THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 13, Issue of April 1, pp. 12967–12975, 2005
Printed in U.S.A.

Aph-1 Contributes to the Stabilization and Trafficking of the γ-Secretase Complex through Mechanisms Involving Intermolecular and Intramolecular Interactions*

Received for publication, August 26, 2004, and in revised form, January 3, 2005
Published, JBC Papers in Press, January 11, 2005, DOI 10.1074/jbc.M409829200

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γ-Secretase cleaves type I transmembrane proteins, including β-amyloid precursor protein and Notch, and requires the formation of a protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2 for its activity. Aph-1 is implicated in the stabilization of this complex, although its precise mechanistic role remains unknown. Substitution of the first glycine within the transmembrane G*XXX*G motif of Aph-1 causes a loss-of-function phenotype in *Caenorhabditis elegans*. Here, using an untranslated region-targeted RNA interference/rescue strategy in *Drosophila* Schneider 2 cells, we show that Aph-1 contributes to the assembly of the γ-secretase complex by multiple mechanisms involving intermolecular and intramolecular interactions depending on or independent of the conserved glycines. Aph-1 binds to nicastrin forming an early subcomplex independent of the conserved glycines within the endoplasmic reticulum. Certain mutations in the conserved G*XXX*G motif affect the interaction of the Aph-1·nicastrin subcomplex with presenilin that mediates trafficking of the presenilin·Aph-1·nicastrin tripartite complex to the Golgi. The same mutations decrease the stability of Aph-1 polypeptides themselves, possibly by affecting intramolecular associations through the transmembrane domains. Our data suggest that the proper assembly of the Aph-1·nicastrin subcomplex with presenilin is the prerequisite for the trafficking as well as the enzymatic activity of the γ-secretase complex and that Aph-1 functions as a stabilizing scaffold in the assembly of this complex.

Mutations in presenilin (PS)[1] genes account for the majority of early onset familial Alzheimer's disease cases, causing an overproduction of those amyloid β peptides (Aβ) ending at position 42 (Aβ42) that most readily form amyloid deposits (1). Aβ is derived from the β-amyloid precursor protein (βAPP) through sequential cleavages by β- and γ-secretases (2). Genetic and biochemical studies suggest that PS is essential for the γ-secretase-mediated intramembrane cleavage of βAPP. Additional type 1 transmembrane proteins (*e.g.* notch, ErbB4, and CD44) also are cleaved by γ-secretase to release intracellular domains that harbor biological activities (3). These data suggest that PS-dependent γ-cleavage is involved in a novel mode of intramembrane proteolysis-dependent signal transduction.

PS is a highly conserved, polytopic integral membrane protein that spans the membrane eight times and undergoes endoproteolysis to generate amino- and carboxyl-terminal fragments (reviewed in Ref. 4). The endoproteolytic fragments of PS form a heterodimer and are then incorporated into a highly stabilized, high molecular weight (HMW) protein complex, whereas the PS holoprotein forms a low molecular weight complex and is rapidly degraded (5, 6). We have shown by a systematic mutational analysis that the stabilization and HMW complex formation of PS are required for γ-secretase activity, which is mediated by the carboxyl terminus of PS (7–9). PS carries two highly conserved intramembrane aspartates that are required for the γ-secretase activity (10). Moreover, solubilized γ-secretase activity is recovered in HMW fractions associated with PS fragments, and transition state analogue γ-secretase inhibitors covalently label PS fragments but not PS holoproteins (11–13). These results suggest that the stabilized HMW PS complex harbors γ-secretase activity and that the paired intramembrane aspartates in PS fragments serve as the active center of the γ-secretase, which functions as a novel type of membrane-bound aspartic protease.

The molecular composition and the mechanism of assembly of the γ-secretase complex have remained elusive. However, recent studies have identified several putative PS complex components. Nicastrin (Nct) is a type 1 transmembrane protein harboring a large ectodomain that is highly glycosylated in mammalian cells (14). Fully glycosylated Nct is selectively associated with PS fragments, and genetic studies indicate a requirement of Nct for γ-secretase activity (15). Genetic studies in *Caenorhabditis elegans* and *Drosophila melanogaster* have revealed two additional polytopic membrane proteins, Aph-1 and Pen-2, that also are required for the γ-secretase activity

* This work was supported by grants-in-aid from the Ministry of Education, Science, Culture and Sports for the 21st Century Center of Excellence Program and by the Program for Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (PMDA), Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a postdoctoral fellowship for research abroad from the Japan Society for the Promotion of Science.

