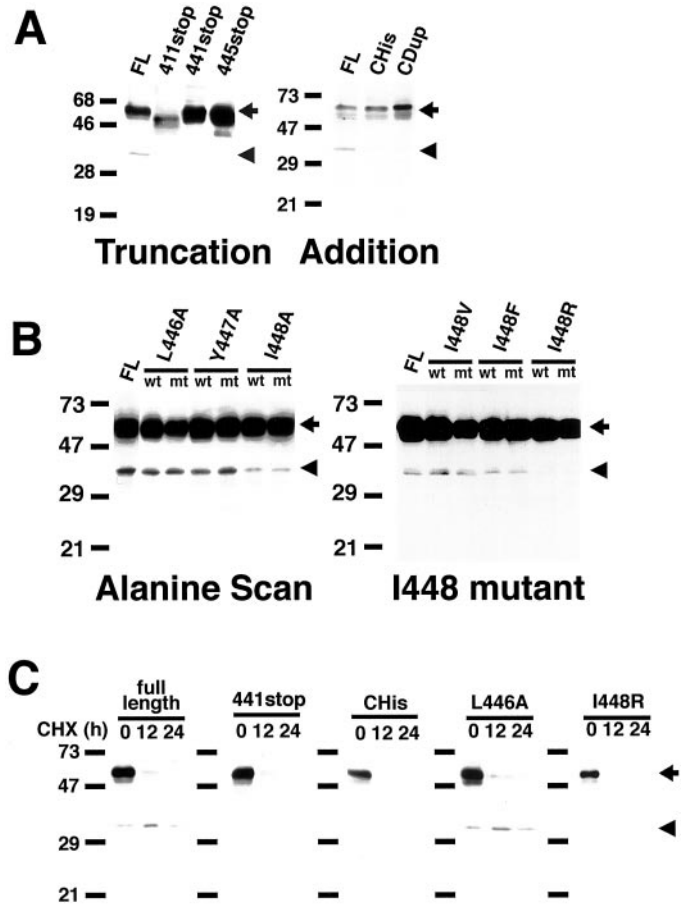

Figure 5. Expression, metabolism, and half-lives of PS2 derivatives in N2a stable cell lines. Western blot analysis of expression of PS2 with C-terminal truncation (*A*, *left panel*), addition (*A*, *right panel*), single amino acid substitution to Ala (*B*, *left panel*), or substitution at Ile448 (*B*, *right panel*) in stably transfected N2a cells. Cell lysates (20 μg protein) from N2a cells transfected with cDNAs encoding full-length (*FL*) or modified PS2 were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-G2N2 antibodies. PS2 cDNAs in *A* are on a wild-type basis, but similar results were obtained in N141I FAD mt PS2 (data not shown). The names of the transfected cDNA constructs are indicated at the *top* of each *lane*. *C*, Analysis of the half-lives of PS2 derivatives. Cells were grown in the presence of cycloheximide (*CHX*, 30 μg/ml) for 0, 12, or 24 hr and then harvested and analyzed as in *A* and *B*. The positions of FL PS2 and endoproteolytic NTF are marked by *arrows* and *arrowheads*, respectively. Molecular mass standards are shown in kilodaltons.

NTF and CTF) of PS are the biologically active forms of these proteins. To investigate the relationship between stabilization and endoproteolysis of the C-terminally modified forms of PS2 studied here and their pathological overproduction of Aβ42, we analyzed the expression and metabolism of these proteins in N2a stable cells by Western blots combined with cycloheximide treatment. All of the C-terminally truncated (i.e., PS2/411stop, PS2/441stop, or PS2/445stop) as well as tagged (i.e., PS2/CHis or PS2/CDup) PS2 that lacked the capacity to promote overproduction of Aβ42 also did not undergo endoproteolysis to give rise to a ~35 kDa NTF and a ~23 kDa CTF normally produced from FL PS2 (Fig. 5*A*, *arrowheads*), although abundant holoproteins of corresponding sizes were expressed (Fig. 5*A*, *arrows*). With respect to mt PS2 with C-terminal single amino acid substitution, mt PS2/L446A, PS2/Y447A, PS2/I448V, or PS2/I448F, which promoted Aβ42 secretion, were cleaved to form endoproteolytic

