Macromolecular complexes containing presenilins (PS), nicastrin (NCT), APH-1, and PEN-2 mediate the γ-secretase cleavage of the β-amyloid precursor protein and Notch. API-1 and NCT stabilize the PS1 holoprotein, whereas PEN-2 is critical for endoproteolysis of PS1. To define the structural domains of PEN-2 that are necessary for mediating PS1 endoproteolysis and γ-secretase activity, we coexpressed APH-1, NCT, and PS1 together with a series of PEN-2 mutants, which harbored deletions in hydrophilic segments, or chimeric PEN-2 molecules that contained heterologous transmembrane domains (TMDs). We now report that with the exception of the PEN-2 variants with deletions proximal to the TMDs, the vast majority of the deletion variants were functional. Mutants that were nonfunctional were also unstable but were rescued by transposition of a heterologous sequence containing conservative amino acid substitutions into the deleted region. Notably, the carboxyl-terminal hydrophilic domain of PEN-2 was dispensable for promoting PS1 endoproteolysis but was critical for stabilizing the resulting PEN-2 derivatives. More importantly, we demonstrated that a chimeric PEN-2 with a replacement of the TMD2 with the TMD1 from sterol regulatory element binding protein 1 (SREBP-1) is fully functional but that a chimeric PEN-2 with a replacement of the TMD1 with the TMD2 from SREBP-1 is not. The function of this latter chimera was rescued by the replacement of the proximal two-thirds of the SREBP-1 TMD2 with the proximal two-thirds of the authentic TMD1 from PEN-2. These results suggest that the proximal two-thirds of the PEN-2 TMD1 is functionally important for endoproteolysis of PS1 holoproteins and the generation of PS1 fragments, essential components of the γ-secretase complex.

The “γ-secretase” complex mediates the intramembranous processing of several type I membrane proteins, including β-amyloid precursor protein (APP) and Notch (for review, see Ref. 1). The components of the γ-secretase complex are presenilins (PS1 and PS2), nicastrin (NCT), APH-1, and PEN-2, and the demonstration that coexpression of these four molecules in Saccharomyces cerevisiae is sufficient to reconstitute γ-secretase activity (2) has confirmed that these four proteins are the essential core components for the catalytic activity.

Several lines of evidence demonstrated 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. 3). Although the precise functional role of the individual components of the γ-secretase complex is unclear, APH-1 and NCT apparently form a stable subcomplex that binds to and stabilizes the PS1 holoprotein (4, 5). This APH-1/NCT-PS1 complex then associates with PEN-2, which induces the proteolytic conversion of the stabilized PS1 holoprotein into the NTF and CTF heterodimer, the preponderant PS-related polypeptides in AD (4–8). Recent studies have revealed that PEN-2 also stabilizes the PS1 heterodimer (9). PEN-2, a 101-amino acid protein, contains two membrane-spanning domains with the NH₂- and carboxyl-terminal domains facing the lumen (10, 11). At present, little information is available pertaining to the mechanism(s) by which PEN-2 promotes the endoproteolysis of PS1.

Identification of the molecular interactions between the individual components of the γ-secretase complex has offered insights into the assembly of the γ-secretase complex and its function. For example, mutational analyses of NCT and APH-1 reveal that the proximal one-third of the lone transmembrane domain (TMD) of NCT and the GXXG motif of the TMD4 of APH-1 are critical for formation of the γ-secretase complex (12–14). In the present study, we systematically introduced mutations in various regions of PEN-2 to determine the molecular domains of PEN-2 required for its function in promoting endoproteolysis of PS1 holoproteins. We previously demonstrated that coexpression of PS1, APH-1, and NCT increases the level of stabilized full-length PS1, but the proteolytic conversion of the stabilized PS1 holoprotein into NTF and CTF only occurs by additional expression of PEN-2 (6). The γ-secretase-mediated production of the APP intracellular domain (AICD) is correspondingly elevated in these cells (6). Based on these results, we tested the function of a series of PEN-2 variants that either have deletions in hydrophilic domains or contain a heterologous TMD by coexpressing these molecules with APH-1, NCT, and PS1 and examining their effects on (1) the endoproteolysis of PS1 and (2) the γ-secretase-mediated generation of the AICD.
AICD derivative. The function of the unstable mutants was rescued by replacing the authentic segment with a heterologous sequence containing conservative amino acid substitutions at each residue. Furthermore, PEN-2 mutants harboring truncations of small segments of the carboxyl-terminal domain or the entire carboxyl terminus were fully functional with respect to the conversion of the PS1 holoprotein to endoproteolytic derivatives, but the resulting PS1 fragments exhibited short half-lives. Finally, and most importantly, we demonstrate that a PEN-2 chimera, in which the TMD2 is replaced with the TMD1 from sterol regulatory element binding protein 1 (SREBP-1), still retains activity. However, a PEN-2 chimera, in which the TMD1 is replaced with a heterologous TMD segment from SREBP-1, is inactive and does not associate with PS1. Finally, a PEN-2 chimera that contains the proximal two-thirds of the PEN-2 TMD1 and the distal one-third of the SREBP-1 TMD2, was functional in promoting PS1 endoproteolysis and, in parallel, enhanced production of the AICD. Thus, we conclude that the proximal two-thirds of the PEN-2 TMD1 is functionally important for endoproteolysis of PS1 holoproteins and the generation of PS1-NTF and -CTF, essential components of the γ-secretase complex.

