Regulated Hyperaccumulation of Presenilin-1 and the “γ-Secretase” Complex

EVIDENCE FOR DIFFERENTIAL INTRAMEMBRANOUS PROCESSING OF TRANSMEMBRANE SUBSTRATES*

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Intramembranous “γ-secretase” processing of β-amyloid precursor protein (APP) and other transmembrane proteins, including Notch, is mediated by a macromolecular complex consisting of presenilins (PSs), nicastrin (NCT), APH-1, and PEN-2. We now demonstrate that in cells coexpressing PS1, APH-1, and NCT, full-length PS1 accumulates to high levels and is fairly stable. Upon expression of PEN-2, the levels of PS1 holoprotein are significantly reduced, commensurate with an elevation in levels of PS1 fragments. These findings suggest that APH-1 and NCT are necessary for stabilization of full-length PS1 and that PEN-2 is critical for the proteolysis of stabilized PS1. In N2a and 293 cells lines that stably overexpress PS1, APH-1, NCT, and PEN-2, PS1 fragment levels are elevated by up to 10-fold over endogenous levels. In these cells, we find a marked accumulation of the APP-CTF (AICD) fragment and a concomitant reduction in levels of both APP-CTFβ and CTFα. Moreover, the production of the γ-secretase-generated Notch S3/NICD derivative is modestly elevated. However, we failed to observe a corresponding increase in levels of secreted Aβ peptides in the medium of these cells. These results lead us to conclude that, although the PS1, APH-1, NCT, and PEN-2 are essential for γ-secretase activity, the proteolysis of APP-CTF and Notch S2/NEXT are differentially regulated and require the activity of additional cofactors that promote production of AICD, NICD, and Aβ.

Presenilins (PS1 and PS2)† are polytopic membrane proteins that are mutated in the majority of pedigrees with early-onset familial Alzheimer’s disease (1). Compelling evidence has emerged to support an essential role for PS in intramembranous “γ-secretase” processing of β-amyloid precursor protein (APP), the developmental signaling receptor, Notch 1, and several additional type 1 transmembrane proteins (2, 3). Intramembranous proteolysis of a set of membrane-tethered APP derivatives (APP-CTFs) leads to the production and subsequent secretion of amyloid β-peptides (Aβs), whereas proteolysis of a membrane-tethered Notch 1 derivative, termed S2/NEXT, results in the release of the Notch intracellular domain (S3/NICD). It has previously been observed that Aβ and S3/NICD production are eliminated in cells derived from mouse blastocysts with compound deletions of PS1 and PS2 (4, 5), and PS1 and PS2 have been demonstrated to photorecross-link to transition-state isosteres that inhibit γ-secretase activity (6–8). In addition, it has been found that two conserved aspartate residues within predicted transmembrane domains 6 and 7 are critical for Aβ and S3/NICD production (9–13). These findings have led to the conclusion that PS are unusual diisopartyl proteases that catalyze intramembranous proteolysis (14).

Full-length PS1 and PS2 are subject to endoproteolysis and accumulate as ~30-kDa N-terminal (NTF) and ~20-kDa C-terminal fragments (CTF) in vivo (15–17). The PS1-NTF and -CTF accumulate in a highly saturable manner (15, 18, 19); overexpression of PS does not elevate the levels of endoproteolytic derivatives (15, 19), but, rather, the vast majority of newly synthesized full-length molecules are rapidly degraded, with only a small fraction of these “precursors” being stabilized as endoproteolytic derivatives (18, 20). Our interpretation of these findings is that endoproteolysis of this fraction of PS precursors only occurs following the association with cellular factors that are expressed at limiting levels (18). PS1-NTF and -CTF remain stably associated (21, 22) along with several additional polypeptides in high molecular mass complexes that range from ~150 kDa to ~2 MDa (22–27). Biochemical studies and genetic screening strategies in Caenorhabditis elegans have led to the identification of three polypeptides that interact with PS1, namely nicastrin (NCT), APH-1, and PEN-2 (28–30). Furthermore, down-regulation of NCT expression by RNAi or genetic ablation impairs γ-secretase cleavage of APP and Notch, commensurate with reduction in the steady-state levels of PS fragments and diminished levels of high molecular weight PS complexes (28, 31–35). Furthermore, down-regulation of NCT expression by RNAi or genetic ablation impairs γ-secretase cleavage of APP and Notch, commensurate with reduction in the steady-state levels of PS fragments and diminished levels of high molecular weight PS complexes (28, 31–35). Furthermore, down-regulation of NCT expression by RNAi or genetic ablation impairs γ-secretase cleavage of APP and Notch, commensurate with reduction in the steady-state levels of PS fragments and diminished levels of high molecular weight PS complexes (28, 31–35). Furthermore, down-regulation of NCT expression by RNAi or genetic ablation impairs γ-secretase cleavage of APP and Notch, commensurate with reduction in the steady-state levels of PS fragments and diminished levels of high molecular weight PS complexes (28, 31–35).