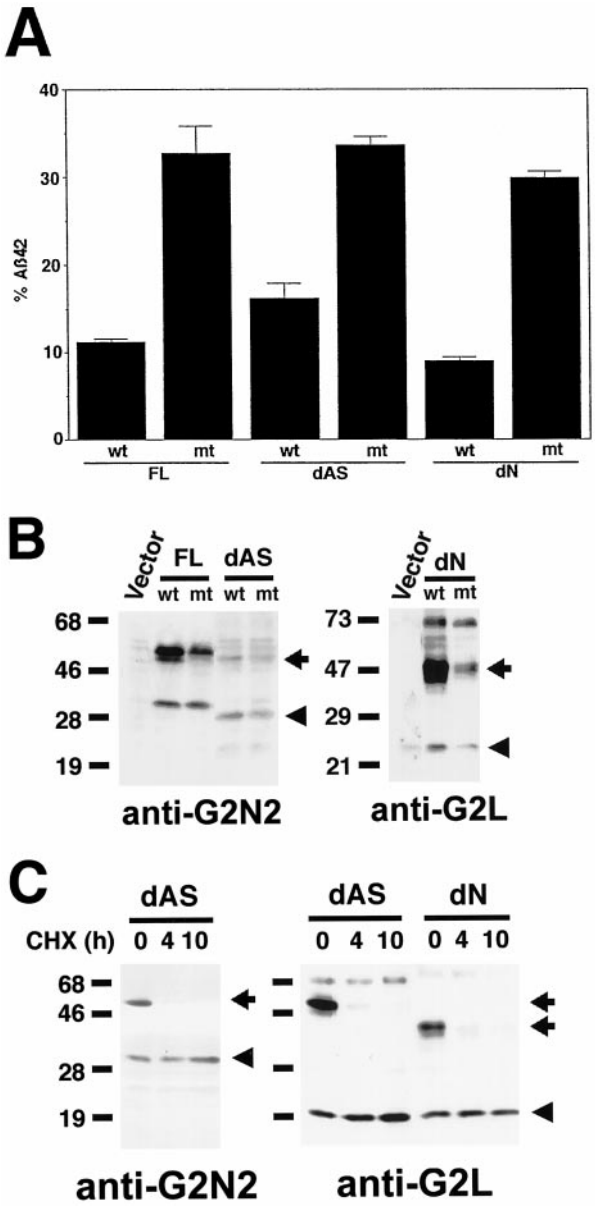

Figure 6. Secretion of Aβ42 and metabolism and stability of PS2 derivatives in cells expressing N-terminally truncated PS2. *A*, Percentages of Aβx-42 as a fraction of total Aβ (= Aβx-40 + Aβx-42) (%Aβ42) secreted from N2a cells stably transfected with N-terminally truncated PS2 genes with (*mt*) or without (*wt*) N141I FAD mutation (*mt*) quantitated by two-site ELISAs. *FL*, Full-length PS2; *dAS*, PS2 lacking the N-terminal acidic stretch corresponding to residues 1–20; *dN*, PS2 lacking the entire N-terminal cytoplasmic domain corresponding to residues 1–75. Mean values ± SE in four independent experiments are shown. Transfected PS2 cDNAs are indicated *below* the *columns*. *B*, Western blot analysis of N-terminally truncated PS2 in stably transfected N2a cells. Cell lysates (20 μg protein) from N2a cells transfected with cDNAs encoding full-length (*FL*) or N-terminally truncated PS2 were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-G2N2 antibodies for dAS (*left panel*) and with anti-G2L for dN, which lacks the epitopes for anti-G2N2 (*right panel*). Note that correspondingly smaller holoproteins (*arrows* in *both panels*) and NTFs (*arrowhead* in the *left panel*) compared with those in cells with FL PS2 were detected and that transfection of dN gave rise to increased levels of CTF (*arrowhead* in *dN*; *right panel*) compared with those of endogenous CTF (*arrowhead* in *Vector*, *right panel*), indicating an effective generation of endoproteolytic fragments. *Vector*, Cells transfected with an empty pcDNA3 vector. *C*, Analysis of the half-lives of N-terminally truncated PS2. Cells were grown in the presence of cycloheximide (*CHX*) for 0, 4, or 10 hr and then harvested and analyzed as in *B*. The positions of holoproteins of PS2/dAS or /dN are marked by *arrows* (*both panels*), and endoproteolytic NTF (*left panel*) and CTF (*right panel*) are marked by *arrowheads*, respectively. The names of the transfected cDNA constructs are indicated at the *top* of each *lane*. Molecular mass standards are shown in kilodaltons.