EXPERIMENTAL PROCEDURES

Constructs—The cDNAs encoding human PS1, NCT, CT11-tagged PEN-2, Myc epitope-tagged APH-1oL, and Myc epitope-tagged Swedish variant APP751swe (APPswe) were described previously (6, 15, 16). Constructs encoding various PEN-2 mutants were generated using PCR-based mutagenesis. All of the PEN-2 cDNAs contain a sequence encoding the last 7 amino acids (RFLEERP) of APLP1 at the carboxyl-terminal end, which is recognized by the CT11 antibody (6). The sequences of each cDNA were verified by sequencing.

Antibodies—The following antibodies described previously (6) were used: PS1Loop, and APPswe antibodies are polyclonal antibodies that recognize residues 1–65 and 320–375 of PS1, respectively; CT11 was generated against a peptide corresponding to the NH2-terminal 26 amino acids of PS1 (a kind gift from Dr. Gopal Thinakaran (7)). CTM2 is a polyclonal antibody that was generated against a peptide of the carboxyl-terminal end of APP fused with a Myc epitope tag (a kind gift from Dr. Gopal Thinakaran).

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells and transformed fibroblasts derived from mouse embryos with homozygous deletions of PS1 (PS1/−) were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Mouse neuroblastoma N2a cells were maintained in 50% Dulbecco's modified Eagle's medium and 50% Opti-MEM supplemented with 5% fetal bovine serum. Cells were transiently transfected with plasmid DNA using Lipofectamine Plus (Invitrogen). Where indicated, cycloheximide (10 μM) was added 6 h after transfection. Subconfluent dishes of stable N2a lines were washed twice in ice-cold phosphate-buffered saline and lysed in CHAPS lysis buffer (1% CHAPS (Calbiochem), 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA). Lysates were subject to centrifugation at 15,000 × g for 20 min at 4 °C, and the resulting supernatant fraction containing detergent-solubilized proteins was used for immunoprecipitation studies.

RESULTS

Functional Effects of PEN-2 Deletion Variants on PS1 Endoproteolysis and AICD Production—We previously demonstrated that stable coexpression of PS1, APH-1, and NCT leads to increased steady-state levels of full-length PS1 and that the endoproteolytic conversion of the stabilized PS1 holoprotein into NTF and CTF is dependent upon added expression of PEN-2 (6). In these settings, the elevated levels of PS1 derivatives are paralleled by an increase in γ-secretase-mediated production of the AICD (6). To determine the functional domains of PEN-2 necessary for promoting PS1 endoproteolysis and γ-secretase-mediated generation of the AICD, we generated cDNAs encoding various PEN-2 deletion mutants (Fig. 1A) and transiently expressed these constructs in HEK293 cells along with cDNAs encoding PS1, APH-1oL, NCT, and APPswe. We chose to assay six deletion variants of PEN-2 that lack hydrophilic segments that are highly conserved from human to Caenorhabditis elegans: two in the NH2-terminal region (Δ3–9 and Δ10–16), two in the hydrophilic loop between the TMD1 and the TMD2 (Δ40–46 and Δ52–60), and two in the carboxyl-terminal region (Δ85–92 and Δ93–100) (Fig. 1A). Wild-type (WT) PEN-2 and the deletion variants harbor a carboxyl-terminal CT11 epitope tag; the CT11-tagged WT PEN-2 promotes endoproteolysis of the PS1 holoprotein and results in “replacement” of endogenous PEN-2 (6). With the exception of the Δ52–60 variant (Fig. 1B, lane 6), and to a lesser degree, the Δ10–16 variant (Fig. 1B, lane 4), all other PEN-2 deletion variants accumulated to comparable levels in HEK293 cells that transiently coexpressed PS1, APH-1, and NCT (Fig. 1B, lanes 3, 5, 7, and 8). Consistent with our previous results (6), coexpression of PS1, APH-1, and NCT led to elevated levels of the PS1 holoprotein (Fig. 1C, lane 1), and additional expression of WT PEN-2 promoted the conversion of the PS1 holoprotein into PS1 fragments (Fig. 1C, lane 2). Among the six mutant PEN-2 constructs tested, coexpression of cDNA encoding Δ3–9, Δ40–46, Δ85–92, or Δ93–100 PEN-2 variants together with APH-1, NCT, and PS1 led to elevated levels of PS1 derivatives that are comparable with the levels achieved in cells expressing WT PEN-2 (Fig. 1C, lanes 3, 5, 7, and 8 versus lane 2). Densitometric quantification failed to reveal any subtle effects of each PEN-2 mutant on PS1 derivatives (data not shown). In contrast, coexpression of Δ10–16 or Δ52–60 PEN-2 variants together with APH-1, NCT, and PS1 failed to increase the levels of PS1 derivatives (Fig. 1C, lanes 4 and 6), and the PS1 holoprotein accumulated in these cells at levels comparable with cells expressing APH-1, NCT, and PS1 alone (Fig. 1C, compare lanes 4 and 6 with lane 1).

