Evidence That Assembly of an Active γ-Secretase Complex Occurs in the Early Compartment of the Secretory Pathway*

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The γ-secretase complex, consisting of presenilins (PS), nicastrin (NCT), APH-1, and PEN-2, catalyzes the intramembranous proteolysis of truncated β-amyloid precursor protein (APP) and Notch derivatives to generate the APP intracellular domain (AICD) and Notch intracellular domain (NICD), respectively. To examine the intracellular sites in which active γ-secretase resides, we expressed NCT variants harboring either an endoplasmic reticulum (ER) retention signal (NCT-ER) or a trans-Golgi network (TGN) targeting motif (NCT-TGN) along with PS1, APH-1, and PEN-2 and examined γ-secretase activity in these settings. In cells expressing NCT-ER and the other components, PS1 fragments hyperaccumulated, but AICD levels were not elevated. On the other hand, upon coexpression of an ER-retained APP variant or a constitutionally active Notch mutant, NÄE, we observed enhanced production of AICD or NICD, respectively, in cells expressing NCT-ER. Moreover, we show that membranes from cells expressing NCT-ER, NCT-TGN, or NCT-WT contain identical levels of PS1 derivatives that can be photoaffinity cross-linked to a biotinylated, benzophenone-derivatized γ-secretase inhibitor. Finally, our cell-free γ-secretase assays revealed nearly equivalent γ-secretase activities in cells expressing PS1, APH-1, PEN-2, and either NCT-WT or NCT-ER. Taken together, we interpret these findings as suggesting that active γ-secretase complex is generated in the early compartments of the secretory pathway but that these complexes are transported to late compartments in which substrates are encountered and subsequently processed within respective transmembrane segments.

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§ The abbreviations used are: APP, β-amyloid precursor protein; Aβ, amyloid β peptides; AICD, APP intracellular domain; CTF, carboxyl terminal fragment; ER, endoplasmic reticulum; NCT, nicastrin; NICD, Notch intracellular domain; NTF, amino terminal fragment; PS, presenilins; TGN, trans-Golgi network; WT, wild type; CHAPSO, 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

We have previously demonstrated that stable coexpression of PS1, APH-1, NCT, and PEN-2 (ANPP) leads to hyperaccumulation of PS1 derivatives to levels ~10-fold higher than endogenous levels. In these cell lines, we observed robust enhancement in the levels of APP intracellular domain (AICD) as well as a modest elevation in the production of Notch intracellular domain (NICD) (8). However, we failed to demonstrate a corresponding increase in the levels of secreted Aβ. We suggested that the differential intracellular distributions of γ-secretase complex and/or its substrates have an impact on the relative levels of products generated in the reactions in vitro. In this regard, the intracellular compartments in which the γ-secretase complex is assembled and stabilized are still somewhat uncertain. To address this issue, we targeted the γ-secretase complex to specific intracellular compartments by modifying the NCT cytoplasmic domain to include signals known to target type I membrane proteins either to the ER or TGN. We now show that coexpression of NCT with an ER retention signal (NCT-ER) with PS1, APH-1, and PEN-2 led to the hyperaccumulation of PS1 derivatives but that the generation of AICD derived from Swedish mutant APP (APP$_{Swe}$) was not elevated in these cells, compared with cells expressing wild type NCT (NCT-WT), PS1, APH-1 and PEN-2. However, when APP harboring the ER retention signal, or a constitutionally active Notch mutant, NÄE, was coexpressed with NCT-ER, PS1, APH-1 and PEN-2, we observed enhanced production of AICD or NICD, comparable with cells expressing NCT-WT, PS1, APH-1, and PEN-2. Indeed, we demonstrate that identical levels of PS1 derivatives can be photoaffinity cross-linked to a bisubtilylated benzophenone-derivatized γ-secretase inhibitor in whole cell membranes from cells expressing PS1, APH-1, PEN-2, and either NCT-WT, NCT-ER, or NCT-TGN. Furthermore, cell-free reconstitution assay using membrane fractions in the presence of an exogenous APP C100 substrate revealed nearly equivalent γ-secretase activities in cells expressing PS1, APH-1, PEN-2, and either NCT-WT or NCT-ER. These results suggest that the “active” γ-secretase complex is generated in the early compartments of the secretory pathway, but that this complex encounters and subsequently facilitates proteolysis of
membrane-tethered substrates only in late compartments of the secretory apparatus.