fragments, i.e., NTF (Fig. 5*B*, *arrowheads*) as well as CTF (data not shown), whereas mt PS2/I448R lacking this property was not cleaved (Fig. 5*B*, *arrowheads*). mt PS2/I448A, which showed intermediate levels of Aβ42 overproduction, yielded moderate levels of endoproteolytic fragments (Fig. 5*B*, *left*, *arrowhead*). Western blots after cycloheximide pretreatment revealed that holoproteins of C-terminally modified PS2 were short-lived with half-lives of <12 hr (Fig. 5*C*, *arrow*), whether endoproteolysis occurs or not. In contrast, the endoproteolytic fragments, if any, derived from transfected PS2 [e.g., NTF indicated by *arrowhead* in L446A of Fig. 5*C*, as well as corresponding CTF (data not shown)] acquired extraordinarily long half-lives of >24 hr, as observed with fragments produced from FL PS2 (Fig. 5*C*). These results strongly suggest that the stable NTF/CTF complexes of mt PS are the pathologically active forms of PS that induce overproduction of Aβ42, and that the integrity of the C-terminal structure of PS is critical for the stabilization of these complexes and the endoproteolysis of PS.

N terminus of mt PS2 is dispensable for production of Aβ42 as well as stabilization and endoproteolysis of PS2

To gain insights into the role of the N terminus of PS2 in Aβ42 production as well as on the stabilization and endoproteolysis of PS2, we constructed cDNAs encoding two types of N-terminally truncated PS2, i.e., dAS lacking the N-terminal 20 residues encompassing the acidic stretch, and dN lacking the entire N-terminal cytoplasmic domain corresponding to residues 1–75. When stably expressed in N2a cells, PS2/dAS as well as PS2/dN with N141I FAD mutation both increased the %Aβ42 at levels similar to that of cells with FL mt PS2 (Fig. 6*A*), although the total amounts of secreted Aβ were not altered (data not shown). Western blot analysis showed that both PS2/dAS and PS2/dN undergo endoproteolysis, yielding smaller NTFs and a ~23 kDa CTF of the same size as that derived from cells with FL PS2 (Fig. 6*B*). Cycloheximide treatment demonstrated that these endoproteolytic fragments have long half-lives (>10 hr) (Fig. 6*C*, *arrowheads*), whereas their corresponding holoproteins are short-lived (Fig. 6*C*, *arrows*). Taken together, we conclude that the N terminus of PS2 is dispensable for overproduction of Aβ42 as well as for stabilization and endoproteolysis of PS2.

Replacement of endogenous PS1 by PS2 derivatives overexpressed in N2a cells

To determine whether various types of PS2 derivatives overexpressed in N2a cells replace endogenous PS, we selected single clonal cell lines stably expressing C- or N-terminally modified PS2 and examined the levels of endogenous PS1 in representative clones (Fig. 7). The amounts of ~30 kDa NTF and ~23 kDa CTF of endogenous mouse PS1 were decreased in cells expressing FL wt or mt PS2, as well as in cells expressing PS2/I446A or PS2/dN, whereas they were maintained at levels similar to those in mock transfectants in cells expressing wt or mt PS2/445stop, PS2/I448R, or PS2/CHis. These results clearly showed that the pathologically active forms of mt PS2 that promote overproduction of Aβ42 can