To examine the effects of WT PEN-2 or PEN-2 variants on the levels of APP-CTFs and γ-secretase-mediated production of the AICD, we analyzed the same lysates by immunoblotting with the CT15 antibody, specific for epitopes in the APP carboxyl terminus. We observed that in all settings wherein PS1 derivatives were elevated, the steady-state levels of the AICD were also elevated and mirrored by a marked reduction in levels of APP-CTFβ and APP-CTFγ (Fig. 1E, lanes 2, 3, 5, 7, and 8). On the other hand, in cells that expressed Δ10–16 or Δ52–60 PEN-2 variants along with APH-1, NCT, and PS1,
wherein the overexpressed PS1 holoproteins failed to undergo endoproteolysis, the levels of APP-CTFs and the AICD were no different from those observed in cells that express APH-1, NCT, PS1, and either hemagglutinin (HA) (lane 1), wild-type PEN-2 (lane 2), Δ3-9 (lane 3), Δ10-16 (lane 4), Δ40-46 (lane 5), Δ52-60 (lane 6), Δ85-92 (lane 7), or Δ93-100 (lane 8) PEN-2 variants. After 48 h, detergent lysates of the cells were analyzed by immunoblotting with CT11 (B), PS1NT (C), CTM2 (D), or CT15 antibodies (E). In B–E, molecular mass markers are shown on the left in kDa. Fl., full-length.

Rescued Function of Unstable PEN-2 Deletion Mutants—The results presented in Fig. 1 could be interpreted to suggest that the hydrophilic segments between amino acids 3–9, 40–46, 85–92, and 93–100 are dispensable for PEN-2-mediated endoproteolysis of PS1 and that the regions encompassing amino acids 10–16 and 52–60 are responsible for its function. Alternatively, it could be argued that PEN-2 harboring deletions of amino acids 10–16 or 52–60 are inherently unstable and fail to associate with the complex. In this regard, it has been shown that large deletions in the ectodomain of NCT disrupt the native protein conformation, resulting in mutant proteins that are unstable and unable to incorporate into mature complexes (13, 21). Indeed, it is quite apparent that the steady-state levels of the Δ10–16 and Δ52–60 PEN-2 variants are lower than WT PEN-2 or other “functional” PEN-2 variants (Fig. 1E, lanes 2–8), suggesting that the “loss of function” phenotype of these mutants may be confounded by several, not mutually exclusive, issues (including alterations in protein folding or disrupted membrane insertion) that lead to enhanced turnover of these variants. Indeed, both segments are immediately proximal to the TMDs, and hence, it is not surprising that in the absence of these segments, the polypeptides may have perturbed membrane insertion. To address this issue directly, we chose to test the function of two additional PEN-2 mutants, sub10–16 and sub52–60, that harbored conserved amino acid substitutions in each of the two regions of interest (Fig. 2A). Coexpression of
either the sub10–16 or the sub52–60 PEN-2 mutants together with APH-1, NCT, and PS1 led to endoproteolytic cleavage of PS1 (Fig. 2, C and G, lane 4), findings that we interpret to suggest that PEN-2 function is largely independent of the exact amino acid sequence in these specific regions.