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† The abbreviations used are: PS1/2, presenilins 1 and 2; APP, β-amyloid precursor protein; CTF, C-terminal fragment; NTF, N-terminal fragment; Aβ, amyloid β-peptide; NCT, nicastrin; RNAi, RNA interference; AICD, APP intracellular domain; RT, reverse transcription; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HEP, human embryonic kidney; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; APPswe, Swedish variant of APPswe, HA, hemagglutinin.
ficking of NCT are severely compromised and levels of APH-1

**RESULTS**

**Steady-state Levels of PS1, APH-1α, NCT, and PEN-2 Are Coordinatey Regulated**—Our initial efforts focused on the effects of APH-1, NCT, and PEN-2 on PS1 accumulation in transiently transfected mouse neuroblastoma N2a cells. APH-1α, NCT, and PEN-2 were epitope-tagged with a sequence, RFLEERP, which is recognized by CT11 antibody (57). We have verified that the CT11-tagged NCT molecule can be coimmunoprecipitated with PS1 fragments in a manner distinguishing from untagged NCT (data not shown). Moreover, we have successfully coimmunoprecipitated PS1 with CT11-tagged APH-1α or CT11-tagged PEN-2 (data not shown), data consistent with previous findings (40, 42, 51).

In transiently transfected N2a cells, we show that, in contrast to cells expressing NCT without APH-1α, the steady-state levels and, particularly, the levels of mature, fully glycosylated NCT are significantly increased in the cells coexpressing NCT and APH-1α (Fig. 1A, lane 1, compare lanes 4 and 6; 8 and 9). Similar results were obtained for PEN-2; the steady-state levels of PEN-2 are higher when coexpressed with APH-1α compared with settings in which only PEN-2 is expressed (Fig. 1A, panel II, compare lanes 5 and 7; 8 and 9). Moreover, the accumulated levels of APH-1α are higher when coexpressed with NCT compared with settings in which APH-1α is expressed without NCT (Fig. 1A, panel III, compare lanes 3 and 6; 7 and 9).

To assess whether APH-1, NCT, and/or PEN-2 are the limiting factor(s) for stabilization of PS1 and could affect the levels of accumulated PS1 fragments, we performed Western blot analysis using PS1\(_{\text{CT}}\) antibody (18). Although the levels of full-length PS1 were elevated in all cases upon transfection of human PS1, the level of PS1-NTF was not changed following coexpression with each component individually (Fig. 1A, panel IV, lanes 2-5). However, in cells that coexpress PS1 and APH-1α with either NCT (Fig. 1A, panel IV, lane 6) or PEN-2 (Fig. 1A, panel IV, lane 7), the levels of PS1-NTF were significantly elevated compared with cells transfected with PS1 alone (Fig. 1A, panel IV, lane 2). Notably, the levels of accumulated full-length PS1 were also much higher in cells expressing PS1, APH-1α, and NCT (Fig. 1A, panel IV, lane 6). Remarkably, when PEN-2 was expressed in addition to PS1, APH-1α, and NCT, the levels of PS1 fragments were elevated even further, commensurate with a marked reduction in levels of full-length PS1 (Fig. 1A, panel IV, lane 9). To verify that the effects of expressing human PS1, NCT, APH-1, and PEN-2 in mouse
neuroblastoma cells could be reproduced in other cell types, we performed an identical series of studies in HEK293 and immortalized fibroblasts from mice with homozygous deletions of PS1 (PS1/-.). We obtained essentially identical results in these cell lines to those observed in N2a cells (Fig. 1B).

The finding that coexpression of PS1, APH-1, and NCT leads to the elevated accumulation of PS1, together with increased levels of mature NCT and APH-1, led us to conclude that these molecules assemble into a stable complex and their steady-state levels are coordinately regulated. Among the components, it would appear that APH-1 serves as a scaffold that stabilizes the complex and, in the case of NCT, promotes (either directly or indirectly) exit of underglycosylated molecules to late compartments where complex oligosaccharide modification occurs. More importantly, the observation that the highest levels of PS1 fragment accumulation are achieved under conditions where PS1, APH-1, and NCT are coexpressed together with PEN-2 suggests that PEN-2 is a critical component of the machinery necessary for PS1 endoproteolysis.