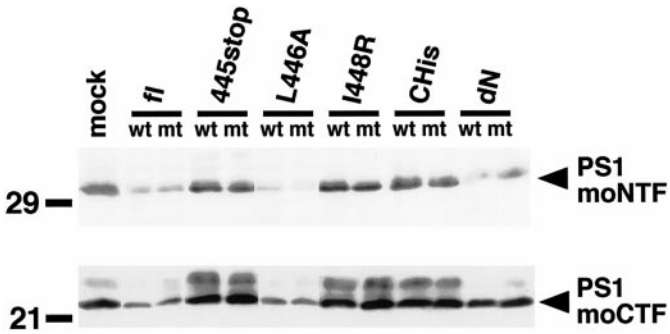

Figure 7. Replacement of endogenous PS1 by PS2 derivatives overexpressed in N2a cells. Western blot analysis of the levels of NTF (*PS1 moNTF*, *top panel*) and CTF (*PS1 moCTF*, *bottom panel*) of endogenous mouse PS1 in N2a cells stably transfected with C- or N-terminally modified PS2. Cell lysates (20 μg protein) from N2a cells transfected with each cDNA were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-PS1N (*top panel*) or anti-G1L3 (*bottom panel*) antibodies. The names of the transfected cDNA constructs are indicated at the *top* of each *lane*. *mock*, Cells transfected with an empty vector alone. Molecular mass standards are shown in kilodaltons.

replace endogenous PS, whereas replacement does not occur with C-terminally modified PS2 lacking the Aβ42-promoting effects.

Integrity of the C terminus of mt PS1 is required for overproduction of Aβ42

To determine whether our conclusions regarding the role of PS C terminus are applicable to PS1, we constructed cDNAs encoding wt and Pro267Ser (P267S) mt PS1 lacking the last seven amino acid residues (PS1/460stop), and FL PS1 with the C-terminal residue Ile467 replaced by Arg (PS1/I467R), and stably expressed them in N2a cells. Consistent with the results obtained with PS2, FL P267S mt PS1 increased the %Aβ42 by ~1.5-fold compared with that of wt PS1, whereas the %Aβ42 was not elevated in cells with mt PS1/460stop nor with mt PS1/I467R (Fig. 8*A*), and the total amounts of secreted Aβ were not altered (data not shown). These C-terminally modified PS1 proteins were expressed as holoproteins (Fig. 8*B*, *FL*) but they were cleaved to produce only trace amounts of endoproteolytic fragments (Fig. 8*B*, *huNTF*). These results confirmed that the integrity of the C-terminal structure of PS1 also is important for the overproduction of Aβ42 as well as for the stabilization and endoproteolysis of this protein similar to PS2.