**EXPERIMENTAL PROCEDURES**

**Constructions**—The cDNAs encoding human NCT, CT11-tagged PEN-2, PS1, Myc epitope-tagged NAT, and Myc epitope-tagged Swedish variant APP695 (pCB6-APP695) were described previously (8, 13). APP695 cDNA (without a Myc epitope tag) was subcloned into pAG3Zeo vector by PCR to generate pAG3-APP695. The construct encoding Myc epitope-tagged APH-1aL was kindly provided by Dr. Gang Yu (12) and subcloned into pAG3Zeo vector. The cDNAs encoding human NCT or APP695 were modified by PCR-based mutagenesis to put additional amino acids (KKLN for the ER retention signal (14) or SDYQRL for the TGN targeting signal (15)) at the carboxyterminal end. The sequences of each cDNA were verified by sequencing.

**Antibodies**—The following antibodies described previously (8) were used for the present study. Polyclonal antibody NCT54 was generated against a glutathione S-transferase fusion protein containing residues 242–546 of human NCT; PS1NT and aPS1Loop antibodies are polyclonal antibodies that recognize residues 1–65 and 320–375 of PS1, respectively; CT15 was generated against a peptide corresponding to the last 15 amino acids of APP; 2D6 is a monoclonal antibody that reacts with Aβ residues 1–12; 9E10 antibody was used to detect Myc epitope-tagged Notch ΔE derivatives.

**Cell Culture, Transfection, and Western Blot**—HEK293 cells or immortalized fibroblasts from mice with homozygous deletions of PS1 (PS1−/−) were transfected using LipofectAMINE Plus (Invitrogen). 48–72 h after transfection, cells were lysed in immunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) supplemented with a protease inhibitor mixture (8). Cell lysates or conditioned medium was subject to SDS-PAGE, and fractionated proteins were transferred to nitrocellulose membrane (Schleicher and Schuell) prior to incubation with selected antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence detection (ECL) system (PerkinElmer Life Sciences).

**Photoaffinity Cross-linking**—Membrane fractions were prepared as described (16) and solubilized with 0.4% CHAPSO. Solubilized membranes were then incubated with 1 μM L-852,505 (17), a photoactive, biotinylated derivative of the aspartyl protease transition state analogue L-685,458, at 4 °C for 1 h followed by irradiation with 365 nm UV light, using a Stratalinker (Stratagene), on ice for 90 min. Biotinylated proteins were captured with streptavidin-agarose beads (Pierce) at 4 °C and eluted by incubation at 95 °C for 5 min in Laemmli-SDS sample buffer. Eluted proteins as well as 1/5th of the input was fractionated by SDS-PAGE and analyzed by Western blot analysis with aPS1Loop antibody.

**In Vitro γ-Secretase Activity Assay**—Membrane fractions were prepared as described (18) and assayed in the presence of 0.25% CHAPSO using recombinant C100-FLAG as a substrate (18). The reactions were terminated by adding radioimmune precipitation buffer and boiling for 5 min. The samples were subject to centrifugation, and Aβ peptides in the supernatant fraction were quantified using an ECL-based assay (18).

