Evidence That the “NF” Motif in Transmembrane Domain 4 of Presenilin 1 Is Critical for Binding with PEN-2*

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Presenilin 1 (PS1) and PS2, nicastrin, anterior pharynx defective phenotype 1 (APH-1), and PS enhancer 2 (PEN-2) mediate the intramembranous, $\gamma$-secretase cleavage of $\beta$-amyloid precursor protein (APP), Notch, and a variety of type 1 membrane proteins. We previously demonstrated that PEN-2 is critical for promoting endoproteolysis of PS1 and that the proximal two-thirds of transmembrane domain (TMD) 1 of PEN-2 is required for binding with PS1. In this study, we sought to identify the structural domains of PS1 that are necessary for binding with PEN-2.

To address this issue, we generated a series of constructs encoding PS1 mutants harboring deletions or replacements of specific TMDs of PS1-NTF, and examined the effects of encoded molecules on interactions with PEN-2, stabilization and endoproteolysis of PS1, and $\gamma$-secretase activity. We now show that PS1 TMDs 1 and 2 and the intervening hydrophilic loop are dispensable for binding to PEN-2. Furthermore, analysis of chimeric PS1 molecules that harbor replacements of each TMD with corresponding transmembrane segments from the sterol regulatory element-binding protein cleavage activating protein (SCAP) revealed that the PS1-SCAP TMD4 mutant failed to coimmunoprecipitate endogenous PEN-2, strongly suggesting that the fourth TMD of PS1 is necessary for interaction with PEN-2.

Further mutational analyses revealed that the “NF” sequence within the TMD4 of PS1 is the minimal motif that is required for binding with PEN-2, promoting PS1 endoproteolysis and $\gamma$-secretase activity.

The $\gamma$-secretase complex mediates the intramembranous processing of a number of type 1 membrane proteins, including $\beta$-amyloid precursor protein (APP) and Notch (for review, see Ref. 1). The $\gamma$-secretase complex consists of presenilins (PS1 and PS2), nicastrin (NCT), anterior pharynx defective phenotype 1 (APH-1), and PS enhancer 2 (PEN-2) (for review, see Ref. 2). The demonstration that coexpression of these four molecules in Saccharomyces cerevisiae, an organism lacking any endogenous $\gamma$-secretase activity, is sufficient to reconstitute $\gamma$-secretase activity (3) has confirmed that these four proteins are the essential core components of the catalytic complex.

Several lines of evidence have emerged to indicate that the steady-state accumulation of each of the components of the complex is coordinately regulated, and in large part, dependent on the expression of other members of the complex (for review, see Ref. 4). Although the precise functional role of the individual components of the $\gamma$-secretase complex is still not clear, APH-1 and NCT apparently form a stable subcomplex that binds to, and stabilizes, newly synthesized PS1 (5, 6). This APH-1-NCT-PS1 complex then associates with PEN-2, resulting in the proteolytic conversion of $\approx42\text{-}50$-kDa PS1 holoprotein into $\approx27\text{-}30$-kDa $\mathrm{NH}_2$-terminal (NTF) and $\approx16\text{-}20$-kDa COOH-terminal (CTF) derivatives (5–9) that are the preponderant PS1-related polypeptides that accumulate in vivo (10–12). Recent studies have revealed that PEN-2 also stabilizes the PS1 heterodimer (13, 14). PEN-2, a 101-amino acid protein, contains two membrane spanning domains with the amino- and carboxyl-terminal domains facing the lumen (15, 16). At present, little information is available pertaining to the mechanism(s) by which PEN-2 promotes the endoproteolysis of PS1.

Identification of the binding sites for the molecular interactions between PS1 fragments and the other complex components have been topics of intense investigation. For example, mutational analyses of NCT and APH-1 have revealed that the proximal one-third of the long transmembrane domain (TMD) of NCT and the GXXXG motif of the TMD4 of APH-1 are critical for formation of the $\gamma$-secretase complex (17–19). In addition, Kaether et al. (20) demonstrated that the carboxy-terminal end of PS1 is necessary for interactions with the TMD of NCT. Very recently, we showed that the proximal two-thirds of the TMD1 of PEN-2 is critical for binding with PS1 and promoting PS1 endoproteolysis (14). However, the domain of PS1 that interacts with PEN-2 has remained unclear.

Presenilins are polytopic membrane proteins with 10 predicted hydrophobic segments. The most widely accepted topology model for PS1 is one in which the molecule spans the membrane eight times with amino and carboxyl termini as well as the large hydrophilic loop between TMDs 6 and 7 being cytosolic (21, 22). However, recent studies have offered support for a 9-TMD model for PS1 in which the carboxyl terminus is luminal (23). Notwithstanding the differences in the topology models, PS1 endoproteolysis unequivocally occurs within the large hydrophilic loop to generate stable NTF and CTF derivatives. Recent studies have revealed that solubilization of the $\gamma$-secretase complex in 1% dodecyl $\beta$-D-maltoside results in the formation of smaller, heterogeneous complexes; one of these contains NCT, APH-1, and PS1-CTF, whereas the other consists of PS1-NTF and PEN-2 (24). These results strongly suggested that PS1-NTF associates with PEN-2.

In the present study, we sought to identify a domain within the PS1-NTF that is required for binding with PEN-2. To address this issue, we generated a series of constructs encoding PS1 mutants harboring deletions or replacements of specific TMDs of PS1-NTF and tested the effects of these variants on the interaction with PEN-2, stabilization and...
endoproteolysis of PS1, and γ-secretase activity. We now show that TMDs 1 and 2 of PS1 and the intervening hydrophilic loop are dispensable for binding to PEN-2. Furthermore, analysis of chimeric PS1 molecules that harbor replacements of each transmembrane segment with topologically oriented transmembrane segments from the SREBP cleavage activating protein (SCAP) revealed that the PS1-SCAP TMD4 mutant failed to coimmunoprecipitate endogenous PEN-2. These results strongly suggest that the fourth TMD of PS1 is required for interaction with PEN-2. We successfully “rescued” PEN-2 binding, PS1 endoproteolysis, and γ-secretase activity of the PS1-SCAP TMD4 mutant by replacing SCAP sequences with varying segments of the authentic PS1 TMD4. These studies culminated with the finding that the “NF” motif within TMD4 of PS1 is critical for binding with PEN-2, promoting PS1 endoproteolysis and γ-secretase activity.

EXPERIMENTAL PROCEDURES

Constructs—The cDNAs encoding human PS1, NCT, CT11 epitope-tagged PEN-2, myc epitope-tagged APH-1-L, myc epitope-tagged Swedish variant APP695 (APP<sub>Swe</sub>), or myc epitope-tagged Notch<sub>ΔE</sub> were previously described (7, 25, 26). Constructs encoding various PS1-SCAP chimeras were generated using PCR-based mutagenesis. The boundaries of TMDs of PS1 and SCAP proteins were determined using the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing. Details of primer sequences and PCR conditions for the TMpred program. The sequences of each mutant cDNA were verified by sequencing.