The Carboxyl Terminus of PEN-2 Is Required for Stabilization of PS1 Derivatives—Our finding that the carboxyl-terminal truncation mutants, Δ85–92 and Δ93–100, appeared to be functional with respect to PS1 endoproteolysis (Fig. 1) seemed somewhat inconsistent with a recent report by Prokop et al. (9) showing that expression of the PEN-2 carboxyl-terminal truncation mutant in PEN-2 knockdown cells does not lead to an elevation in steady-state levels of PS1 fragments. To clarify this issue, we tested a mutant PEN-2 molecule with a truncation of the entirety of the carboxyl-terminal domain of 17 amino acids (Δ85–101), coexpressed this construct with cDNA encoding APH-1, NCT, and PS1 in HEK293 cells (Fig. 3 A). In these cells, the Δ85–101 PEN-2 mutant promoted proteolytic conversion of the PS1 holoprotein to PS1-NTF (Fig. 3 A, lane 6) at levels similar to those observed in cells coexpressing WT PEN-2 and the other three components of the γ-secretase complex (Fig. 3 A, lane 4). Realizing that our constructs harbor a CT11 epitope tag that may have influenced the function of the

**Fig. 2.** Effects of conserved amino acid substitutions within segments encompassing residues 10–16 or 52–60 of PEN-2 on PS1 endoproteolysis and the γ-secretase processing of APP. A, amino acid sequences of residues 10–16 of wild-type PEN-2 and the sub10–16 PEN-2 variant (left) and of residues 52–60 of wild-type PEN-2 and the sub52–60 PEN-2 variant (right). B–I, HEK293 cells were transiently cotransfected with APPswe, APH-1, NCT, PS1, and either HA (lane 1), wild-type PEN-2 (lane 2), Δ10–16 (B–E, lane 3), sub10–16 (B–E, lane 4), Δ52–60 (F–I, lane 3), or sub52–60 (F–I, lane 4) PEN-2 variants. After 48 h, detergent lysates of the cells were analyzed by immunoblotting with CT11 (B and F), PS1NT (C and G), CTM2 (D and H), or CT15 antibodies (E and I). Molecular mass markers are shown on the left in kDa. Fl., full-length.
carboxyl-terminal deletion variant, we tested an untagged Δ85–101 PEN-2 variant. The behavior of this PEN-2 mutant, with respect to promoting PS1 endoproteolysis, was indistinguishable from the epitope-tagged Δ85–101 PEN-2 variant (Fig. 3A, lanes 6 and 7).

Perplexed with the discrepancies between our data and those from Prokop et al. (9) that were performed in RNA interference-mediated PEN-2 knockdown lines, we considered the possibility that in our studies, endogenously expressed PEN-2 in HEK293 cells could contribute to the stabilization of PS1 fragments. Hence, we repeated the same experiments in PS1-deficient fibroblasts in which PEN-2 mRNA levels are unaltered. However, the steady-state levels of endogenous PEN-2 are markedly diminished in these cells due to the absence of the stabilizing influence of PS1 (22). Similar to HEK293 cells (Fig. 3A), expression of APH-1, NCT, and PS1 in PS1-deficient fibroblasts resulted in the accumulation of the PS1 holoprotein without any further increase in levels of PS1 fragments, compared with cells expressing PS1 alone (Fig. 3B, compare lane 3 versus 2). Additional expression of either WT PEN-2 or the Δ85–101 PEN-2 mutant in these cells led to a reduction in levels of accumulated full-length PS1 (Fig. 3B, lanes 4 and 6). However, the accumulated levels of PS1-NTF in cells expressing the Δ85–101 PEN-2 mutant were clearly lower than the PS1-NTF levels in cells expressing WT PEN-2 (Fig. 3B, compare lane 6 versus 4). To test whether the differences in steady-state levels of PS1 fragments in these two settings were a result of differences in protein stability, we treated cells with cycloheximide to block de novo protein synthesis. Consistent with several earlier reports (reviewed in Ref. 1), PS1 fragments were quite stable in cells that coexpress PS1 with APH-1, NCT, and WT PEN-2, whereas the PS1 holoprotein was rapidly degraded (Fig. 3C, lanes 1–3). Most notable is the demonstration that the PS1 fragments in cells expressing PS1, APH-1, NCT, and the Δ85–101 PEN-2 variant were also relatively unstable (Fig. 3C, lanes 4–6), compared with the PS1-NTF that accumulates in cells expressing WT PEN-2, PS1, NCT, and APH-1 (Fig. 3C, lanes 1–3). These data are fully consistent with the data presented by Prokop et al. (9) showing that the half-life of PS1 derivatives in cells expressing carboxyl-terminal deletion variants of PEN-2 is considerably reduced compared with cells expressing WT PEN-2.