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[1] The abbreviations used are: PS, presenilin; Aβ, amyloid β peptide; βAPP, β-amyloid precursor protein; CDS, coding sequence; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CHX, cycloheximide; dsRNA, double-stranded RNA; ER, endoplasmic reticulum; FL, full-length or holoprotein; GFP, green fluorescent protein; HMW, high molecular weight; mt, mutant; Nct, nicastrin; RNAi, double-stranded RNA-mediated interference; SC100, the carboxyl-terminal 99 amino acid fragment of βAPP fused to a signal peptide of rat preproenkephalin cDNA; TGN, *trans*-Golgi network; TMD, transmembrane domain; UTR, untranslated region; wt, wild-type.

(16, 17). We and others have shown that overexpression of Aph-1 together with Nct facilitates the formation of a stabilized but γ-secretase-inactive HMW complex containing PS holoproteins and that Pen-2 subsequently facilitates the formation of PS fragments, conferring γ-secretase activity (18–22). Biochemical studies support the notion that a membrane protein complex comprised of these four proteins represents the minimal framework of γ-secretase (23, 24). Recently, it was reported that Aph-1 preferentially interacts with immature Nct to form an intermediate subcomplex during the early biosynthetic process of γ-secretase in mammalian cells (25–27), suggesting that the Aph-1·Nct subcomplex functions as a stabilization factor for the γ-secretase complex.

To investigate the mechanistic roles of Aph-1 in the formation of the PS complex, we focused on the conserved glycine residues within transmembrane domain 4 (TMD4) that form a G*XXX*G motif. In *C. elegans*, a glycine to aspartate substitution of the initial glycine residue of this motif causes the *aph-1(or28)* allele, which is a recessive, maternal effect mutation (16). *Aph-1* mutant worms lack the anterior pharynx through a defect in Notch signaling, suggesting that this glycine is obligatory for Aph-1 function. It was shown that the G*XXX*G motif of mammalian Aph-1a is critical for the assembly and activity of γ-secretase (28, 29). However, the specific protein-protein interactions and stoichiometry of individual components as well as the molecular basis for the stabilizing effect of Aph-1, including the mechanistic role of the G*XXX*G motif, remain unknown. Notably, the amount of exogenous γ-secretase component proteins that can acquire proper functions is limited, because they need to compete with the endogenous proteins for binding with other components in a proper stoichiometry and acquire stability to form a functional γ-secretase complex (30, 31). Furthermore, the presence of multiple homologues/isoforms of Aph-1 in the mammalian genome that form different complexes containing either PS1 or PS2 (32) may confound the results of overexpression experiments of Aph-1. To overcome these technical obstacles, we developed a complementation assay using 3′-untranslated region (UTR)-targeted RNAi knockdown followed by transfection of the coding sequence of mutant Aph-1 with artificial UTR derived from an expression vector in *Drosophila* S2 cells, the latter harboring single presenilin (*Psn*) or *Aph-1* genes, respectively, to minimize the effect of endogenous proteins. Using this system, we analyzed the role of Aph-1 in γ-secretase complex formation, as well as the effect of mutations in the G*XXX*G motif on the function and metabolism of Aph-1.

MATERIALS AND METHODS

Construction of Expression Plasmids—Expression plasmids for Psn, Nct-V5, Aph-1-FLAG, hemagglutinin-Pen-2, and the carboxyl-terminal 99-amino acid fragment of βAPP fused to a signal peptide of rat preproenkephalin cDNA (SC100) were generated as described previously (9, 19). cDNAs encoding Aph-1/G112D, Aph-1/G112F, Aph-1/G112A, Aph-1/G112L, Aph-1/G116D, Aph-1/G116A and Aph-1/G116L were generated by the long-PCR protocol. All constructs were sequenced using Thermosequenase (Amersham Biosciences) on an automated sequencer (Li-Cor, Lincoln, NE).