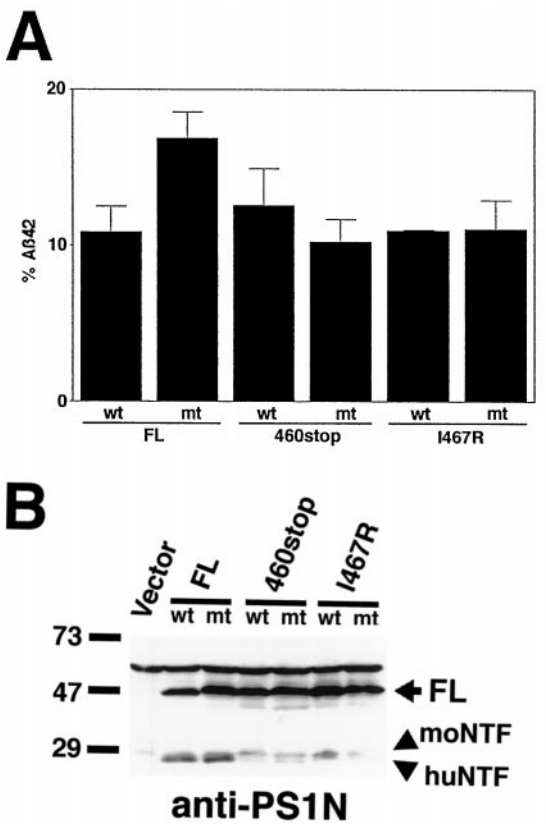

Figure 8. Secretion of Aβ42 and metabolism of PS1 derivatives in cells expressing C-terminally modified PS1. *A*, Percentages of Aβx-42 as a fraction of total Aβ (= Aβx-40 + Aβx-42) (%Aβ42) secreted from N2a cells stably transfected with cDNAs encoding full-length (*FL*) PS1, PS1 lacking the C-terminal 7 amino acid residues (*460stop*), or PS1 replaced at residue Ile467 with Arg (*I467R*) with (*mt*) or without (*wt*) P267S FAD mutation quantitated by two-site ELISAs. Mean values ± SE in four independent experiments are shown. Transfected PS1 cDNAs are indicated below the columns. *B*, Western blot analysis of C-terminally modified PS1 in stably transfected N2a cells. Cell lysates (20 μg protein) from N2a cells transfected with cDNAs encoding full-length (*FL*), C-terminally truncated (*460stop*) or substituted (*I467R*) PS1 were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-PS1N antibody. The approximate position of holoproteins is marked by *arrow* (*FL*), and endogenous murine NTF (*moNTF*) and human NTF (*huNTF*) derived from transfected PS1 with a slightly faster mobility are marked by *arrowheads*, respectively. The names of the transfected cDNA constructs are indicated at the *top* of each *lane*. Molecular mass standards are shown in kilodaltons.

DISCUSSION

In this study, we have clearly shown that (1) the integrity of the C-terminal structure of PS is required for the ability of FAD mt PS to increase secretion of amyloidogenic Aβ42; (2) subtle modifications of the C terminus of PS, especially those eliminating the hydrophobicity of the C-terminal Ile residue, abrogate the endoproteolysis of PS; (3) the pathological activity of FAD mt PS to increase Aβ42 is most likely mediated by stabilized complexes of endoproteolytic fragments of PS; and (4) the N terminus of PS, in contrast to the C terminus, is dispensable for the overproduction of Aβ42, as well as for the stabilization or endoproteolysis of PS.

The mechanisms whereby PS proteins mediate their physiological as well as pathological functions remain elusive. Here we showed a strict parallel between the overproduction of Aβ42 and the stabilization and endoproteolysis of PS in a series of PS proteins harboring subtle modifications at the C terminus. Taken together with recent observations on PS1 (Steiner et al., 1998) or chimeric molecules of PS1NTF–PS2CTF (Saura et al., 1999), our data support the notion that stabilized fragments of mt PS are responsible for the pathological augumentation of Aβ42 production, although the precise role of endoproteolytic cleavage in PS function remains unknown. FAD-associated mt PS1 lacking exon 9, which escapes cleavage within the sixth loop domain, is stabilized (Ratovitski et al., 1997), incorporated into high molecular weight stable complexes (Capell et al., 1998), and acquires the abnormal ability to promote overproduction of Aβ42 caused by an amino acid substitution at the splice junction site (Steiner et al., 1999), suggesting that endoproteolysis merely represents a molecular signature that indicates the occurrence of stabilization but is not mandatory for the function of PS.

Very recently, it was reported that mutating either of the two conserved Asp residues in the sixth and seventh TM domains of PS1 substantially reduces γ-cleavage of βAPP as well as PS1 endoproteolysis, whereas these Asp-mutated PS1 species can