Antibodies—The following antibodies previously described (7) were used for the study; PS1<sub>NT</sub> and αPS1loop antibodies are polyclonal antibodies that recognize residues 1–65 and 320–375 of PS1, respectively (10, 11); PNT-2 antibody was raised against a peptide corresponding to the NH<sub>2</sub>-terminal 26 amino acids of PEN-2 (a kind gift from Dr. Gopal Thinakaran (8)); CTM2 is a polyclonal antibody that was generated against a peptide of the carboxyl-terminal end of APH-1 fused with a myc-epitope tag (a kind gift from Dr. Gopal Thinakaran (14)); antibody 369, raised against a peptide corresponding to amino acids 649–695 of APP<sub>695</sub> (27).

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells and transformed fibroblasts derived from mouse embryo with homozygous deletions of PS1 (PS1<sup>+/−</sup>) (28) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen). Mouse neuroblastoma N2a cells were maintained in 50% Dulbecco’s modified Eagle’s medium and 50% Opti-MEM (Invitrogen) supplemented with 5% fetal bovine serum. Cells were transiently transfected with plasmid DNA using Lipofectamine PLUS (Invitrogen). cDNA encoding viral hemagglutinin (HA) was used to adjust the amount of transfected DNA. Where indicated, cycloheximide (30 µg/ml) was added for 0–6 h. To generate stable cell lines expressing either wild type or various PS1 mutants, PS1-deficient fibroblasts were cotransfected with 10 µg of transgenes (pAG32Zeo vector) and 100 ng of pIREShyg (Invitrogen) using the calcium phosphate method (29).

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RESULTS

PS1 TMDs 1 and 2 Are Dispensable for PEN-2 Binding—Using blue native-PAGE analysis, Fraering <em>et al.</em> (24) showed that solubilization of the γ-secretase complex with 1% dodecyl β-D-maltoside resulted in the generation of four heterogeneous complexes. Second-dimensional analysis of the complexes revealed that one complex consisted of nicas- trin, APH-1, and PS1-CTF, whereas the other contained PS1-NTF and PEN-2 (24). These results strongly suggested that the PS1-NTF is involved in binding with PEN-2.

To identify a domain within the PS1-NTF to which PEN-2 binds, we first examined the PS1 D<sub>M1,2</sub> mutant that harbors a deletion of TMDs 1 and 2 of PS1 and the intervening hydrophilic loop (Fig. 1A). This mutant has been reported to have a dominant-negative effect on γ-secretase activity (31, 32); APP-CTFs accumulate and γ-secretase cleavage of Notch is inhibited. We explored the possibility that the loss-of-function phenotype of the D<sub>M1,2</sub> mutant might be because of inability of this mutant to associate with PEN-2. To test this hypothesis, we performed coimmunoprecipitation experiments using stable N2a or 293 cell lines that stably express wild type PS1, the D<sub>M1,2</sub> PS1 mutant (32), or the D385A PS1 mutant (30). CHAPS-solubilized lysates of these cell lines were subject to immunoprecipitation with either PS1<sub>NT</sub> antibody (11) or the PNT-2 antibody, raised against a peptide corresponding to the NH<sub>2</sub>-terminal 26 amino acids of PEN-2 (8). As expected, PS1<sub>NT</sub> antibody immunoprecipitated both full-length PS1 and PS1-NTF from cells expressing wild type PS1 (Fig. 1B, lanes 5 and 7). Moreover, both D<sub>M1,2</sub> and D385A PS1 mutants accumulated as holoproteins and these mutant proteins were also immunoprecipitated by the PS1<sub>NT</sub> antibody (Fig. 1B, lanes 6 and 8). As previously described (8, 14), endogenous PEN-2 was coinmunoprecipitated with wild type PS1 under these conditions (Fig. 1C, lanes 5 and 7). Notably, endogenous PEN-2 was also coinmunoprecipitated with either the D<sub>M1,2</sub> or D385A PS1 mutants (Fig. 1C, lanes 6 and 8). We confirmed these findings using the PNT-2 antibody for immunoprecipitation. We show that PNT-2 antibody coimmunoprecipitates not only wild type PS1 holoprotein, and PS1-NTF (Fig. 1B, lanes 9 and 11), but also the unprocessed D<sub>M1,2</sub> and D385A PS1 mutant proteins (Fig. 1B, lanes 10 and 12, respectively). To establish the specificity of the coimmunoprecipitation assay, we examined the association of PEN-2 with the D<sub>M1,2</sub> or D385A PS1 mutants using an antibody, termed ABrI, raised against a sequence contained within BRI, an unrelated membrane protein. We now show that the ABrI antibody coimmunoprecipitated neither endogenous PEN-2 nor the D<sub>M1,2</sub> PS1 mutant (Fig. 1D, lane 3), suggesting that the observed
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FIGURE 1. Coimmunoprecipitation of the ΔM1,2 PS1 mutant with endogenous PEN-2. A, schematic diagram of the ΔM1,2 PS1 mutant that harbors deletion of TMDs 1 and 2 of PS1 and the intervening hydrophilic loop. B and C, HEK293 cells stably expressing wild type PS1 (lanes 1, 5, and 9) or the ΔM1,2 PS1 mutant (lanes 2, 6, and 10) or N2a cells expressing wild type PS1 (lanes 3, 7, and 11) or the D385A PS1 mutant (lanes 4, 8, and 12) were lysed in 1% CHAPS, and aliquots of lysates were immunoprecipitated with either PS1NT (lanes 5–8) or PNT-2 antibodies (lanes 9–12). The resulting immunoprecipitates (IP) as well as total detergent lysates corresponding to 1/20 of the volume used for immunoprecipitation (lanes 1–4; input) were resolved on 16.5% Tris/Tricine SDS-PAGE and probed with PS1NT antibodies (B) or PNT-2 antibodies (C). D, HEK293 cells stably expressing the ΔM1,2 PS1 mutant was lysed in 1% CHAPS and aliquots of lysates were immunoprecipitated with either PS1NT (lane 2) or unrelated Abri antibodies (lane 3). The immunoprecipitates and 10% of the input were resolved on Tris/Tricine SDS-PAGE and probed with PS1NT (upper panel) or PNT-2 antibodies (lower panel). In B–D, molecular mass markers are shown on the left in kDa. WB, Western blot; Fl., full-length.
interaction between the ΔM1,2 PS1 mutant and PEN-2 is not a result of nonspecific hydrophobic interactions between these molecules. Collectively, these results indicate that a domain of PS1 that includes TMDs 1 and 2 and the intervening hydrophilic loop are dispensable for binding to PEN-2.