Cell Culture and Transfection—Maintenance of *Drosophila* Schneider (S2) cells, production of the double-stranded RNA (dsRNA), and transient transfection were done as described previously (9, 19). Short interfering RNA was designed by the siDirect program (33, 34) using the primers 5′-GCUUUUGUAUAACAUUAUAAA-3′ (sense) and 5′-UAUAAUGUUAUACAAAAGCUA-3′ (antisense). 0.1 nmol of RNA duplex (per well in a 6-well plate) was transfected into S2 cells in short interfering RNA experiments. Stable transformants in S2 cells were generated by transfection of cDNAs together with pCoHygro or pCoBlast vector (Invitrogen) (ratio of transfected cDNAs was 2:0.1 μg) using Cellfectin and selected in S2 medium containing hygromycin (Wako) or blastcidin (Invitrogen) at 250 or 5 μg/ml, respectively.

Antibodies and Immunochemical Analyses—Two rabbit polyclonal antibodies to Psn, anti-GDN1 and anti-GDL1, were generated as described (9). The rabbit polyclonal antibody against the carboxyl-terminal region of *Drosophila* Nct, SdN2, was raised against a KLH-conjugated synthetic peptide (CRSEVLFEDLPA) and affinity-purified. The rabbit polyclonal antibodies C4 against the cytoplasmic carboxyl terminus of human βAPP, MLO7 against GM130, and JSEE1 against syntaxin 5 were kindly provided by Drs. Y. Ihara (University of Tokyo) and M. Lowe (The University of Manchester), respectively. The other antibodies were purchased from Invitrogen (anti-V5 monoclonal), Sigma (anti-V5 polyclonal, anti-FLAG M2 monoclonal, and anti-FLAG polyclonal), Stressgen (anti-KDEL), Transduction Laboratories (anti-Rab8, anti-syntaxin 6, and anti-adaptin-γ), and Roche Diagnostics (anti-HA 3F10 monoclonal). Sample preparations and analyses, including immunoblot analysis and co-immunoprecipitation, were performed as described previously (7–9, 19, 35–40).

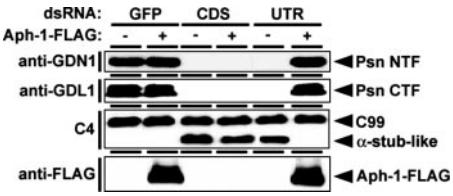

FIG. 1. **UTR-targeted RNAi in S2 cells.** Immunoblot analysis of S2 cells stably expressing SC100 transfected with dsRNAs against GFP, Aph-1 CDS, or UTR with (+) or without (−) co-transfection of a cDNA encoding wild-type Aph-1 tagged with FLAG (as indicated at the *top*). Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with each antibody (as indicated at the *left*). Note that the decrease in the levels of Psn fragments (*arrowheads*) as well as the accumulation of the βAPP carboxyl-terminal stub (*α-stub-like*) was observed in cells transfected with dsRNA against the CDS or the UTR regions of Aph-1. Exogenous Aph-1 was expressed in UTR-targeted knockdown cells, whereas CDS-targeted RNAi abolished the expression of exogenous proteins.

RESULTS

Establishment of a Complementation Assay by 3′-UTR-targeted RNAi Knock-down in Drosophila S2 Cells—To minimize the effect of endogenous Aph-1 proteins in the analyses of the exogenous mutant Aph-1, we generated short interfering RNAs targeted to the 3′-UTR of the *Drosophila Aph-1* gene using a computer-based prediction program (33, 34). UTR-targeted RNAi in S2 cells expressing SC100, the carboxyl-terminal 99-amino acid fragment of βAPP fused to a signal peptide of rat preproenkephalin cDNA, decreased the levels of Psn fragments (*i.e.* amino-terminal fragment or carboxyl-terminal fragment) in a similar manner to that observed in cells treated with coding sequence (CDS)-targeted RNAi (Fig. 1). Furthermore, a robust accumulation of APP carboxyl-terminal stubs (*i.e.* the α-stub-like; see Fig. 1) and a significant reduction in Aβ secretion (24.9 ± 1% of those with GFP-RNAi) were observed (data not shown; see Fig. 2*B*). These data suggest that the UTR-targeted RNAi against the *Aph-1* gene caused a reduction in the levels of endogenous Aph-1 proteins, thereby leading to the breakdown of the γ-secretase complex and inhibition of its activity. We next expressed the wild-type Aph-1 protein that is encoded by an expression plasmid containing the CDS of *Aph-1* with a vector-derived artificial UTR. As expected, exogenous proteins were expressed in cells despite UTR-targeted RNAi, whereas these proteins were abolished by the CDS-targeted RNAi (Fig. 1). These data were consistent with the previous reports demonstrating that transitive RNAi is absent in *Drosophila* (41, 42). Moreover, in UTR knockdown cells, the levels of Psn fragments as well as the γ-secretase activity were completely restored by the exogenous wild-type (wt) Aph-1 protein (Fig. 2, *A* and *B*). These data suggested that the complementation assay in cells treated with UTR-targeted RNAi enables us to analyze the activity and metabolism of exogenous Aph-1 proteins under the Aph-1-null phenotype in *Drosophila* S2 cells.