Stable Overexpression of PS1 with APH-1, NCT, and PEN-2—To assay the effects of overexpressing components of the γ-secretase complex on the production of Aβ peptides and Notch S3/NICD, we generated N2a and HEK293 cell lines that stably express PS1 and its complex components. Equimolar amounts of cDNAs encoding human PS1, APH-1α, CT11-tagged PEN-2, and NCT were mixed and transfected into an N2a cell line, Sw.10 (55, 58), that constitutively expresses APPswe harboring Swedish mutation with a C-terminal myc epitope tag (APPswe). We obtained cell lines that express various combinations of transgenes. For example, in one line (ANPP.5), we detected high levels of NCT, elevated levels of both full-length PS1 and PS1 fragments, but no PEN-2 (Fig. 2A, lane 4). In the absence of available antibodies, we cannot assay the levels of APH-1α in the cell lines. However, RT-PCR analysis confirmed the expression of human APH-1α mRNA in ANPP.5 line (Fig. 2A, panel IV, lane 4). Hence, stable overexpression of NCT and APH-1 without PEN-2 in the N2aANPP.5 line, led to the accumulation of full-length PS1 and endoproteolytic derivatives, a result fully consistent with the transient transfection assays (Fig. 1). More importantly, the cell lines that stably express PS1, NCT, PEN-2, and APH-1α exhibit marked elevation in accumulated PS1 fragments, and very low levels of full-length PS1 (ANPP.1 and ANPP.7, Fig. 2A, lanes 2 and 5).

To confirm the observations in stable N2a cell lines, we stably transfected cDNAs encoding PS1, APH-1α, CT11-tagged PEN-2, and NCT into an HEK293 cell line, 293Sw.3, that constitutively expresses APPswe. We identified one line, ANPP.8, that hyperaccumulates PS1 fragments to extremely high levels, in parallel with abundant levels of accumulated mature NCT, PEN-2, and APH-1α (Fig. 2B, lane 2). In addition,
line ANP.24, expressing APH-1α, NCT, and PS1 but not PEN-2, exhibited high levels of both full-length PS1 and PS1 fragments (Fig. 2B, lane 4), results similar to those obtained in transient transfection assays (Fig. 1B) and in the N2aANP.5 line (Fig. 2A). Interestingly, reprobing the blot with PNT-2 antibody, which recognizes epitopes within the N-terminal domain of PEN-2 (51), revealed that in PP.22 cells that overexpress PS1 and CT11-tagged PEN-2, expression of endogenous PEN-2 was almost completely eliminated (Fig. 2A, panel IV, compare lanes 1 and 3). This “replacement” phenomenon is similar to those reported for PS1 fragments, mature NCT, and APH-1 (15, 33, 45) and further supports the notion that levels of these proteins are coregulated by association with other “limiting” components of the complex. It should be noted that in contrast to PP.22 cells, we did not observe replacement of endogenous PEN-2 in ANPP.8 cells (Fig. 2B, panel IV, lane 2). Our interpretation of this finding is that, in ANPP cells that overexpress all components of the PS1 complex, the factors are now not limiting and, hence, able to stabilize both endogenous and exogenous PEN-2. Consistent with this interpretation, we observed that in ANP.24 lines that overexpress APH-1α, NCT, and PS1, endogenous PEN-2 accumulated to even higher levels (Fig. 2B, panel IV, compare lanes 1 and 4). Thus, we posit that the stability and, hence, steady-state accumulation of PEN-2 are in large part dependent on the coexpression of PS1, APH-1, and NCT.

Our demonstration that full-length PS1 accumulates in cells that overexpress PS1 along with APH-1α and NCT suggested that APH-1α might serve as a scaffold that “primes” PS1 for endoproteolytic cleavage, whereas expression of additional PEN-2 is necessary for promoting endoproteolysis. One prediction from this model is that both full-length PS1 and endoproteolytic derivatives might be stable in cells overexpressing PS1, APH-1α, and NCT. To test this possibility, we treated HEK293 lines ANPP.8 and ANP.24 with the protein synthesis inhibitor, cycloheximide, and assessed the levels of full-length PS1 and