replace endogenous PS1 fragments, thereby eliminating the activity of PS1 in cells (Wolfe et al., 1999). It is conceivable that the two Asp residues are essential for the ability of PS1 to activate (or alternatively, to work as a) γ-secretase that is mediated by the stabilized form of PS, whereas the C terminus of PS plays a critical role in the formation of the stabilized complexes of PS, which in turn leads to increased secretion and deposition of Aβ42 in the FAD brains and cells. In this regard, it is particularly interesting that some of the loss-of-function SEL-12 mutants in *C. elegans* (Levitan and Greenwald, 1995) or *Drosophila* PS mutants (Struhl and Greenwald, 1999), which are incapable of facilitating Notch signaling, are truncated at the C terminus (e.g., within the putative seventh loop domain in the *ar133* mutant of SEL-12) (Levitan and Greenwald, 1995). Further studies will be needed to determine whether these C-terminally truncated PS homologs are stabilized, and whether stabilized complex of PS is required for γ (-like) cleavage of Notch or βAPP to release NICD or Aβ, respectively.

What then is the mechanistic role of the PS C terminus in the stabilization and function of PS proteins? Holoproteins of C-terminally modified PS polypeptides studied here were robustly expressed, and as we have previously confirmed with NTFs of PS2, they were inserted into membranes and localized to endoplasmic reticulum [Tomita et al. (1998) and our unpublished observations], despite their relatively short half-lives. One possible mechanism would be that the highly hydrophobic cytoplasmic tails of PS (i.e., -Leu-Tyr-Ile for PS2 and -Phe-Tyr-Ile for PS1) serve as the binding domain of some interacting proteins that are required for the stabilization of PS. Notably, PDZ domain-containing proteins are known to bind to the C terminus of transmembrane proteins with specific motifs such as Ser/Thr-X-Val/Ile (for group I PDZ domains) or hydrophobic amino acids at positions −2 and 0 (for group II) (Songyang et al., 1997), the latter being very similar to those of the PS C terminus noted above. In fact, deletion, mutation, or addition of C-terminal amino acid residues abolishes their binding to PDZ domains (Saras et al., 1997). Taken together with the fact that the stabilization of PS is regulated by competition for limiting cellular factor(s), it is tempting to speculate that interacting proteins that bind to the C terminus of PS are the determinants for the stabilization and replacement of PS that are vital to the function of PS.

Another possibility would be that the C terminus of PS per se plays an important role in the proper folding or conformation of PS required for the stabilization and/or function of PS proteins. Although little is known about the roles of different domains of polytopic membrane proteins in the stabilization of polypeptides, data from deletion studies in *lac* permease of *Escherichia coli* may have interesting implications for our findings. *Lac* permease is a polytopic membrane protein that spans the membrane 12 times, with the N and C termini oriented to the cytoplasmic side. Kaback and colleagues (Roepe et al., 1989) have shown that a total ablation of the 17 amino acid C-terminal cytoplasmic tail of *lac* permease does not have any decremental effects on the stabilization and function of this protein, whereas additional deletion of five more amino acids constituting the C-terminal portion of the 12th TM domain drastically destabilized the protein after insertion into the membrane. It was also reported that removal of the C-terminal tails of two other polytopic membrane proteins, bacteriorhodopsin (Huang et al., 1981) or melibiose permease (Botfield and Wilson, 1989), did not affect their functions. Thus, it is highly likely that the C terminus of PS, especially the hydrophobic C-terminal residues, plays a unique role in the stabilization of PS as a polytopic membrane protein. The precise mechanism whereby the C terminus stabilizes PS is unknown at present; it may bind to some important domain within the TM or loop structures of PS to maintain a structure required for stabilization or function, or alternatively, the hydrophobic C terminus may interact with, or is anchored within, membranes ensuring the proper conformation of PS. It is also possible that the conformation of the whole protein maintained by the C terminus allows the binding of a limiting factor (protein) with some portion(s) of PS outside the C terminus.

The hydrophobic C terminus of PS could be a therapeutic target for the treatment of FAD because the development of small compounds that bind to the C terminus of PS and mimic these modifications may destabilize and reduce the total amount of "functional" mt PS proteins, which promote overproduction of Aβ42, in the brains of patients with FAD linked to PS mutations. Future studies of the roles of the C terminus of PS will pave the way for understanding the pathomechanisms as well as for the development of novel therapeutic strategies for FAD and possibly sporadic AD.

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