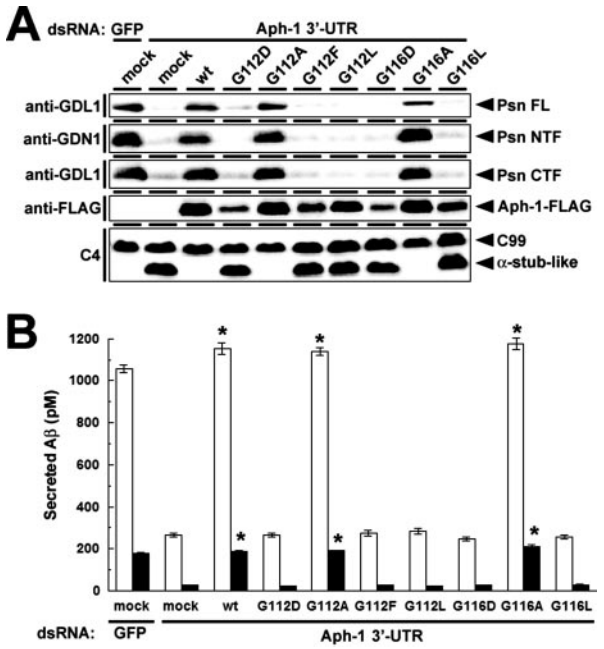

FIG. 2. **Effect of glycine substitutions in the G*XXX*G motif of Aph-1 on γ-secretase complex formation and γ-secretase activity.** *A*, immunoblot analysis of S2 cells transiently transfected with cDNAs encoding wt or mt Aph-1 (as indicated at the *top*) together with dsRNAs. Cell lysates were analyzed by immunoblotting with each antibody (as indicated at the *left*). *B*, enzyme-linked immunosorbent assay quantitation of Aβ40 (*open columns*) and Aβ42 (*filled columns*) secreted from knockdown SC100 cell lines expressing wt or mt Aph-1. *Bars* represent the mean ± S.E. in four independent experiments, and the names of transfected Aph-1 cDNAs and dsRNAs are indicated *below the columns*. Aβ secretion was significantly increased exclusively in knockdown cells expressing wt Aph-1 (*asterisk* represents statistically significant compared with mock-transfected knockdown cell ($p < 0.001$) as analyzed by Dunnett test) but not mt Aph-1.