**Identification of PS1 TMD4 as the PEN-2 Interacting Domain**—Having ruled out TMDs 1 and 2 of PS1 as PEN-2 binding sites, we turned our attention to TMDs 3–6 in the PS1-NTF. For these studies, we generated constructs that encode chimeras in which each TMD of PS1 was replaced with a corresponding domain of an unrelated, polytopic membrane protein, SCAP. Maintaining the topological orientation of each TMDs, we replaced PS1 TMD3 or TMD5 with the SCAP TMD2 and substituted PS1 TMD4 or TMD6 with the SCAP TMD1 (see Fig. 2A).

We stably transfected these PS1-SCAP TMD replacement mutant constructs (TMD3, TMD4, TMD5, and TMD6), as well as wild type human PS1 cDNA into PS1-deficient fibroblasts. Western blot analyses of

![FIGURE 2. Stable expression of PS1-SCAP TMD mutants. A, schematic diagram of PS1 and SCAP. To maintain the topological orientation, TMD3 or TMD5 of PS1 was replaced with the SCAP TMD2, whereas TMD4 or TMD6 of PS1 was substituted with the SCAP TMD1. B and C, stable cell lines expressing wild type PS1 (lane 2), TMD3 (lane 3), TMD4 (lane 4), TMD5 (lane 5), or TMD6 PS1-SCAP mutants (lane 6) were generated in PS1-deficient fibroblasts. Detergent lysates of these cell lines as well as parental cells (lanes 1 and 7) were prepared and analyzed by immunoblot with PS1NT (B) or PNT-2 antibodies (C). In B and C, molecular mass markers are shown on the left in kDa. WB, Western blot; FL, full-length.](image-url)
The failure of the PS1-SCAP TMD4 or TMD5 mutants to rescue PEN-2 levels could be explained by the instability of these variants. To test this notion, we treated parallel dishes of cells from each stable PS1-SCAP TMD5 mutant holoprotein was degraded rapidly, whereas the endoproteolytic derivatives were quite stable (Fig. 3A, lanes 5–8). In contrast, both the PS1-SCAP TMD6 and the PS1-SCAP TMD4 mutant proteins that accumulate as unprocessed, full-length species, were quite stable (Fig. 3A, lanes 9–12 and B, lanes 4–6). However, and for reasons that are presently unclear, the unprocessed PS1-SCAP TMD5 mutant was extremely unstable (Fig. 3A, lanes 13–16). Nevertheless, this result would explain the fact that PEN-2 levels are only slightly elevated in cells expressing this mutant (see Fig. 2C).

Earlier studies have shown that in the absence of the stabilizing effects of PS1, the steady-state levels of endogenous PEN-2 are markedly diminished in PS1-deficient mouse fibroblasts, and this phenotype is rescued by expression of human PS1 (33). To examine whether expression of PS1-SCAP TMD replacement mutants could also “rescue” the loss of PEN-2 expression in PS1-deficient fibroblasts, we examined the PEN-2 levels in the same detergent lysates of these stable cell lines by immunoblot analysis with PNT-2 antibody. Consistent with earlier reports that the PS1-nicastrin interaction is mediated by a domain of PS1 very near the carboxyl terminus (20), the PS1NT antibody immunoprecipitated both wild type PS1 and PS1 fragments over time (Fig. 3). Consistent with several earlier reports (reviewed in Ref. 34), wild type PS1-NTF are very stable, whereas full-length PS1 holoproteins are short-lived (Fig. 3A, lanes 1–4). Similarly, the TMD3 PS1-SCAP mutant holoprotein was degraded rapidly, whereas the endoproteolytic derivatives were quite stable (Fig. 3A, lanes 5–8). In contrast, both the PS1-SCAP TMD6 and the PS1-SCAP TMD4 mutant proteins that accumulate as unprocessed, full-length species, were quite stable (Fig. 3A, lanes 9–12 and B, lanes 4–6). However, and for reasons that are presently unclear, the unprocessed PS1-SCAP TMD5 mutant was extremely unstable (Fig. 3A, lanes 13–16). Nevertheless, this result would explain the fact that PEN-2 levels are only slightly elevated in cells expressing this mutant (see Fig. 2C). We should note that in the stable cell lines, all four PS1-SCAP mutants were coimmunoprecipitated with antibodies raised against NCT (data not shown), findings consistent with earlier reports that the PS1-nicastrin interaction is mediated by a domain of PS1 very near the carboxyl terminus (20).

To examine the interaction of the PS1-SCAP mutants with endogenous PEN-2, we performed coimmunoprecipitation experiments. CHAPS-solubilized lysates of each stable cell line were subject to immunoprecipitation with either PS1NT or PNT-2 antibodies (Fig. 4). As expected, the PS1NT antibody immunoprecipitated both wild type PS1 and all the PS1-SCAP TMD mutants (Fig. 4A, lanes 6–10), and as we showed earlier, endogenous PEN-2 was coimmunoprecipitated with wild type PS1 under these conditions (Fig. 4B, lane 6). Moreover, endogenous PEN-2 was also coimmunoprecipitated by PS1NT antibody in cells expressing the PS1-SCAP TMD3 or TMD6 mutants (Fig. 4B, lanes 9–12).
TMD4 of PS1 Is Required for Binding with PEN-2

FIGURE 4. Coimmunoprecipitation of PS1-SCAP TMD mutants with PEN-2. A, PS1-deficient fibroblasts stably expressing wild type PS1 (lanes 1, 6, 11, and 16), TMD3 (lanes 2, 7, and 12), TMD4 (lanes 3, 8, and 13), TMD5 (lanes 4, 9, and 14), or TMD6 PS1-SCAP mutants (lanes 5, 10, and 15) were lysed in 1% CHAPS and aliquots of lysates were immunoprecipitated with PS1\textsubscript{NT} (lanes 6–10), PEN-2 (lanes 11–15), or unrelated ABri antibodies (lane 16). The resulting immunoprecipitates as well as detergent lysates corresponding to the 1/20 of the volume used for immunoprecipitation (IP) (lanes 1–5; input) were resolved on 16.5% Tris/Tricine SDS-PAGE and probed with αPS1Loop (A) or PNT-2 antibodies (B). A longer exposure of the input lanes in A was also shown in the lower panel. An asterisk indicates immunoglobulin bands. Molecular mass markers are shown on the left in kDa. WB, Western blot; FL, full-length.