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**Fig. 2. Stable overexpression of PS1/APH-1/NCT/PEN-2.** A, detergent lysates of stable N2a cell lines expressing APH-1α/NCT/PEN-2/PS1 (ANPP.1 and 7; lanes 2 and 5), PS1 (P.2; lane 3), APH-1/NCT/PS1 (ANP.5; lane 4), NCT/PEN-2 (NP.8; lane 6), or APH-1α/NCT (AN.22; lane 7) as well as parental cell line (Sw.10; lane 1) were analyzed by immunoblotting with PS1NT (panel I), NCT54 (panel II), or CT11 (panel III) antibody. For APH-1α expression, cytoplasmic RNA was isolated and amplified by RT-PCR with human APH-1α-specific primers (panel IV). B, detergent lysates of stable HEK293 cell lines expressing APH-1α/NCT/PEN-2/PS1 (ANPP.8; lane 2), PS1/PEN-2 (PP.22; lane 3), or APH-1α/NCT/PS1 (ANP.24; lane 4), as well as parental cell line (Sw.3; lane 1) were analyzed by immunoblotting with PS1NT (panel I), NCT54 (panel II), CT11 (panel III), or PNT-2 antibody (panel IV). Expression of APH-1α in these cells was analyzed by RT-PCR (panel V). C, stable HEK293ANPP.8 (lanes 1–6) or ANP.24 lines (lanes 7–12) were treated with cycloheximide (30 μg/ml) for the intervals indicated. Detergent lysates were prepared and analyzed by immunoblotting with PS1NT antibody. Molecular mass markers are in kDa.
PS1 fragments over time. In contrast to the very short (<1.5 h) half-life of full-length PS1 in line ANPP.8 (Fig. 2C, lanes 1–6), we now find that the half-life of full-length PS1 in line ANP.24 is apparently extended to ~6 h (Fig. 2C, lanes 7–12). We speculate that the degree to which full-length PS1 can be "stabilized" will be determined by the relative levels of coexpressed NCT and APh-1 in those cells. Unfortunately, we were not able to generate multiple independent lines expressing differing levels of PS1, APh-1, and NCT, and, hence, this proposal remains to be verified. In any event, our results offer the suggestion that APh-1 serves as a scaffold protein that stabilizes full-length PS1 and NCT and that PEN-2 is an essential component of the apparatus that is responsible for PS1 endoproteolysis.

Analysis of APP Processing in Stable Cell Lines—The finding that PS1 fragments hyperaccumulate in ANPP lines leads to the prediction that PS1-mediated γ-secretase processing of APP-CTFs should be elevated in these cells. To test this hypothesis, we performed metabolic labeling and immunoprecipitation studies to evaluate APP processing in the stable cell lines.

Because the transgenes were introduced into clonal N2a or 293 lines that harbor integrated copies of APPswe, these "lines" now consist of populations of cells that express the human APPswe polypeptide at differing levels. As expected, the synthetic rate of human APPswe (by 10 min pulse-labeling) and steady-state levels (after 4 h labeling) are variable between the N2a lines (Fig. 3A, panels I and II). Immunoprecipitation analysis of radiolabeled cell lysates using the CT15 antibody, revealed a significant reduction of APP-CTFmycβ and APP-CTFmycα in lysates of ANPP.1 and ANPP.7 lines (Fig. 3A, panel III, lanes 2 and 4; quantified in Fig. 3B, panels I and II). Interestingly, we observed a striking elevation in the levels of ~7 kDa APP-CTFmycγ-, or AICD in these cells (Fig. 3A, panel III, lanes 2 and 4; quantified in Fig. 3B, panel III). Despite the differences in levels of intracellular AICD polypeptides, however, the levels of secreted Aβ peptides in the conditioned medium of ANPP lines 1 and 2, when normalized to APP synthetic levels, were not significantly different from the parental Sw.10 line, or other lines that express PS1 and varying combinations of other components (Fig. 3A, panel V; quantified in Fig. 3B, panel IV).

We also observed some variation in levels of newly synthesized APPswe (10 min pulse) between the 293 cell lines (Fig. 3C, panel I). However, in lysates of 293ANPP.8 line, that expresses the highest levels of PS1 fragments, we observed high levels of AICD and marked diminution in levels of APP-CTF-

It has been reported that transient expression of APP-CTFβ (or C99) along with PEN-2 in a Drosophila S2 cell line that stably expresses PS1, APh-1, and NCT (S2 ANP cells) leads to an elevation of secreted Aβ peptides compared with S2 ANP cells that transiently express C99, alone (41). To validate this finding in mammalian cells, we transiently transfected 293ANP.24 cells with APPswe cDNA in combination with either cDNA encoding PEN-2, or a control cDNA encoding influenza hemagglutinin (HA). Compared with 293ANP.24 cells that express HA, we observed a small increase in the levels of PS1 fragments and a corresponding decrease in levels of full-length PS1 in 293ANP.24 cells transiently expressing PEN-2 (Fig. 4A, lanes 1 and 2). In parallel, we observed a marked increase in the levels of AICD that is accompanied by a marked reduction in levels of APP-CTFβ and CTFs in these cells (Fig. 4B, lanes 1’ and 2’). Despite this, the levels of Aβ peptides in the medium are not elevated in any significant way (Fig. 4C, lanes 1’ and 2’; quantified in Fig. 4D). Thus, in stable 293 ANPP cells that express all four components of the complex, or 293 ANP cells transiently coexpress PEN-2, we fail to demonstrate an elevation in levels of secreted Aβ peptides.