Certain Mutations of the Conserved Glycines in TMD4 of Aph-1 Abolish the Formation of the Active γ-Secretase Complex—Amino acid sequence alignment of Aph-1 in different organisms showed that all known Aph-1 proteins contain a highly conserved amino acid motif within the proximal portion of TMD4, which is comprised of the GL*XX*GX*X*(S/H)(G/A) sequence (16, 28). The substitution of the first glycine residue of this sequence to aspartate (G122D) has been linked to a loss-of-function mutation in *C. elegans* (16). To gain insight into the role of this glycine residue in the metabolism of the γ-secretase complex, we transiently transfected Aph-1/G112D, which is mutated at an equivalent residue to the G122D mutant of *C. elegans*, into S2 cells deficient in endogenous Aph-1. Immunoblot analysis revealed that Aph-1/G112D failed to restore Psn fragments, suggesting that substitution of the first glycine of the G*XXX*G motif abolished the formation of γ-secretase complex (Fig. 2*A*). Conserved residues in TMD4 comprise two G*XXX*G motifs, known as a transmembrane helix-helix packing motif (43, 44). Previous data from a TOXCAT assay that investigated TMD association in biological membranes using artificial TMD fused with transcription factors indicated that the replacement of glycine with small side-chain amino acids (*e.g.* alanine or serine) in the G*XXX*G motif facilitated the oligomerization of TMDs, whereas bulky amino acids (*e.g.* tyrosine or phenylalanine) or hydrophilic residues destabilized the TMD association (45). Furthermore, large aliphatic residues such as leucine, isoleucine, or valine are disfavored at the positions of the glycines within the G*XXX*G motif, although these are the most frequent residues in TMDs (46). To elucidate the roles of glycines in the G*XXX*G motif of TMD4 of Aph-1, we further transfected Aph-1 harboring G112A, G112F, G112L, G116D, G116A, or G116L substitutions into the Aph-1 knockdown cells. Immunoblot analysis revealed that G112F, G112L, G116D, or G116L mutant (mt) Aph-1 failed to rescue the Psn fragments just as G112D mt Aph1 failed, whereas mt Aph-1 harboring the alanine substitution (*i.e.* G112A or G116A) restored the levels of Psn fragments to a similar extent as those restored by wt Aph-1 (Fig. 2*A*). In addition, the steady-state levels of Aph-1 polypeptides were lower in G112D, G112F, G112L, G116D, or G116L mutants as compared with those in wt or alanine mt Aph-1. To further characterize the effect of the glycine mutations of Aph-1 on γ-secretase activity, we analyzed the metabolism of SC100 and quantitated the levels of Aβ secreted from knockdown cells transiently expressing SC100. Mutants that were unable to rescue the Psn fragments (*e.g.* G112D) also failed to diminish the accumulation of the α-stub-like by knockdown of *Aph-1*, whereas alanine mutants rescued these fragments in a similar manner as that of wt Aph-1 (Fig, 2*A*). Similarly, overexpression of wt Aph-1 as well as the alanine mutants led to a complete rescue of Aβ secretion (Fig. 2*B*) (*e.g.* 109.0 ± 1.0% for wt Aph-1, 107.9 ± 1.8% for G112A, and 111.2 ± 2.5% for G116A, respectively, in Aβ40 secretion compared with those with GFP-RNAi). In contrast, the levels of secreted Aβ from Aph-1 knockdown cells transfected with mt Aph-1 (*i.e.* G112D, G112F, G112L, G116D, and G116L) were not recovered (25.3 ± 0.9, 25.8 ± 1.4, 26.7 ± 1.2, 23.4 ± 0.9, and 24.0 ± 0.8%, respectively, in Aβ40 secretion as compared to those with GFP-RNAi), suggesting that these mutations cause a loss-of-function in γ-secretase activity. These results suggest that the small size and hydrophobic nature of the conserved glycine residues within TMD4 of Aph-1 are important for the function of Aph-1 to facilitate the generation of the active γ-secretase complex.

Certain Mutations of the Conserved Glycines of Aph-1 Abolish the Formation of a Functional γ-Secretase Complex through Assembly with Psn or Pen-2 but Not with Nct—We next established S2 cell lines stably expressing wt or mt Aph-1 (*i.e.* G112D, G112F, G112L, G116D, or G116L) together with Nct and Pen-2 (wt or mt ANPen cells, respectively). Overexpression of wt Aph-1 together with other components resulted in a significant increase in the levels of Psn fragments as described previously (19). However, the levels of the Psn fragments, Nct, and Pen-2, in mt ANPen cells were lower than those in wt ANPen cells (Fig. 3*A*, *left, lysate*) as observed in transient rescue experiments in Aph-1 knockdown cells (Fig. 2*A*). To gain more insight into the roles of these mutations of the conserved glycines, we performed co-immunoprecipitation assays in ANPen stable cell lines (Fig. 3*A*, *right*). Endogenous Psn as well as exogenously transfected Nct or Pen-2 were co-precipitated with FLAG-tagged wt Aph-1, consistent with our previous results (19). However, none of the glycine mt Aph-1 interacted with endogenous Psn or transfected Pen-2, whereas mt Aph-1 polypeptides were stably associated with Nct regardless of the glycine mutations. We further treated ANPen cells by UTR-targeted RNAi and confirmed that overexpression of mt Aph-1 failed to restore the levels of Psn under these conditions (data not shown). To examine whether Psn interacts with mt Aph-1, we transiently transfected Psn into mt ANPen stable cell lines together with UTR-targeted RNAi to knockdown endogenous Aph-1. Despite a robust accumulation of the Psn holoprotein (also called full length or FL) upon overexpression of Psn with UTR-targeted Aph-1 RNAi, the levels of Psn fragments, Nct, Aph-1, or Pen-2 were not altered in mt ANPen cells, whereas Psn fragments were increased in wt ANPen cells upon the transfection of Psn (Fig. 3*B*, *left, input*; compare the wt columns without (−) and with (+) Psn). We then performed co-immunoprecipitation assays on lysates of these cells; for these exper-