7 and 10), despite the fact that the PS1-SCAP TMD6 mutant is not subject to endoproteolytic processing. In any event, these latter results indicate that TMD3 and TMD6 of PS1 are dispensable for binding to PEN-2. Interestingly, the PS1-SCAP TMD5 mutant, although highly unstable, still retained the ability to bind a small fraction of PEN-2 (Fig. 4B, lane 9). More significantly, we did not observe coimmunoprecipitation of the PS1-SCAP TMD4 mutant with endogenous PEN-2 (Fig. 4B, lane 8), despite the fact that this mutant protein appears to be highly stable (see Fig. 3). Taken together, these results strongly suggest that the TMD4 of PS1 is required for binding with PEN-2.

γ-Secretase Activity of PS1-SCAP Mutants—Having examined the interaction of the PS1-SCAP mutants with PEN-2, we then examined the activity of the mutants in promoting γ-secretase-mediated proteolytic conversion of APP-CTFs and NotchΔE to AICD and NICD, respectively. We transiently expressed a myc epitope-tagged Swedish variant APP\textsubscript{695} cDNA into each stable fibroblast line and analyzed the levels of APP-CTFs in detergent lysates of these cells by Western blot analysis using the 369 antibody, specific for epitopes in the cytoplasmic domain of APP (27). As previously reported (35), overexpression of human PS1 in PS1-deficient fibroblasts leads to a marked reduction in APP-CTF\textsubscript{mycβ} and CTF\textsubscript{mycα} that is accompanied by an elevation in levels of AICD (Fig. 5B, compare lane 3 versus 2). Similarly, the levels of AICD were elevated in cells stably overexpressing the endoproteolyis-competent PS1-SCAP TMD3 variant (Fig. 5B, lane 4). However, expression of the three other PS1-SCAP chimeras (TMD4, TMD5, and TMD6) failed to promote γ-secretase processing of APP-CTFs (Fig. 5B, lanes 5–7). We confirmed these results in the stable cell lines by examining the γ-secretase processing of a transiently expressed, myc epitope-tagged NotchΔE polypeptide, a constitutively active form of Notch. As has been shown earlier (for review, see Ref. 36), expression of wild type PS1 in PS1-deficient cells enhances the production of NICD (Fig. 5C, compare lane 3 versus 2). Similarly, the levels of NICD generated in cells expressing the PS1-SCAP TMD3 mutant were also elevated, compared with parental cells (Fig. 5C, compare lane 4′ versus 2′), albeit not to the levels observed in cells expressing wild type PS1 (Fig. 5C, compare lane 4′ versus 3′). Again, expression of the three other PS1-SCAP mutants failed to increase the production of NICD (Fig. 5C, compare lanes 5′–7′ versus 2′).

Identification of a Domain within PS1 TMD4 That Binds to PEN-2—Having established that the PS1 TMD4 is required for association with PEN-2, we asked whether we could narrow down a minimal domain within PS1 TMD4 that was necessary for binding. Our strategy was to test the capacity of additional chimeras that contained either one- or two-thirds of PS1 TMD4 to rescue the deficit in PEN-2 binding and γ-secretase activity seen in cells expressing the PS1-SCAP TMD4 mutant. For this, we generated constructs in which the proximal two-thirds (PPC), first and last thirds (PCP), or distal two-thirds (CPP) of the authentic PS1 TMD4 chimera were replaced with sequences from the respective PS1 TMD3 variant. For this, we generated constructs in which the proximal two-thirds (PPC), first and last thirds (PCP), or distal two-thirds (CPP) of the PS1-SCAP TMD4 chimera were replaced with sequences from the authentic PS1 TMD4 chimera. To evaluate the endoproteolysis and
γ-secretase activity of these mutants, we cotransfected these mutant cDNAs with cDNA encoding Notch ΔE into PS1-deficient fibroblasts. Among the three new chimeras (PPC, PCP, or CPP), only the CPP (Fig. 6B, lane 6) or PCP (Fig. 6B, lane 5) mutants were subject to endoproteolysis. However, we noted that the steady-state levels of NTF from these mutants were considerably lower than the NTF derived from wild type PS1 (Fig. 6B, compare lanes 5 and 6 versus 2). Moreover, expression of these two mutants in PS1-deficient cells only partially rescued the γ-secretase processing of Notch ΔE (Fig. 6C, lanes 5 and 6), but at levels that reflected the accumulated levels of NTF, compared with NTF derived from wild type PS1 (Fig. 6B, compare lanes 5 and 6 versus 2). The inefficient endoproteolysis of the PCP mutant in PS1-deficient cells was interesting, and we suspected it was probably the result of altered α-helicity imposed by the presence of the proline residue present in the SCAP TMD1 (see Fig. 6A). Indeed, we observed that the PCP mutant harboring a proline to alanine substitution was subject to endoproteolysis at levels no different from cells expressing wild type PS1 (data not shown). More importantly, the PPC mutant, containing the proximal two-thirds of the authentic PS1 TMD4 and distal third of the SCAP TMD1, accumulated as a holoprotein (Fig. 6B, lane 4) and was inactive in promoting γ-secretase processing of Notch ΔE when expressed in PS1−/− fibroblasts (Fig. 6C, lane 4).