**RESULTS AND DISCUSSION**

To gain a better understanding of cellular sites of γ-secretase assembly, we directed NCT to different subcellular compartments by introducing discrete modifications into its cytoplasmic tail. The addition of a dilysine (KK) motif at the −3 and −4 amino positions from the end of type I membrane proteins effectively retains these proteins in the ER (14), whereas the addition of SDYQRL signal from TGN-38 targets membrane proteins to late compartments of the secretory apparatus (15). We generated DNA-encoded mutant NCT containing these modifications and transfected either NCT-WT or these mutant constructs into HEK293 cells together with cDNAs encoding PS1, APH-1, and PEN-2. Immunoblot analysis of detergent lysates of transfected cells with NCT54 antibody (8) revealed that NCT-WT and NCT-TGN migrated as an ~110-kDa endoglycosidase H-sensitive immature form and heterogeneous ~120-kDa endoglycosidase H-resistant mature forms (Fig. 1A, lanes 4 and 6). However, NCT-ER failed to undergo complex glycosylation to generate a mature form but instead mostly accumulated as an immature form (Fig. 1A, lane 5), suggesting that the NCT-ER mutant does not reach the late compartments where complex glycosylation occurs. As we have shown previously (8), coexpression of PS1, APH-1, PEN-2, and NCT-WT leads to the hyperaccumulation of PS1 fragments (Fig. 1B, lane 4) compared with cells expressing PS1 alone (Fig. 1B, lane 3). Notably, in cells coexpressing NCT-ER or NCT-TGN with PS1, APH-1, and PEN-2, accumulated levels of PS1-CTF (Fig. 1B, upper panel, lanes 4 and 6) and NTF (Fig. 1B, lower panel, lanes 5 and 6) are not markedly elevated. The extent of hyperaccumulation of PS1 fragments was not significantly different among cells expressing NCT-WT, NCT-ER, or NCT-TGN with the three other components, suggesting that the stabilization and endoproteolysis of PS1 can occur in early compartments of the secretory pathway.

Next, we examined whether the complexes consisting of PS1, APH-1, PEN-2, and either NCT-WT, NCT-ER, or NCT-TGN could promote γ-secretase-mediated proteolytic conversion of APP-CTFs to AICD. Consistent with our earlier studies (8), coexpression of PS1, APH-1, PEN-2, and NCT-WT led to the form (Fig. 1A, lane 1), suggesting that the NCT-ER mutant does not reach the late compartments where complex glycosylation occurs. As we have shown previously (8), coexpression of PS1, APH-1, PEN-2, and NCT-WT leads to the hyperaccumulation of PS1 fragments (Fig. 1B, lane 4) compared with cells expressing PS1 alone (Fig. 1B, lane 3). Notably, in cells coexpressing NCT-ER or NCT-TGN with PS1, APH-1, and PEN-2, accumulated levels of PS1-CTF (Fig. 1B, upper panel, lanes 4 and 6) and NTF (Fig. 1B, lower panel, lanes 5 and 6) are not markedly elevated. The extent of hyperaccumulation of PS1 fragments was not significantly different among cells expressing NCT-WT, NCT-ER, or NCT-TGN with the three other components, suggesting that the stabilization and endoproteolysis of PS1 can occur in early compartments of the secretory pathway.
marked reduction of APP-CTFmycβ and CTFmycα and significant elevation of AICD (Fig. 1C, lower panel, compare lanes 3 and 4). The levels of AICD were also higher in cells overexpressing NCT-TGN and the other components of the complex (Fig. 1C, lane 6), suggesting that post-Golgi compartments are sites at which APP-CTFs are processed to generate AICD. In contrast, coexpression of NCT-ER with PS1, APH-1, and PEN-2, failed to increase the level of AICD (Fig. 1C, lane 5), despite the fact that PS1 fragments hyperaccumulated to similar levels to that observed in cells expressing NCT-WT and the complex components. The levels of secreted Aβ peptides in vivo were not increased with expression of PS1, APH-1, NCT, and PEN-2 (Fig. 1D, compare lanes 3 and 4) as we described previously (8) and were not significantly different between cells expressing the different NCT targeting variants (Fig. 1D, lanes 4–6).