APP-CTFβ Is a Substrate for γ-Secretase-mediated Generation of Aβ in ANPP Cells—Having failed to observe elevations in secreted Aβ in ANPP cells, we considered the possibility that the population of membrane-tethered APP-CTFβ, the substrates for γ-secretase-mediated production of Aβ, is limiting. To examine this issue, we transiently transfected 293ANPP.8, or 293PP.22 cell lines with cDNA encoding HA as a control, or APPswe, and examined the levels of AICD and secreted Aβ. As we have shown above (Fig. 3C), Western blot analysis with CT15 antibody revealed that the levels of AICD are elevated and those of CTFmycα and CTFmycβ are reduced in ANPP.8 cells compared with PP.22 cells (Fig. 5A, lanes 1 and 2). After transient overexpression of APPswe, the level of AICD is elevated in both cell lines but still much higher in ANPP.8 cells compared with PP.22 cells (Fig. 5A, lanes 3 and 4). Parallel Western blot analysis of conditioned medium using the 26D6 antibody revealed that steady-state levels of secreted Aβ peptides are considerably elevated in medium of both cell lines transfected with APPswe cDNA (Fig. 5C, lanes 1 and 2) compared with the same cell lines transfected with cDNA encoding HA (Fig. 5C, lanes 1, 2, 1’, and 2’). Thus, in ANPP cells, membrane-tethered APP-CTFs are fully competent to serve as substrates to generate more Aβ peptides. However, the relative level of secreted Aβ in medium of ANPP.8 versus PP.22 lines following transient expression of APPswe is different than that observed in medium of HA transfected cells (Fig. 5C, compare lanes 3 and 4 with 1’ and 2’, respectively; quantified in Fig. 5D). Hence, under conditions of substrate (i.e. APP-CTFβ) excess, the γ-secretase activity in ANPP.8 cells is capable of generating additional Aβ peptides, but despite the marked elevation in steady-state PS1 levels in these cells, we did not observe a commensurate increase in levels of secreted Aβ peptides.

Modest Enhancement of γ-Secretase Processing of Notch in ANPP Lines—In view of our demonstration that AICD is elevated in lysates of cells that hyperaccumulate PS1 fragments and components of the PS1 complex, and the similarity in the relative positioning of this processing event to the scissile sites responsible for γ-secretase processing of Notch (46–48), we evaluated intramembranous processing of Notch in these cell lines. HEK293 or N2a cell lines were transiently transfected with APPswe cDNA (Fig. 5C, lanes 3 and 4) versus PP.22 lines following transient expression of APPswe is different than that observed in medium of HA transfected cells (Fig. 5C, compare lanes 3 and 4 with 1’ and 2’, respectively; quantified in Fig. 5D). Hence, under conditions of substrate (i.e. APP-CTFβ) excess, the γ-secretase activity in ANPP.8 cells is capable of generating additional Aβ peptides, but despite the marked elevation in steady-state PS1 levels in these cells, we did not observe a commensurate increase in levels of secreted Aβ peptides.