We also examined the effects of these mutants on the γ-secretase processing of APP-CTFs by coexpressing wild type PS1 or each of these chimeras, together with APH-1, NCT, PEN-2, and APPSwe in HEK293 cells. As we had shown earlier (7, 14, 25), transient coexpression of APH-1, NCT, PEN-2, and wild type PS1 (ANPP) led to the hyperaccumulation of PS1 derivatives relative to cells transiently expressing PS1 alone (Fig. 6D, compare lanes 4 and 3, respectively). Commensurate
FIGURE 6. NFGVGM motif in the PS1 TMD4 is necessary for PEN-2 binding. A, amino acid sequences of the TMD4 of PS1, the TMD1 of SCAP, and the TMD4 of various PS1-SCAP chimeras in which distal (PPC), middle (PCP), or proximal (CPP) one-third of the authentic PS1 TMD4 was replaced with the corresponding sequences from the SCAP TMD1. B and C, PS1-deficient fibroblasts were transiently cotransfected with myc epitope-tagged NotchΔE cDNA and HA (lane 1), wild type PS1 (lane 2), the PS1-SCAP TMD4 mutant (lane 3), PPC (lane 4), PCP (lane 5), or CPP mutant constructs (lane 6). After 48 h, detergent lysates of the cells were prepared and analyzed by immunoblot with PS1NT (B) or 9E10 (C) antibodies. D and E, HEK293 cells were transiently cotransfected with APPswe cDNA and HA (lane 2), wild type PS1 (lanes 3 and 4), TMD4 PS1-SCAP mutant (lanes 5 and 6), PPC (lanes 7 and 8), PCP (lanes 9 and 10), or CPP variant constructs (lanes 11 and 12). Each PS1 variant construct was cotransfected with HA (lanes 3, 5, 7, 9, and 11) or APH-1 (lanes 4, 6, 8, 10, and 12). After 48 h, detergent lysates of the cells were prepared and analyzed by immunoblot with PS1NT (D) or 9E10 (E) antibodies.
with an elevation in PS1 fragments in cells expressing ANPP, we observe an increase in γ-secretase-mediated production of AICD (7) (Fig. 6E, compare lanes 3 and 4). In contrast, transient coexpression of the PS1-SCAP TMD4 mutant together with APH-1, NCT, and PEN-2 led to only a slight increase in levels of PS1 endoproteolytic derivatives and AICD (Fig. 6, D and E, lanes 5 and 6). This latter finding was curious, particularly in view of our demonstration that expression of the PS1-SCAP TMD4 mutant in PS1−/− fibroblasts did not generate any PS1 fragments (Fig. 2B, lane 4; Fig. 3A, lane 9; Fig. 4A, lane 3; Fig. 5A, lane 5). How do we resolve this apparent discrepancy? We argue that the small increase in levels of PS1-NTF and AICD in 293 cells cotransfected with the PS1-SCAP TMD4 mutant, APH-1, NCT, and PEN-2, is a result of the stabilization and subsequent endoproteolysis of endogenous human PS1 by excess levels of exogenously overexpressed APH-1, NCT, and PEN-2, the factors that would normally be present at limiting levels. Consistent with the results obtained in PS1−/− cells, upon coexpression with APH-1, NCT, and PEN-2, the CPP (Fig. 6D, lanes 11–12) and PCP (Fig. 6D, lanes 9–10) mutants were subject to endoproteolysis and hyperaccumulated levels of PS1-NTF (Fig. 6D, lanes 9–12), that was paralleled by elevated levels of AICD (Fig. 6E, lanes 9–12). In contrast, coexpression of the PPC mutant with APH-1, NCT, and PEN-2 led to a slight increase in the levels of PS1-NTF and AICD and this increase was comparable with that observed in cells coexpressing the PS1-SCAP TMD4 "loss-of-function" mutant with the other members of the γ-secretase complex (Fig. 6, D and E, lanes 7–8 versus 5–6), indicating that the PPC mutant is not subject to endoproteolysis and unlikely to promote γ-secretase processing of APP-CTFs.

To assess the binding of the PPC, PCP, and CPP mutants with PEN-2, we stably expressed these chimeras in PS1−/− fibroblasts, prepared 1% CHAPS solubilized lysates from these cell lines, and performed coimmunoprecipitation studies. PS1-specific PS1NT antibody communoprecipitated endogenous PEN-2 in PS1−/− deficient fibroblasts expressing human wild type PS1 (Fig. 6G, lane 6) and as we showed earlier, the PS1-SCAP TMD4 mutant failed to bind PEN-2 (Fig. 6G, lane 7). Interestingly, endogenous PEN-2 was also communoprecipitated by PS1NT antibody in cells expressing CPP and to a lesser degree, PCP variants (PCP.17 and CPP.24, respectively; Fig. 6G, lanes 9 and 10, respectively). We should note that it is presently not certain whether endogenous PEN-2 binds to the full-length, or endoproteolytic derivatives of the CPP and PCP variants, but this is a moot point because the domain of interaction with PEN-2 appears to be within the distal segment of PS1 TMD4. In support of this argument, we did not observe communoprecipitation of endogenous PEN-2 with the PPC mutant (line PPC.5; Fig. 6G, lane 8), similar to the results obtained for the original PS1-SCAP TMD4 mutant (Fig. 6G, lane 7). Taken together, these results indicate that the "NFGVVGM" motif in the distal one-third of the PS1 TMD4 is critical for binding with PEN-2 and that the proximal two-thirds of PS1 TMD4 is dispensable for this function.

A Minimal Motif within PS1 TMD4 for Interaction with PEN-2—To further narrow down a minimal motif in the TMD4 of PS1, we generated five additional constructs encoding PS1-SCAP chimeras in which 3–4 amino acids in the middle or distal regions of the PS1 TMD4 were replaced by the corresponding sequences from SCAP TMD1 (Fig. 7A). In designing these constructs, we replaced the PS1 sequence VAL with ILA, instead of the analogous sequence from SCAP TMD1 (IPL) to preclude potential structural perturbations within the TMD induced by the presence of the proline residue (see above). In addition to the original TMD4 sequence, we also generated a construct in which the ISI segment at the very carboxyl terminus of the predicted PS1 TMD4 was replaced with LTL (Fig. 7A).

Coexpression of wild type PS1, VAL to ILA, LIW to VTT, VGM to FAY, or ISI to LTL mutants with NotchΔE in PS1−/− deficient fibroblasts led to the generation of PS1-NTF (Fig. 7B, lanes 3, 5, 6, 8, and 9, respectively) and elevated levels of NICD to varying degrees (Fig. 7C, lanes 3, 5, 6, 8, and 9, respectively), indicating that the nonoverlapping 3-residue segments within PS1 TMD4 are largely dispensable for promoting PS1 endoproteolysis and γ-secretase activity. In contrast, the NFGV to YIIL variant accumulated as an unprocessed PS1 chimera (Fig. 7B, lane 7) and failed to promote proteolysis of NotchΔE to generate NICD (Fig. 7C, lane 7), phenotypes that mimic the observations made in cells expressing the loss-of-function PS1-SCAP TMD4 chimera (Fig. 7, B and C, lane 4). In addition, transient coexpression of wild type PS1, VAL to ILA, LIW to VTT, VGM to FAY, or ISI to LTL mutants together with APH-1, NCT, and PEN-2 in HEK293 cells resulted in hyperaccumulation of PS1 fragments (Fig. 7D, lanes 3–4, 7–8, 9–10, 13–14, and 15–16, respectively) and elevated levels of AICD (Fig. 7E, lanes 3–4, 7–8, 9–10, 13–14, and 15–16, respectively). However, upon transient coexpression of the NFGV to YIIL variant together with APH-1, NCT, and PEN-2, we only observed accumulation of the unprocessed PS1 chimera (Fig. 7D, lanes 11 and 12) and failed proteolysis of APP-CTFs to generate NICD (Fig. 7E, lanes 11 and 12).