Why do we observe an elevation in PS1 derivatives in cells expressing the carboxyl-terminal PEN-2 deletion variants, whereas Prokop et al. (9) do not? We propose that in contrast to PS1-deficient knockdown cells, in which PEN-2 mRNA are depleted, the steady-state levels of PEN-2 mRNA are not altered in PS1-deficient cells relative to wild-type fibroblasts. Therefore, the expression of PS1, NCT, and APH-1 can serve to stabilize newly synthesized endogenous mouse PEN-2 molecules. Thus, a fraction of transiently expressed PS1 holoproteins is subject to endoproteolysis, and the resulting PS1 derivatives assemble into stable complexes with mouse PEN-2 and coexpressed NCT/APH-1 polypeptides. Once the limiting levels of endogenously expressed PEN-2 molecules are “saturated,” the remaining fraction of PS1 holoproteins that is stabilized by endogenous and exogenous APH-1/NCT is then subject to endoproteolysis by virtue of the expression of mutant PEN-2 molecules.

Taken together with the results provided by Prokop et al. (9), our results suggest that the carboxyl-terminal hydrophilic region of PEN-2 (residues 85–101) is dispensable for promoting endoproteolysis of PS1 holoproteins but is critical for stabilizing the resulting PS1 derivatives.

The TMD1 of PEN-2 Is Indispensable for Endoproteolysis of PS1 Holoproteins—Having established that deletions and/or substitutions of the vast majority of hydrophilic segments in PEN-2 sequences have little impact on promoting PS1 endoproteolysis, we turned our attention to the TMDs of PEN-2. In this regard, the proximal one-third of the NCT TMD and the GXXG motif of the TMD4 of APH-1 have been shown to be critical for assembly of the γ-secretase complex (12–14). For this purpose, we first generated cDNAs encoding chimeric PEN-2 molecules, in which the TMD1 and the TMD2 from human PEN-2 were replaced with the TMDs from an unrelated protein, the human SREBP-1, which also contains two membrane-spanning domains (23). Considering the fact that the
transmembrane topology of SREBP-1 is reversed relative to PEN-2 (23), we generated constructs encoding human PEN-2 in which the PEN-2 TMD1 was substituted with the TMD2 from SREBP-1 (TMD1-SREBP) or in which the PEN-2 TMD2 was substituted with the TMD1 from SREBP-1 (TMD2-SREBP) (Fig. 4A). Coexpression of the TMD2-SREBP PEN-2 mutant with APH-1, NCT, and PS1 led to PS1 endoproteolysis (Fig. 4C, lane 4) and elevated steady-state levels of the AICD (Fig. 4E, lane 4), but the TMD1-SREBP mutant PEN-2 protein exhibited a loss of function phenotype with respect to these latter parameters (Fig. 4, C and E, lane 3) despite expression at levels comparable with the TMD2-SREBP mutant (Fig. 4B, lanes 3 and 4). At first approximation, these results suggested that although the TMD2 of PEN-2 is dispensable for mediating PS1 endoproteolysis, the PEN-2 TMD1 plays a critical role in mediating PS1 endoproteolysis.

Although these latter conclusions seemed plausible, it was also conceivable that the failure of the TMD1-SREBP PEN-2 mutant to facilitate PS1 endoproteolysis was simply due to misfolding and/or improper membrane insertion of the chimeric protein. As there is presently no available structural information to examine the issue of protein misfolding, we chose to employ a well established protease protection assay to compare the topology of the TMD1-SREBP PEN-2 mutant to WT PEN-2 (Fig. 5). For these studies, 100,000/x100 g membrane fractions were prepared from cells expressing either WT PEN-2 (Fig. 5, lanes 1–6) or the TMD1-SREBP PEN-2 variant (Fig. 5, lanes 7–12), and parallel aliquots of these preparations were incubated with increasing concentrations of proteinase K. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride, and the reaction products were analyzed by Western blot with the CT11 antibody, specific for the CT11 epitope tag placed at the carboxyl terminus, or the PNT-2 antibody that is generated against the NH2-terminal region of PEN-2 (7).
Proteinase K treatment of membranes from cells expressing WT PEN-2 resulted in the appearance of a ~6-kDa, CT11-reactive, carboxyl-terminal derivative (Fig. 5A, lanes 5 and 6, asterisk) and an ~4-kDa, PNT-2-positive, NH2-terminal fragment (Fig. 5B, lanes 5 and 6, double asterisks), findings consistent with those described previously (11). The fact that these shortened fragments are protected from proteinase K digestion confirms that the NH2- and carboxyl-terminal ends of PEN-2 are located within the lumen. Similarly, proteinase K treatment of membranes from cells expressing the TMD1-SREBP PEN-2 mutant protein led to the accumulation of an ~6-kDa, CT11-immunoreactive derivative (Fig. 5A, lanes 10 and 11, asterisk) and an ~4-kDa, PNT-2-immunoreactive fragment (Fig. 5B, lanes 10 and 11, double asterisks) that are virtually indistinguishable from the protected fragments that arise from WT PEN-2. These studies indicate that the TMD1-SREBP mutant indeed adopts a topology identical to WT PEN-2, and hence, the failure of this mutant to promote PS1 endoproteolysis is not simply a result of a confounding artifact related to improper membrane insertion.