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APP-CTFβ is a substrate for γ-secretase-mediated generation of Aβ in ANPP cells—Having failed to observe elevations in secreted Aβ in ANPP cells, we considered the possibility that the population of membrane-tethered APP-CTFβ, the substrates for γ-secretase-mediated production of Aβ, is limiting. To examine this issue, we transiently transfected 293ANPP.8, or 293PP.22 cell lines with cDNA encoding HA as a control, or APPswe, and examined the levels of AICD and secreted Aβ. As we have shown above (Fig. 3C), Western blot analysis with CT15 antibody revealed that the levels of AICD are elevated and those of CTFmycα and CTFmycβ are reduced in ANPP.8 cells compared with PP.22 cells (Fig. 5A, lanes 1 and 2). After transient overexpression of APPswe, the level of AICD is elevated in both cell lines but still much higher in ANPP.8 cells compared with PP.22 cells (Fig. 5A, lanes 3 and 4). Parallel Western blot analysis of conditioned medium using the 26D6 antibody revealed that steady-state levels of secreted Aβ peptides are considerably elevated in medium of both cell lines transfected with APPswe cDNA (Fig. 5C, lanes 1 and 2) compared with the same cell lines transfected with cDNA encoding HA (Fig. 5C, lanes 1, 2, 1’, and 2’). Thus, in ANPP cells, membrane-tethered APP-CTFs are fully competent to serve as substrates to generate more Aβ peptides. However, the relative level of secreted Aβ in medium of ANPP.8 versus PP.22 lines following transient expression of APPswe is different than that observed in medium of HA transfected cells (Fig. 5C, compare lanes 3 and 4 with 1’ and 2’, respectively; quantified in Fig. 5D). Hence, under conditions of substrate (i.e. APP-CTFβ) excess, the γ-secretase activity in ANPP.8 cells is capable of generating additional Aβ peptides, but despite the marked elevation in steady-state PS1 levels in these cells, we did not observe a commensurate increase in levels of secreted Aβ peptides.
Fig. 3. Metabolic labeling of stable cells expressing PS1/APH-1/NCT/PEN-2. A, stable N2a lines were metabolically labeled with \[^{35}\text{S}\]methionine for 10 min (panel I) or 4 h (panels II–V) and cell lysates (panels III–IV) or conditioned medium (panels IV–V) were immunoprecipitated with P2-1 (panel I), CT15 (panels II and III), or 26D6 antibody (panels IV–V) and resolved on 16.5% Tris/Tricine SDS-PAGE. The radioactive bands were visualized by phosphorimaging. B, levels of APP-CTFs and secreted A\(\beta\) were quantified by phosphorimaging and normalized to the synthetic level of APP. The normalized values are expressed as a percentage of those in parental Sw.10 line and the average (\(\pm\) S.E.) of four (lines NP8 and AN.22) to seven (other lines) separate samples are shown. C, stable HEK293 lines were metabolically labeled with \[^{35}\text{S}\]methionine for 10 min (panel I) or 4 h (panels II–IV) and cell lysates (panels I–II) or conditioned medium (panels III–IV) were immunoprecipitated with P2-1 (panel I), CT15 (panel II), or 26D6 antibody (panels III–IV) and resolved on 16.5% Tris/Tricine SDS-PAGE. The radioactive bands were visualized by phosphorimaging. D, levels of APP-CTFs and secreted A\(\beta\) were quantified by phosphorimaging and normalized to the synthetic level of APP in each line. The normalized values are expressed as a percentage of those in parental Sw.3 line and the average (\(\pm\) S.E.) of seven separate samples are shown. In A and C, molecular mass markers are in kDa.
NICD in ANPP lines is not as robust as the conversion of APP-CTFs to AICD. In view of earlier reports showing that NICD is susceptible to proteasomal degradation (60, 61), we also analyzed NAE levels in the presence of lactacytin. The relative increase in accumulated NICD in the presence of lactacytin was not appreciably different between the lines (Fig. 6E), arguing against differences in the stability of NICD between cell lines examined. These findings would argue that, although AICD production is elevated in ANPP cells that hyperaccumulate PS1 fragments, APH-1, NCT, and PEN-2, processing of Notch is only modestly elevated.
FIG. 6. Effects of hyperaccumulated PS1 fragments on the γ-secretase processing of NotchΔE. A, parallel dishes of HEK293Sw.3 (lanes 1 and 2), ANPP.8 (lanes 3 and 4), and PP.22 (lanes 5 and 6) lines transiently transfected with NotchΔE cDNA were pulse-labeled with [35S]methionine for 20 min and either harvested immediately (lanes 1, 3, and 5) or chased for 2 h with excess cold methionine (lanes 2, 4, and 6). Detergent lysates were prepared and immunoprecipitated with the myc15 antibody, and the radioactive bands were visualized by phosphorimaging. B, levels of NICD and NotchΔE after chase period were quantified by phosphorimaging and expressed as NICD/(NICD + NotchΔE). The normalized values are expressed as a percentage of those in parental Sw.3 line and the average (± S.E.) of three (PP.22) or five (Sw.3 and ANPP.8 lines) separate samples are shown. C, parallel dishes of N2aSw.10 (lanes 1 and 2), ANPP.1 (lanes 2 and 7), P.2 (lanes 3 and 8), ANPP.7 (lanes 4 and 9), and NP.8 (lanes 5 and 10) lines were transiently transfected with NotchΔE cDNA and pulse-labeled with [35S]methionine for 20 min and either harvested immediately (lanes 1–5) or chased for 2 h (lanes 6–10). Detergent lysates were immunoprecipitated with the myc15 antibody, and the radioactive bands were visualized by phosphorimaging. D, levels of NICD and NotchΔE after the chase period were quantified by phosphorimaging and expressed as NICD/(NICD + NotchΔE). The normalized values are expressed as a percentage of those in parental Sw.10 line and the average (± S.E.) of three (P.2 and NP.8) or four (other lines) separate samples are shown. E, parallel dishes of 293Sw.3 (lanes 1–6), 293ANPP.8 (lanes 7–12), N2aNP.8 (lanes 13–15), or N2aANPP.7 (lanes 16–18) lines were transiently transfected with NotchΔE cDNA, pulse-labeled with [35S]methionine for 20 min, and harvested immediately (lanes 1, 2, 7, 8, 13, and 16) or chased for 1 h (lanes 3, 4, 9, 10, 14, 15, 17, and 18) or 2 h (lanes 5, 6, 11, and 12). Detergent lysates were immunoprecipitated with the myc15 antibody, and the radioactive bands were visualized by phosphorimaging. Lactacystin was added to a final concentration of 10 μM during pulse-labeling and chase media. F, levels of NICD and NotchΔE after the chase period were quantified by phosphorimaging and expressed as NICD/(NICD + NotchΔE). In A, C, and E, molecular mass markers are in kDa.
It is now well established that the macromolecular complex that includes PS1 is responsible for intramembranous processing of APP-CTFs and Notch S2/NEXT to generate Aβ peptides and S3/NICD, respectively. Three additional components of the γ-secretase complex, NCT, APH-1, and PEN-2, have been identified (28–30). Genetic, or RNAi-mediated reduction in expression of these components reduces the levels of other factors in the complex and results in compromised production of Aβ peptides and S3/NICD (24, 29, 39, 40, 51). In addition, all three components have been shown to coexist with PS1 in high molecular weight assemblies (24, 41, 42, 45). In the present report, we offer several important insights into the regulated assembly of the PS1 complex and the role of this assembly on γ-secretase processing of APP-CTFs and Notch S2/NEXT.