To examine whether these mutants associate with PEN-2, we stably expressed these chimeras in PS1−/− fibroblasts, prepared 1% CHAPS-solubilized lysates from these cell lines, and performed communoprecipitation experiments. As we showed earlier, PS1NT antibody communoprecipitated endogenous PEN-2 in PS1−/− deficient fibroblasts expressing human wild type PS1 (Fig. 8B, lane 8), whereas the PS1-SCAP TMD4 mutant failed to associate with PEN-2 (Fig. 8B, lane 9). Interestingly, endogenous PEN-2 was also communoprecipitated by PS1NT antibody in cells expressing either VAL to ILA (lane VAL.23; Fig. 8B, lane 10), LIW to VTT (lane LIW.19; Fig. 8B, lane 11), VGM to FAY (lane VGM.3; Fig. 8B, lane 13), or ISI to LTL variants (lane ISI.3; Fig. 8B, lane 14). In contrast, we did not observe communoprecipitation of endogenous PEN-2 with the NFGV to YIIL mutant (lane NFGV.8; Fig. 8B, lane 12), similar to the results obtained for the original PS1-SCAP TMD4 mutant (Fig. 8B, lane 9) or the PPC mutant (Fig. 6G, lane 8).

In view of the latter finding that the NFGV sequence plays an important role in binding with PEN-2, we tested two additional constructs encoding PS1-SCAP chimeras in which either the NF or GV residues of the TMD4 of PS1−/− fibroblasts. Coexpression of the GV to IL variant with NotchΔE led to the generation of PS1-NTF (Fig. 9B, lane 8), commensurate with increased production of NICD (Fig. 9C, lane 8), similar to that seen in cells expressing wild type PS1 (Fig. 9, B and C, lane 3). In contrast, the PS1-SCAP chimera harboring a replacement of NF with YI in TMD4 resulted in a loss-of-function phenotype (Fig. 9, B and C, lane 7), very similar to that observed with the PS1-SCAP TMD4 mutant (Fig. 9, B and C, lanes 4), and the NFGV mutant (Fig. 9, B and C, lanes 5); the NF to YI polypeptide accumulated as a full-length species (Fig. 9B, lane 7) and failed to promote proteolytic conversion of NotchΔE to generate NICD (Fig. 9C, lane 7). In addition, coexpression of the GV to IL mutant with APH-1, NCT, and PEN-2 in HEK293 cells led to elevated levels of PS1−/− fibroblasts were prepared after 72 h and analyzed by immunoblot with PS1NT (D) or CTM2 (E) antibodies. F and G, PS1−/− deficient fibroblasts stably expressing wild type PS1 (lanes 1 and 6), TMD4 (lanes 2 and 7), PPC (lanes 3 and 8), PCP (lanes 4 and 9), or CPP variants (lanes 5 and 10) were lysed in 1% CHAPS and aliquots of lysates were immunoprecipitated (IP) with PS1NT antibody (lanes 6–10). Recovered materials as well as 5% of the input (lanes 1–5) were resolved on 16.5% Tris/Tricine SDS-PAGE and probed with PS1NT (F) or PNT-2 (G) antibodies. An asterisk indicates immunoglobulin bands. In B–G, molecular mass markers are shown on the left in kDa. WB, Western blot; FL, full-length.
NFGV motif in the PS1 TMD4 is critical for PS1 endoproteolysis and γ-secretase activity.

A. Amino acid sequences of TMD4 of wild type PS1 and various PS1-SCAP chimeras in which 3–4 amino acids in the middle or distal regions of PS1 TMD4 were replaced by the corresponding sequences from TMD1 of SCAP. B and C, PS1-deficient fibroblasts were transiently cotransfected with myc epitope-tagged NotchΔE cDNA and HA (lane 2), wild type PS1 (lane 3), the PS1-SCAP TMD4 (lane 4), VAL to ILA (lane 5), LIW to VTT (lane 6), NFGV to YIIL (lane 7), VGM to FAY (lane 8), or ISI to LTL variant constructs (lane 9). After 48 h, detergent lysates of the cells were prepared and analyzed by immunoblot with PS1NT (B) or 9E10 (C) antibodies. D and E, HEK293 cells were transiently cotransfected with APPswe cDNA and HA (lanes 1 and 2), wild type PS1 (lanes 3 and 4), PS1-SCAP TMD4 mutant (lanes 5 and 6), VAL to ILA (lane 7 and 8), LIW to VTT (lanes 9 and 10), NFGV to YIIL (lanes 11 and 12), VGM to FAY (lanes 13 and 14), or ISI to LTL variant constructs (lanes 15 and 16). Each PS1 variant construct was cotransfected with HA (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or APH-1, NCT, and PEN-2 (ANP; lanes 2, 4, 6, 8, 10, 12, 14, and 16). Detergent lysates were prepared after 48 h and analyzed by immunoblot with PS1NT (D) or 369 (E) antibodies. In B–E, molecular mass markers are shown on the left in kDa. WB, Western blot; FL, full-length.
CTF and increased production of AICD (Fig. 9, D and E, lanes 11 and 12). However, coexpression of the NF to YI mutant with APH-1, NCT, and PEN-2 failed to promote proteolytic conversion of APP-CTFs to full-length. Therefore, we examined the effects of a series of PS1 mutants harboring structural motifs that contribute to binding with PEN-2. With this information, we discovered that the PS1-SCAP TMD4 mutant (Fig. 9, D and E, lanes 3 and 4), and the NFGV mutant (Fig. 9, D and E, lanes 5 and 6).

Furthermore, endogenous PEN-2 was coimmunoprecipitated by PS1NT antibody in PS1−/− fibroblasts that stably express the GV to IL mutant (line GV.4: Fig. 10B, lane 12), whereas the NF to YI mutant failed to associate with PEN-2 (line NF.16: Fig. 10B, lane 11), similar to the results obtained for the original PS1-SCAP TMD4 mutant (Fig. 10B, lane 8), the PPC mutant (Fig. 6G, lane 8) and the NFGV to YIIL mutant (Fig. 10B, lane 9). Collectively, these results indicate that the NF motif in the TMD4 of PS1 is critical for binding with PEN-2, promoting PS1 endoproteolysis and γ-secretase activity.

DISCUSSION

In the present study, we sought to identify the molecular domains of PS1 that are required for binding with PEN-2, resulting in endoproteolytic processing of the PS1 holoprotein. We chose to concentrate our efforts on the transmembrane domains of PS1-NTF because Fraering et al. (24) demonstrated that disruption of the γ-secretase complex in mild detergents resulted in the production of smaller, heterogeneous complexes, one of which contained PS1-NTF and PEN-2. With this information, we examined the effects of a series of PS1 mutants harboring deletions or replacements of specific TMDs of PS1-NTF on interactions with PEN-2, stabilization and endoproteolysis of PS1, and γ-secretase activity. The present studies now offer several important insights relevant to the domains in PS1 that are critical for interacting with PEN-2, promoting conversion of PS1 holoproteins to endoproteolytic derivatives, and γ-secretase-mediated processing of APP and Notch.