If the topology of the TMD1-SREBP mutant mirrors that of wild-type PEN-2, why does the chimeric molecule fail to promote endoproteolysis? One scenario that we considered was that this mutant does not associate with PS1. To examine this issue, we generated independent N2a cell lines that constitutively express human WT PS1 (line N2aWT.11 (17)) and either human WT PEN-2 or a panel of human PEN-2 mutants, including TMD1-SREBP. The set of PEN-2 deletion mutants, including Δ3–9, Δ40–46, Δ85–92, or Δ93–100, were deliberately chosen because expression of these variants promoted PS1 endoproteolysis and, hence, would be expected to bind to PS1. CHAPS-solubilized lysates of each cell line were subjected to coimmunoprecipitation analysis with either PS1NT or CT11 antibodies (Fig. 6). PS1NT antibody immunoprecipitated both full-length PS1 and PS1-NTF from all cell lines as expected (Fig. 6A, lanes 7–12). Indeed, WT PEN-2 and all functional PEN-2 deletion variants were coimmunoprecipitated with PS1 under these conditions (Fig. 6B, lanes 7–11). Importantly, we did not observe coimmunoprecipitation of the TMD1-SREBP PEN-2 mutant with PS1 (Fig. 6B, lane 12) despite the fact that this mutant is expressed at levels comparable with WT PEN-2 and the PEN-2 deletion variants (Fig. 6B, compare lane 6 with lanes 1–5). We confirmed these latter findings using the CT11 antibody for coimmunoprecipitation studies. We show that the CT11 antibody immunoprecipitates WT PEN-2 and all PEN-2 mutants as expected (Fig. 6B, lanes 13–18) and that the PS1 holoprotein is coimmunoprecipitated from cells expressing other WT PEN-2 or the functional PEN-2 deletion mutants (Fig. 6A, lanes 13–17). However, we did not observe coimmunoprecipitation of the PS1 holoprotein in cells expressing TMD1-SREBP (Fig. 6A, lane 18). Collectively, our results strongly suggest but do not formally prove that the failure of the TMD1-SREBP PEN-2 mutant to facilitate endoproteolysis of the PS1 holoprotein is largely because of the inability of the mutant molecule to associate with PS1 holoproteins.

Identification of a Domain in TMD1 Critical for PEN-2 Function—Having established that the mutant TMD1-SREBP PEN-2 fails to associate with PS1 and does not promote endoproteolysis of PS1 holoproteins, one could argue that the PEN-2 TMD1 is necessary for function. To lend support to the latter conclusion, we then asked whether we could “rescue” the function of the TMD1-SREBP chimera by testing an additional series of chimeras in which the TMD2 from SREBP-1 is replaced by one-third (PSS, SPS, SSP) or two-thirds (SPP, PSP, PPS) of the authentic PEN-2 TMD1 (Fig. 7A). Coexpression of these chimeras together with APH-1, NCT, and PS1 revealed that the PPS mutant, containing the proximal two-thirds of the PEN-2 TMD1 and the distal one-third of the SREBP-1 TMD2, was the only chimeric mutant that was functional in promoting PS1 endoproteolysis and in parallel, enhanced production of the AICD at levels comparable with those seen in cells expressing WT PEN-2 (Fig. 7, C and E, lane 5 versus lane 2). All other chimeras failed to promote PS1 endoproteolysis and AICD production (Fig. 7, C and E, lanes 3 and 4 and 6–8) despite being expressed at essentially identical steady-state levels to WT PEN-2 or the PPS PEN-2 mutant (Fig. 7B). Collectively, these results indicate that the proximal two-thirds of the TMD1 of PEN-2 is functionally important for promoting PS1 endoproteolysis and activity of the γ-secretase complex.
probed with PS1NT (lysates corresponding to the 5% of the volume used for immunoprecipitation, input) were resolved on 16.5% Tris/Tricine SDS-PAGE and lanes 1–6 wild-type PEN-2, lanes 7–12 Δ52–60, lanes 13–18 Δ85–92, and lanes 19 Δ93–100. The resulting immunoprecipitates as well as total detergent extracts corresponding to the 5% of the volume used for immunoprecipitation (lanes 1–6, input) were resolved on 16.5% Tris/Tricine SDS-PAGE and probed with PS1NT (A) or CT11 (B) antibody. Fl., full-length. Molecular mass markers are shown on the left in kDa. IP, immunoprecipitate.