First, and in view of the demonstration that PS1 is subject to endoproteolytic processing and that the accumulation of resulting fragments is highly regulated and saturable, we tested the prediction that the steady-state levels of PS1 derivatives are governed by the association with molecules expressed in limiting levels (15, 18–20). To this end, we assessed the extent to which coexpression of PS1 with APH-1, NCT, and PEN-2 singly, or in combination, modulates the levels of PS1 derivatives. We report that coexpression of PS1 with APH-1 and NCT or APH-1 and PEN2, leads to elevated levels of PS1 derivatives. Furthermore, coexpression of APH-1 increases the steady-state level of NCT, especially the mature, fully glycosylated form, and PEN-2, suggesting that APH-1 is a critical limiting factor to stabilize the PS1 complex. Notably, when PS1 is coexpressed with APH-1 and NCT, full-length PS1 also accumulates to a higher level, and these polypeptides exhibit stability not much different from PS1 fragments. Interestingly, when PEN-2 is additionally expressed, the levels of full-length PS1 are significantly reduced, commensurate with a marked elevation in levels of PS1 fragments. These results suggest that APH-1 and NCT are involved in the stabilization of full-length PS1 and that PEN-2 plays a critical role in the endoproteolytic cleavage of stabilized PS1 protein, a conclusion that was independently reached by Luo et al. (51) and Takasugi et al. (41) using RNAi approaches while this report was in preparation. The one or more mechanisms by which PEN-2 facilitates endoproteolytic cleavage of PS1 are not clear at present. Because PEN-2 does not contain domains homologous to known proteases, it is likely to play an accessory role in the endoproteolysis of PS1. For example, PEN-2 could serve as a critical cofactor for the “presenilinase” enzyme or modify the conformation of the PS1 complex to render the scissile bond more accessible to presenilinase. Our findings confirm our earlier prediction that the regulated, saturable accumulation of PS1-NTF and CTF is mediated by the association with cellular factors that are expressed at limiting levels. In addition, although it remains possible that additional components of the complex are yet to be identified, a tentative conclusion from these studies is that APH-1, NCT, and PEN-2 are components of the minimal complex necessary for establishing the steady-state levels of PS1 fragments.

Second, we demonstrate that the levels of PEN-2 are regulated in a very similar manner as that described for PS1, mature NCT, and APH-1 (15, 33, 45). In this case, stable overexpression of an epitope-tagged human PEN-2 results in reduced levels of accumulated endogenous PEN-2. This replacement phenomenon for PEN-2 further supports the notion that levels of these proteins are coregulated by association with other limiting components of the complex. Interestingly, in ANPP cells that coexpress PS1, APH-1, NCT, and epitope-tagged PEN-2, we did not observe replacement of endogenous PEN-2. We interpret these findings to suggest that, in ANPP cells that overexpress all components of the PS1 complex, the factors are now not limiting and, hence, able to stabilize both endogenous and exogenous PEN-2. Consistent with this interpretation, in ANP cells that overexpress PS1, APH-1α, and NCT, we observed that endogenous PEN-2 accumulates to higher levels than cells expressing endogenous levels of the complex.