First, we show that a PS1 mutant lacking TMDs 1 and 2 and the intervening hydrophilic loop (PS1ΔM1,2) is capable of binding to PEN-2. However, this mutant accumulates as an unprocessed, full-length protein, suggesting that the binding of PS1 with PEN-2 is necessary, but not sufficient to promote PS1 endoproteolysis.

Second, our analysis of four chimeric molecules that harbor replacements of each of PS1 TMD 3–6 with a corresponding transmembrane segment from SCAP revealed several important features (summarized in TABLE ONE). Similar to wild type PS1, the PS1-SCAP TMD3 variant associated with PEN-2, was stabilized, and promoted γ-secretase processing of APP and Notch. However, the PS1-SCAP TMD4, TMD5, and TMD6 chimeras accumulated as full-length holoproteins and failed to promote proteolysis of APP-CTF and NotchΔE. The TMD5 mutant exhibited a short half-life, and was coimmunoprecipitated with PEN-2, albeit at much lower levels than wild type PS1. As it is widely believed that stabilization of full-length PS1 precedes the interaction with PEN-2 and subsequent endoproteolysis, inefficient binding of the TMD5 mutant was most likely because of instability of this protein. However, because of the inherent instability of the PS1-SCAP TMD5 chimera, we cannot completely rule out the possibility that the TMD5 of PS1 also harbors structural motifs that contribute to binding with PEN-2.

Most importantly, we demonstrate that the PS1-SCAP TMD4 mutant is stable, but fails to interact with PEN-2, findings strongly suggesting that the PS1 TMD4 is the binding site for PEN-2. In this regard, Drs. Takeshi Iwatsubo and Taisuke Tomita (37) and their respective laboratories have also discovered that the PS1 TMD4 is necessary for PEN-2 binding. These latter studies were performed by...
FIGURE 9. NF motif in the PS1 TMD4 is required for PS1 endoproteolysis and \( \gamma \)-secretase activity. A, amino acid sequences of TMD4 of wild type PS1 and various PS1-SCAP chimeras. B and C, PS1-deficient fibroblasts were transiently cotransfected with myc epitope-tagged Notch\( \Delta E \) cDNA and HA (lane 2), wild type PS1 (lane 3), the PS1-SCAP TMD4 mutant (lane 4), NFGV to YIIL (lane 5), VGM to FAY (lane 6), NF to GV (lane 7), or GV to IL mutant cDNAs (lane 8). After 48 h, detergent lysates of the cells were prepared and analyzed by immunoblot with PS1\( \text{NT} \) (B) or 9E10 (C) antibodies. D and E, HEK293 cells were transiently cotransfected with APP\( \text{swe} \) cDNA and wild type PS1 (lanes 1 and 2), TMD4 PS1-SCAP mutant (lanes 3 and 4), NFGV to YIIL (lanes 5 and 6), VGM to FAY (lanes 7 and 8), NF to YI (lanes 9 and 10), or GV to IL variant constructs (lanes 11 and 12). Each PS1 variant construct was cotransfected with either HA (lanes 1, 3, 5, 7, 9, and 11) or APH-1xL, NCT, and PEN-2 cDNAs (ANP; lanes 2, 4, 6, 8, 10, and 12). Detergent lysates were prepared after 60 h and analyzed by immunoblot with \( \alpha \)PS1Loop (D) or 369 (E) antibodies. In B–E, molecular mass markers are shown on the left in kDa. WB, Western blot; FL, full-length.
analysis of a series of chimeras in which the TMDs of PS1 were swapped with the TMDs of a novel membrane-bound collagen, CLAC-P/collagen type XXV (CLAC-P), a type II membrane protein, or CD4, a type I membrane protein (37). Collectively, our results and those from Drs. Iwatsubo and Tomita and co-workers (37) that were obtained by analysis of chimeras that contained transmembrane segments from entirely unrelated proteins strongly reinforce our conclusion that the TMD4 of PS1 harbors the information that is required for interaction with PEN-2.

Third, analysis of three chimeras in which distal (PPC), middle (PCP), or proximal (CPP) one-third of the PS1 TMD4 was replaced with the corresponding sequences from the TMD1 of SCAP revealed that only the latter two mutants (PCP and CPP) were subject to endoproteolysis and promoted \( \beta\)-secretase processing of APP and Notch. In contrast, the PPC mutant containing the proximal two-thirds of the authentic PS1 TMD4 and the distal third of the SCAP TMD1, accumulated as a holoprotein and was inactive in promoting \( \beta\)-secretase-mediated generation of AICD and NICD. In addition, this mutant failed to associate with endogenous PEN-2, similar to the original PS1-SCAP TMD4 mutant. These data strongly suggest that the NFGVVGM motif in the distal one-third of the PS1 TMD4 is critical for binding with PEN-2 and that the proximal two-thirds of PS1 TMD4 are dispensable for this function.

Finally, we have narrowed down a domain within the PS1 TMD4 that is necessary for the interaction with PEN-2. These results indicate that the NF motif in the TMD4 of PS1 is critical for PEN-2 binding. However, it should be noted that when coexpressed with APH-1, NCT, and PEN-2, the NF to YI mutant was subject to endoproteolysis, albeit very inefficiently, and retained partial \( \beta\)-secretase activity (see Fig. 9, D and E). Therefore, it is still conceivable that in addition to the NF motif, surrounding amino acids also contribute to PEN-2 binding, PS1 endoproteolysis, and \( \beta\)-secretase activity.

In summary, we have identified a minimal motif within the PS1 TMD4 that is critical for PEN-2 binding, promoting PS1 endoproteolysis, and \( \beta\)-secretase activity. It will be critical to define the molecular mechanism(s) by which PEN-2 promotes PS1 endoproteolysis and to develop an understanding of the structural and conformational alterations in PS1 that render it susceptible to endoproteolysis.

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| TABLE ONE |
| --- |
| Data summary of PS1-SCAP TMD mutants |
| | Stabilization | Coimmunoprecipitation with PEN-2 | Rescue of PEN-2 levels | Endoproteolysis | AICD production | NICD production |
| PS1 | + + | + + | + + | + + | + + | + + |
| TMD3 | + + | + + | + + | + + | + + | + + |
| TMD4 | + + | - | - | - | - | - |
| TMD5 | - | - | + | + | + | + |
| TMD6 | + + | + + | + + | + + | + + | + + |

FIGURE 10. NF motif in the PS1 TMD4 is critical for PEN-2 binding. A and B, PS1-deficient fibroblasts stably expressing wild type PS1 (lanes 1 and 7), PS1-SCAP TMD4 mutant (lanes 2 and 8), NFGV to YI1 (lanes 3 and 9), VGM to FAY (lanes 4 and 10), NF to YI (lanes 5 and 11), or GV to IL mutants (lanes 6 and 12) were lysed in 1% CHAPS and aliquots of lysates were immunoprecipitated with PS1NT antibody (lanes 1–6). Recovered materials as well as 5% of the input (lanes 1–6) were resolved on 16.5% Tris/Tricine SDS-PAGE and probed with aPS1Loop (A) or PNT-2 (B) antibodies. A longer exposure of the input lanes in A was also shown in the lower panel. Molecular mass markers are shown on the left in kDa. WB, Western blot; FL, full-length.
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REFERENCES