DISCUSSION

It is now well established that the macromolecular complex containing PS1, APH-1, NCT, and PEN-2 is responsible for intramembranous γ-secretase processing of APP-CTFs and Notch S2/NEXT to generate the AICD and S3/NICD, respectively (5–7, 24). Among these components, APH-1 and NCT form a stable subcomplex that stabilizes full-length PS1, whereas PEN-2 is required for proteolytic conversion of the stabilized PS1 holoprotein into functional PS1 fragments.

Identifying the molecular domain(s) of individual components that are responsible for the generation of the γ-secretase complex is essential for clarifying the mechanism(s) of γ-secretase complex maturation and function and potentially offers new targets for mechanism-based therapeutics. In the present study, we investigated the molecular domains of PEN-2 required for function in promoting PS1 endoproteolysis. We demonstrated previously that coexpression of PS1, APH-1, and NCT increases the level of stabilized full-length PS1 holoprotein into NTFs and CTFs only occurs by additional expression of PEN-2 (6). The γ-secretase-mediated production of the AICD is correspondingly elevated in cells that overexpress all four components of the γ-secretase complex (6). Based on these results, we assayed the function of various PEN-2 deletion and substitution variants by coexpressing these molecules with PS1, APH-1, and NCT and assessed the effects on the endoproteolysis of PS1 and the γ-secretase-mediated generation of the AICD.

The present studies now offer several important insights relevant to the domains in PEN-2 that are critical for mediating conversion of PS1 holoproteins to endoproteolytic derivatives and γ-secretase-mediated processing of APP-CTFs.

First, with the exception of the Δ10–16 and Δ52–60 mutants, expression of a variety of PEN-2 mutants that harbor small deletions (7–9 amino acids) of evolutionarily conserved regions promoted PS1 endoproteolysis, commensurate with an elevation in steady-state levels of the γ-secretase-generated AICD derivative. The Δ10–16 and Δ52–60 mutants were intrinsically unstable, a phenotype likely to reflect altered membrane insertion and/or folding because of the removal of charged amino acids contained within these segments. Supporting this notion, the steady-state levels of these mutants were successfully rescued by replacing the authentic segment with an artificial sequence that included conservative amino acid substitutions at each residue. In turn, we observed that expression of these “substitution” PEN-2 variants fully rescued PS1 endoproteolysis and production of the AICD at levels comparable with settings in which WT PEN-2 was coexpressed with PS1, APH-1, and NCT.

Second, PEN-2 mutants harboring truncations of small segments of the carboxyl-terminal domain or the entire carboxyl terminus were fully functional with respect to the conversion of the PS1 holoprotein to endoproteolytic derivatives and AICD production. However, in cells expressing the PEN-2 variant lacking the entire carboxyl terminus, we observed rapid decay of the endoproteolytic derivatives of PS1. These findings are entirely consistent with the recent report from Prokop et al. (9) that described the effects of the PEN-2 carboxyl-terminal deletion variant on PS1 endoproteolysis and stability of PS1 derivatives in PEN-2 knockout cells. In contrast, Hasegawa et al. (25) have reported that residues 90–94 and the overall length of carboxyl terminus of PEN-2 are required for binding to the other components of the γ-secretase complex, PS1 endoproteolysis, and γ-secretase activity. Although the latter study may seem discordant from the data presented by Prokop et al. (9) and those presented herein, we offer an alternative interpreta-
tion of the data in the report by Hasegawa et al. (25). In this latter study, the authors showed that PS1 endoproteolysis is rescued in PEN-2 knockdown cells by WT PEN-2 but not by the Δ90–94 PEN-2 mutant (Fig. 7). In this study, it is quite clear that expression of the Δ90–94 PEN-2 mutant in PEN-2 knockdown cell lines (#13 and #19), which show the highest levels of accumulated PS1 holoproteins and very low levels of PS1 fragments, leads to a consistent reduction in steady-state levels of PS1 holoproteins relative to the levels seen in each knockdown cell line. We interpret these data by suggesting that the Δ90–94 PEN-2 mutant is capable of promoting endoproteolysis of PS1 derivatives but that the resultant derivatives are unstable, a scenario that is entirely consistent with the data obtained by Prokop et al. (9) and ourselves. A very similar scenario can account for the data presented in Fig. 8 by Hasegawa et al. (25). In this case, expression of PEN-2 harboring larger deletions of the carboxyl terminus or the entire carboxyl terminus clearly shows lowered steady-state levels of PS1 holoproteins compared with the levels in “mock” PEN-2 knockdown cells. What is not apparent in the studies by Hasegawa et al. (25) and Prokop et al. (9) is an elevation in steady-state levels of PS1 derivatives in the PEN-2 knockdown lines transfected with the PEN-2 carboxyl-terminal deletion mutants. In contrast to these studies in PEN-2 knockdown cells, wherein PEN-2 mRNA are depleted, we clearly observe slightly higher levels of PS1 derivatives in PS1-deficient fibroblast cells coexpressing PS1, NCT, and APH-1 and the carboxyl-terminally truncated Δ85–101 PEN-2 variant compared with PS1-deficient fibroblasts expressing WT PS1 alone. In the setting of PS1-deficient fibroblasts, the steady-state levels of PEN-2