Third, we have tested the hypothesis that elevated levels of the protease, be it PS1 alone or in a complex, enhance production of Aβ peptides, AICD, and S3/NICD. In stable ANPP cell lines that coexpress APH-1, NCT, PEN-2, and PS1, wherein exogenous PS1 fragment levels are elevated by up to 10-fold over endogenous levels, there is a consistent and dramatic increase in the production of AICD accompanied by marked reduction of APP-CTFβ and CTFs. Furthermore, γ-secretase-mediated proteolysis of S2/NEXT to S3/NICD was also elevated, although the extent of increase is not as robust as that of AICD observed in these cells. These results strongly suggest that hyperaccumulated PS1 complex does indeed reach the compartments where membrane-tethered substrates (APP-CTFs and S2/NEXT) reside and that the complex plays an essential role in catalyzing proteolysis of these substrates to generate AICD and NICD, respectively. Therefore, it appears that APH-1, NCT, PEN-2, and PS1 constitute the minimal core of the γ-secretase enzyme complex to generate AICD and NICD. Surprisingly, we have failed to observe a corresponding increase in levels of secreted Aβ peptides in the conditioned medium of these cell lines. These results are in striking contrast to three very recent reports (27, 41, 62) showing the increased generation of Aβ peptides upon overexpression of four components of the PS1 complex. It should be noted that two of these studies employed an in vitro assays consisting of exogenous substrates added to detergent-solubilized membrane preparations from stably transfected 293 cells, or a yeast strain that coexpresses all four components (27, 62). However, neither study reported on the levels of secreted Aβ from living cells. On the other hand, Takasugi and colleagues (41) revealed that transient coexpression of PEN-2 and C99 in Drosophila S2 cells that stably overexpress PS1, APH-1, and NCT lead to an increase of ~2-fold in secretion of Aβ peptides. Because the levels of APP-CTFs, the penultimate substrates of Aβ, are markedly reduced in our ANPP cells, we explored the possibility that these substrates are limiting because of excessive proteolysis by hyperaccumulated PS1/γ-secretase that generates AICD. To examine this issue, we provided additional levels of APP substrates by transiently transfecting APPswe. However, whereas the levels of secreted Aβ peptides are markedly increased in APP-transfected ANPP cells compared with untransfected cells, there is no difference in the extent to which Aβ peptides are elevated over cells that express PS1 at endogenous levels. Finally, and to ask whether we could reproduce the findings of Takasugi et al. (41) in mammalian cells, we transiently transfected PEN-2 CDNA into 293ANP.24 cells. In this setting, we show that the levels of PS1 fragments are elevated and that the levels of AICD are markedly elevated, findings very similar to those seen in stable ANPP cells. However, we failed to detect an elevation in the levels of secreted Aβ peptides in the medium of these transiently transfected cells. At present, we can neither offer a satisfying explanation for the apparently discordant data on Aβ production obtained from the in vitro solubilized membrane preparations (27, 62) and the in vivo assay system (this report), nor can we find an appealing scenario to accommodate our failure to replicate, in mammalian cells, the data in Drosophila S2 ANP cells showing an elevation in Aβ peptide production by transient overexpression of PEN-2 (41).
Collectively, our findings support the model that overexpression of PS1 fragments and associated components of the complex have differential effects on production of Aβ, NICD, and AICD. The significance of this observation in relation to the potential physiological function(s) of APP and AICD, or the pathogenesis of Alzheimer’s disease, is not known. However, our present demonstration that the generation of AICD can be dissociated from the production of Aβ peptides strongly argues that Aβ and AICD production are not temporally linked events but rather two separately regulated, proteolytic events. These findings also suggest that, although molecular components constituting the minimal core of the γ-secretase complex have been identified, we consider it likely that additional cofactors are required that modulate γ-secretase activity in a substrate-selective manner. It is conceivable that these cofactors could alter γ-secretase activity at the level of post-translational modifications, conformational alterations of individual components of the complex, or subunit stoichiometries. In this regard, recent studies by Phiel and colleagues (63) have demonstrated that in both in vitro and in vivo settings, inhibition of glycosyl synthase kinase 3α activity leads to dramatic reductions in secretion of Aβ peptides, without any apparent alteration in γ-secretase processing of Notch S2/NEXT to generate S3/NICD.

In any event, our proposal offers the attractive hypothesis that our present demonstration that the generation of AICD can be selectively inhibited in vivo, and that AICD production are not temporally linked events but rather two separately regulated, proteolytic events. These findings also suggest that, although molecular components constituting the minimal core of the γ-secretase complex have been identified, we consider it likely that additional cofactors are required that modulate γ-secretase activity in a substrate-selective manner. It is conceivable that these cofactors could alter γ-secretase activity at the level of post-translational modifications, conformational alterations of individual components of the complex, or subunit stoichiometries. In this regard, recent studies by Phiel and colleagues (63) have demonstrated that in both in vitro and in vivo settings, inhibition of glycosyl synthase kinase 3α activity leads to dramatic reductions in secretion of Aβ peptides, without any apparent alteration in γ-secretase processing of Notch S2/NEXT to generate S3/NICD. In any event, our proposal offers the attractive hypothesis that the therapeutic modalities designed to selectively inhibit γ-secretase-mediated production of Aβ peptides might be identified that do not incur potentially detrimental consequences of inhibiting intramembranous processing of other substrates that are essential for modulating important physiological functions during development and in aging.

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