Why is the complex consisting of NCT-ER and the other components incapable of promoting γ-secretase processing of APP-CTFs to generate AICD? A simple explanation is that the γ-secretase substrates, APP-CTFβ and CTFα, known to be generated in late compartments of the secretory pathway, are not accessible to the active γ-secretase complex localized in the ER. To examine this issue, we chose to provide the NCT-ER complex with an APP substrate that is also ER-retained. Here, we coexpressed the APP Swedish variant (APPSwe) with the dilsine ER targeting signal (APPSwe-ER) together with NCT-ER and the other components. Although APPSwe is subject to β-secretase processing in the Golgi apparatus, we speculated that extended residence of this substrate in the ER would lead to enhanced processing by catalytically active pro-BACE1 (19) and, perhaps, newly synthesized TACEα/secretase and subsequent generation of CTFβ and CTFα. Indeed, we show that the levels of APP-CTFs corresponding to CTFβ11 and CTFα are elevated in cells expressing APPSwe-ER (Fig. 2A, lane 6 versus 1). Consistent with previous results (Fig 1C), the levels of AICD were markedly elevated in cells expressing PS1, APH-1, PEN-2, and either NCT-WT or NCT-TGN when coexpressed with wild type nontagged APPSwe or TGN-targeted APPSwe compared with cells expressing PS1 alone (Fig. 2A, lanes 3 and 5 versus 2; lanes 11 and 13 versus 10), whereas coexpression of NCT-ER with the other three components of the complex failed to elevate production of AICD (Fig. 2A, lanes 4 and 12). When we coexpressed APPSwe-ER with NCT-WT and the three other components of the γ-secretase complex, the levels of AICD generated were much lower compared with cells expressing wild type APPSwe and NCT-WT, PS1, APH-1, and PEN-2 (Fig. 2A, lane 7 versus 3). Interestingly, the levels of AICD that accumulate in cells that coexpress APPSwe-ER, NCT-ER, PS1, APH-1, and PEN-2 were much higher than those seen in cells coexpressing APPSwe-ER and PS1 (Fig. 2A, compare lanes 6 and 8), suggesting that the retention of the γ-secretase complex (NCT-ER, PS1, APH-1, and PEN-2) and the substrate (APPSwe-ER) in early compartments leads to enhanced proteolysis of APP-CTFs to generate AICD. In fact, in cells that coexpress NCT-WT, NCT-ER, or NCT-TGN in combination with the three remaining components and APPSwe-ER, the levels of AICD generated were extremely comparable (Fig. 2A, compare lanes 7–9).

To determine whether intramembranous processing of APP-CTFs by the ER-retained γ-secretase complex was unique to this substrate, and to ask whether our results could be extended to another cell type, we evaluated the intramembranous processing of a Myc epitope-tagged version of the Notch-related membrane-tethered substrate NΔE in PS1-deficient fibroblasts. As γ-secretase processing of the truncated Notch derivative occurs in a ligand-independent, “constitutive” manner (20), we reasoned that this molecule would encounter the γ-secretase complex in the ER and during transit to the plasma membrane. As we showed earlier (8, 21), expression of PS1 in these cells enhances production of NICD, and these levels are further elevated upon coexpression of PS1, APH-1, PEN-2, with NCT-WT (Fig. 2B, compare lanes 4 and 3). Remarkably, the level of NICD generated in cells expressing PS1, APH-1, PEN-2, and NCT-ER was comparable with that in cells expressing NCT-WT or NCT-TGN with the other three components of the complex (Fig. 2B, compare lanes 4–6). These data on the processing of NΔE were indistinguishable from those observed in transfected HEK293 cells (data not shown). Collectively, these results indicate that the γ-secretase complex assembled in the ER is fully capable of promoting intramembranous proteolysis of APP-CTFs and NΔE to generate AICD and NICD, respectively, as long as the substrates are accessible to the enzyme complex.