Fig. 7. The proximal two-thirds of PEN-2 TMD1 is necessary for PS1 endoproteolysis and the γ-secretase activity. A, amino acid sequences of TMDs of human PEN-2, SREBP-1, and various chimeras in which one-third (SPP, PSP, PPS) or two-thirds (PSS, SPS, SSP) of the authentic PEN-2 TMD1 was replaced with the corresponding sequences from the TMD2 of SREBP-1. B–E, HEK293 cells were transiently transfected with APPswe, APH-1, NCT, PS1, and either HA (lane 1), wild-type PEN-2 (lane 2), or indicated PEN-2 variants (lanes 3–8). Detergent lysates of the cells were analyzed by immunoblotting with CT11 (B), αPS1Loop (C), CTM2 (D), or CT15 antibodies (E). In B–E, molecular mass markers are shown on the left in kDa. Fl., full-length.
mRNA are not altered relative to wild-type fibroblasts and are translationally competent. Therefore, the expression of PS1, NCT, and APH-1 can serve to stabilize newly synthesized endogenous mouse PEN-2 molecules. Thus, a fraction of transiently expressed PS1 holoproteins are subject to endoproteolysis, and the resulting PS1 derivatives assemble into stable complexes with mouse PEN-2 and coexpressed NCT/APH-1 polypeptides. Once the limiting levels of endogenously expressed PEN-2 molecules are saturated, the remaining fraction of PS1 holoproteins that is stabilized by endogenous and exogenous APH-1/NCT is then subject to endoproteolysis by virtue of expression of mutant PEN-2, molecules that we and Prokop et al. (9) would argue are functional with respect to mediating PS1 endoproteolysis. In this latter instance, however, the resulting derivatives exhibit shortened half-lives.

Third, and perhaps most notable, is our finding that a PEN-2 chimera, in which the TMD2 is replaced by the TMD1 from SREBP-1, still retains activity but that a PEN-2 mutant, in which the TMD1 is replaced by the TMD2 from SREBP-1, is inactive. We demonstrate that the failure of this latter chimera to promote endoproteolysis of PS1 holoproteins is not simply because of altered topology of the chimera relative to WT PEN-2. However, this chimera fails to be coimmunoprecipitated with PS1, suggesting that the TMD1 of PEN-2 is essential for the interaction of PEN-2 with PS1 holoproteins. Formal proof of this model will clearly require higher resolution structural analysis.

Finally, we have identified a region that includes the proximal two-thirds of TMD1 that is critical for PEN-2-mediated endoproteolysis of PS1 holoproteins. Although compelling, we should caution that the results of our studies using PEN-2-SREBP1 chimeras may be influenced by the context of the choice of the proteins, and further analysis with additional chimeras will be necessary to verify our findings. Despite the strengths of our conclusions, our results do not offer a clear picture of the molecular mechanism(s) by which PEN-2 promotes PS1 endoproteolysis. PEN-2 does not contain any consensus protease motifs and, hence, likely exerts its effects in more indirect ways. For example, PEN-2 could bind to PS1 directly or other members of the complex and modify the conformation of PS1 to render the cleavage site more accessible to the “presenilinase” enzyme. Alternatively, PEN-2 could serve as a critical cofactor for the presenilase or perhaps recruit the presenilase to the stabilized PS1 holoprotein. In this regard, antibodies raised against the NH2-terminal domain of PEN-2 only immunoprecipitate very minor amounts of PEN-2 in n-dodecyl β-d-maltoside-solubilized membranes from stable cell lines (22), suggesting that the NHz-terminal domain might be normally masked by associated protein(s).

In summary, our findings lead us to conclude that PEN-2 has two independent functions (9) that are dependent on two different regions of the polypeptide. First, the PEN-2 TMD1 plays a critical role in binding to PS1 (and/or NCT and APH-1) and that the proximal two-thirds of this domain is essential for promoting endoproteolysis of PS1 holoproteins. Second, and supporting the conclusions by Prokop et al. (9), the carboxyl-terminal hydrophilic region of PEN-2 plays an important role in stabilizing the resulting PS1 NTF/CTF heterodimer after the cleavage. It will be critical to identify the regions in PS1, NCT, or APH-1 to which PEN-2 binds and to develop an understanding of the structural and conformational alterations in PS1 that render it susceptible to endoproteolysis.

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