1. Sisodia, S. S., and St. George-Hyslop, P. H. (2002) Nat. Rev. Neurosci. 3, 281–290
2. De Strooper, B. (2003) Neuron 38, 9–12
3. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) Nat. Cell. Biol. 5, 486–488
4. Periz, G., and Fortini, M. E. (2004) J. Neurosci. Res. 77, 399–322
5. Lavoie, M. J., Fraering, P. C., Ostaszewski, B. L., Ye, W., Kimberly, W. T., Wolfe, M. S., and Selkoe, D. J. (2003) J. Biol. Chem. 278, 37213–37222
6. Takasugi, N., Tomita, T., Hayashi, L., Tsuuroka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) Nature 422, 438–441
7. Kim, S. H., Ikeuchi, T., Yu, C., and Sisodia, S. S. (2003) J. Biol. Chem. 278, 33992–34002
8. Luo, W. J., Wang, H., Li, H., Kim, B. S., Shah, S., Lee, H. J., Thinakaran, G., Kim, T. W., Yu, G., and Xu, H. (2003) J. Biol. Chem. 278, 7850–7854
9. Hu, Y., and Fortini, M. E. (2003) J. Cell. Biol. 161, 685–690
10. Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Rato-vitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1996) Neuron 17, 181–190
11. 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
12. Ratovitski, T., Slunt, H. H., Thinakaran, G., Price, D. L., Sisodia, S. S., and Borchelt, D. R. (1997) J. Biol. Chem. 272, 24536–24541
13. Prokop, S., Shirotani, K., Edbauer, D., Haass, C., and Steiner, H. (2004) J. Biol. Chem. 279, 23255–23261
14. Kim, S. H., and Sisodia, S. S. (2005) J. Biol. Chem. 280, 1992–2001
15. Francis, R., McGrath, G., Zhang, J., Ruddy, D. A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Filmes, C. S., Hiebsch, R., Bubley, C., Nye, J. S., and Curtis, D. (2002) Dev. Cell. 3, 85–97
16. Crystal, A. S., Morais, V. A., Pierson, T. C., Pijak, D. S., Carlin, D., Lee, V. M., and Doms, R. W. (2003) J. Biol. Chem. 278, 20117–20123
17. Capell, A., Kaether, C., Edbauer, D., Shirotani, K., Merkl, S., Steiner, H., and Haass, C. (2003) J. Biol. Chem. 278, 52519–52523
18. Morais, V. A., Crystal, A. S., Pijak, D. S., Carlin, D., Costa, J., Lee, V. M., and Doms, R. W. (2003) J. Biol. Chem. 278, 41284–41291
19. Lee, S. F., Shah, S., Yu, C., Wigley, W. C., Li, H., Lim, M., Pedersen, K., Han, W., Thomas, P., Lundkvist, J., Hao, Y. H., and Yu, G. (2004) J. Biol. Chem. 279, 4144–4152
20. Kaether, C., Capell, A., Edbauer, D., Winkler, E., Novak, B., Steiner, H., and Haass, C. (2004) EMBO J. 23, 4738–4748
21. Doan, A., Thinakaran, G., Borchelt, D. R., Slunt, H. H., Ratovitsky, T., Podlisny, M., Selkoe, D. J., Seeger, M., Gandy, S. E., Price, D. L., and Sisodia, S. S. (1996) Neuron 17, 1023–1030
22. Li, X., and Greenwald, I. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7109–7114
23. Laudon, H., Hansson, E. M., Melen, K., Bergman, A., Farmery, M. R., Winblad, B., Lendahl, U., von Heijne, G., and Naslund, J. (2005) J. Biol. Chem. 280, 35352–35360
24. Fraering, P. C., Lavoie, M. I., Ye, W., Ostaszewski, B. L., Kim, S. H., Selkoe, D. J., and Wolfe, M. S. (2004) Biochemistry 43, 323–333
25. Kim, S. H., Yin, Y. I., Li, Y. M., and Sisodia, S. S. (2004) J. Biol. Chem. 279, 48615–48619
26. Thinakaran, G., Teplow, D. B., Siman, R., Greenberg, B., and Sisodia, S. S. (1996) J. Biol. Chem. 271, 9390–9397
27. Buxbaum, J. D., Gandy, S. E., Cicchetti, P., Ehrlich, M. E., Czernik, A. J., Fraccaso, R. P., Ramabhadran, T. V., Unterbeck, A. J., and Greengard, P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6003–6006
28. Martyis-Zage, J. L., Kim, S. H., Berechid, B., Bingham, S. J., Chu, S., Sklar, J., Nye, J., and Sisodia, S. S. (2000) J. Mol. Neurosci. 15, 189–204
29. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
30. Kim, S. H., Leem, J. Y., Lah, J. J., Slunt, H. H., Levey, A. I., Thinakaran, G., and Sisodia, S. S. (2001) J. Biol. Chem. 276, 43343–43350
31. Murphy, M. P., Uljon, S. N., Fraser, P. E., Fauq, A., Lookingbill, H. A., Findlay, K. A., Smith, T. E., Lewis, P. A., McLendon, D. C., Wang, R., and Golde, T. E. (2000) J. Biol. Chem. 275, 26277–26284
32. Leem, J. Y., Saura, C. A., Pietrzik, C., Christianson, I., Wanamaker, C., King, L. T., Veselits, M. L., Tomita, T., Gasparini, L., Iwatsubo, T., Xu, H., Green, W. N., Koo, E. H., and Thinakaran, G. (2002) Neurobiol. Dis. 11, 64–82
33. Steiner, H., Winkler, E., Edbauer, D., Prokop, S., Basset, G., Yamazaki, A., Kostka, M., and Haass, C. (2002) J. Biol. Chem. 277, 39062–39065
34. Price, D. L., and Sisodia, S. S. (1998) Annu. Rev. Neurosci. 21, 479–505
35. Yu, C., Kim, S. H., Ikeuchi, T., Xu, H., Gasparini, L., Wang, R., and Sisodia, S. S. (2001) J. Biol. Chem. 276, 43756–43760
36. Selkoe, D., and Kopen, R. (2003) Annu. Rev. Neurosci. 26, 565–597
37. Watanabe, N., Tomita, T., Sato, C., Kitamura, T., Morohashi, Y., and Iwatsubo, T. (2005) J. Biol. Chem. 280, 41967–41975