Although these latter data supported the notion that the γ-secretase complex assembled in the ER can promote intramembranous proteolysis of APP-CTFs and NΔE, it is known that only a small fraction of PS1 complexes participates in γ-secretase processing (22). Hence, it was conceivable that in the setting of hyperaccumulation of the complex, small increases in active γ-secretase are sufficient to drive conversion of substrates to completion. To examine the levels of active γ-secretase in cells expressing PS1, APH-1, PEN-2, and
NCT-WT or NCT targeting variants, we used two independent assays. In the first, we prepared membranes from cells that coexpress PS1, APH-1, and PEN-2 and either NCT-WT, NCT-ER, or NCT-TGN and incubated these preparations at 4 °C for 60 min with 0.25% CHAPSO and 1 mM L-685,458 (23). Mixtures were subject to photoactivation at 350 nm on ice for 90 min, and biotinylated polypeptides were recovered with immobilized streptavidin and subjected to Western blot analysis with αPS1Loop antibody. Significantly elevated levels of PS1-CTF are labeled by the photoprobe in membranes from cells expressing PS1 alone (Fig. 3A, lane 7 versus 6). Importantly, the levels of PS1-CTF that were recovered with streptavidin are not significantly different among membranes from cells expressing NCT-WT, NCT-ER, or NCT-TGN (Fig. 3A, lanes 7–9), suggesting that the levels of active γ-secretase in each complex are independent of the intracellular sites in which they reside. Second, and to further confirm that the γ-secretase complex present in the ER is fully active as an enzyme, we measured the in vitro γ-secretase activity using recombinant C100-FLAG as a substrate. Membrane fractions prepared from cells overexpressing PS1 alone, PS1-APH-1-PEN-2-NCT-WT, or PS1-APH-1-PEN-2-NCT-ER were incubated with C100-FLAG peptides with or without the γ-secretase inhibitor L-685,458, and the levels of Aβ peptides were measured as described (18). Coexpression of PS1, APH-1, PEN-2, and NCT-WT led to a marked increase of in vitro γ-secretase activity, which was fully inhibited by the specific γ-secretase inhibitor L-685,458 (Fig. 3B). More importantly, membranes from cells expressing PS1, APH-1, PEN-2, and NCT-ER exhibited in vitro γ-secretase activity comparable with that from cells expressing NCT-WT with the other three components of the γ-secretase complex (Fig. 3B), further supporting our view that the γ-secretase complex assembled in the ER is active in promoting intramembranous proteolysis of APP-CTFs.

In the present study, we offer several important insights into the assembly and activity of γ-secretase. First, we show that expression of NCT-ER with PS1, APH-1, and PEN-2 leads to the hyperaccumulation of active PS1 fragments and that the levels of γ-secretase activity in membranes from these cells are equivalent to those observed in cells that coexpress NCT-WT, PS1, APH-1, and PEN-2. These findings indicate that the stabilization and assembly of the γ-secretase complex can occur in early compartments of the secretory pathway. However, we failed to observe the expected increase in the proteolytic conversion of APP-CTFs to AICD, an observation we interpret as suggesting that the substrates are not accessible to γ-secretase localized in the ER. In contrast, when coexpressed with an ER-targeted APP mutant or a constitutionally active Notch variant, NÆ, we observed that γ-secretase complex in the ER is capable of catalyzing proteolytic conversion of APP-CTFs and NÆ to AICD and NICD, respectively. Indeed, cell-free assays using recombinant C100-FLAG revealed that the γ-secretase complex assembled in the ER is active as the enzyme. It is not presently clear whether active γ-secretase complex assembled in the ER contributes to the generation of Aβ peptides under standard conditions. However, a fraction of the amyloidogenic Aβ42 peptides are known to be generated in the ER, but these peptides are retained in intracellular compartments and are generated independent of PS expression (24). On the other hand, overwhelming evidence has accrued to suggest that γ-secretase cleavage of APP-CTFs, Notch S2/NEXT, and presumably other γ-secretase substrates occurs in late compartments of the secretory pathway, including the TGN, endosomes, and plasma membrane (for review, see Ref. 1). Thus, although the bulk of PS1 is localized to the ER in mammalian cells, and presumably is associated with other components of complex, these species are largely “inert” because the substrates are unavailable to be processed. Indeed, we show that by forcing membrane-tethered substrates to encounter the PS1 complex in the ER, these molecules are subject to intramembranous proteolysis. Taken together with studies showing that a fraction of PS1 is engaged in an active γ-secretase complex (22), that low levels of endogenous PS1 are detectable on the plasma membrane (25), and the compelling demonstration that in addition to expression in the ER, PS1 and complex components are present in “lipid raft” microdomains in endosomes and post-Golgi compartments (26), our study suggests that active γ-secretase complex is likely assembled in early compartments of the secretory pathway but that this complex must be transported to late compartments in order to access those substrates that are subject to intramembranous proteolysis. It
will be critical to identify the mechanism(s) by which PS and complex components are transported to late compartments and the biochemical and cellular events responsible for the encounter between \( \gamma \)-secretase and its substrates.

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