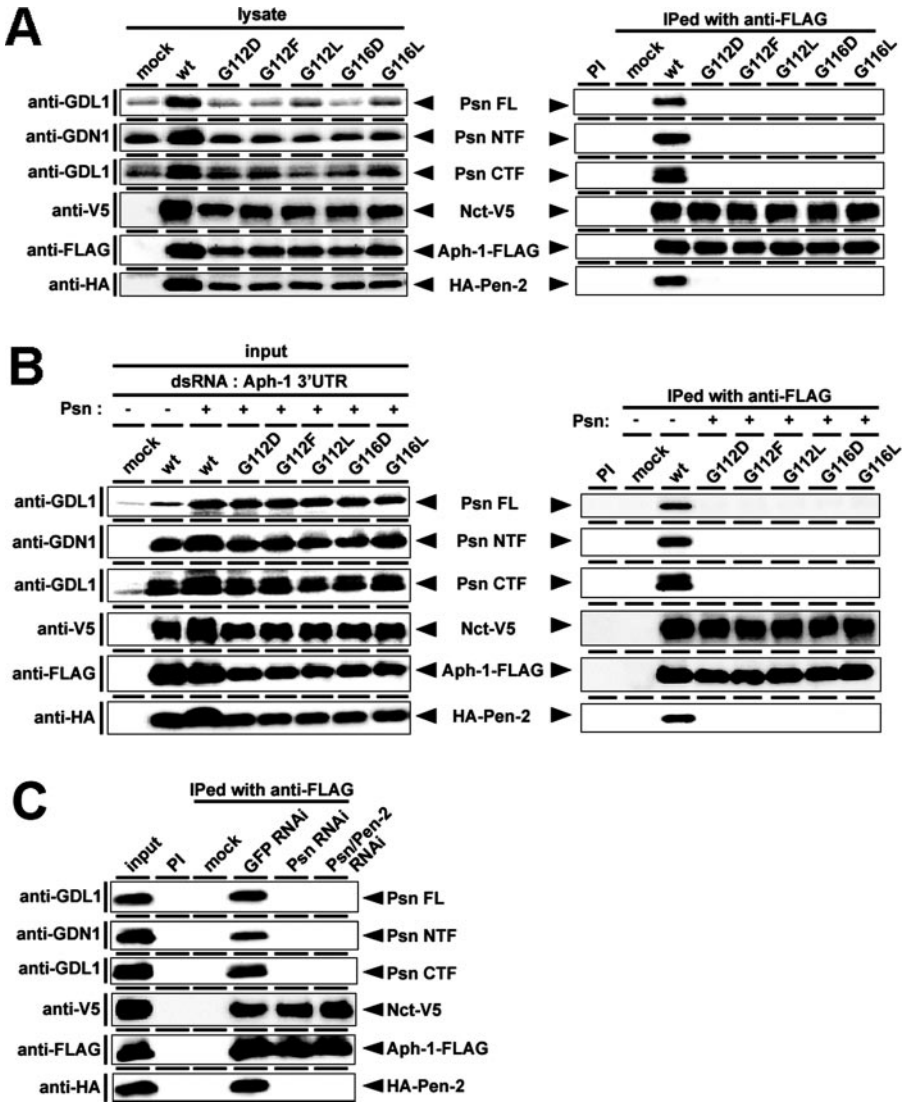

FIG. 3. **Effects of glycine mutation of Aph-1 on the interaction of γ-secretase complex components.** *A*, co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from ANPen cells stably expressing wt or mt Aph-1 (as indicated at the *top of each section*) without RNAi knockdown of endogenous Aph-1. Cell lysates containing equal levels of wt or mt Aph-1 proteins (cell lysates show the steady-state level of wt Aph-1 in ANPen cells; *left section*, labeled *lysate*) were precipitated by preimmune serum (*PI*) or anti-FLAG antibody and then analyzed by immunoblotting (*right section*, labeled *IPed* (immunoprecipitated) *with anti-FLAG*) with each antibody as indicated. *B*, co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from wt or mt ANPen cells transiently transfected with dsRNA for UTR sequences of Aph-1 with (+) or without (−) the concomitant transfection of Psn cDNA. Immunoblot analysis of input samples for immunoprecipitation is shown in the *left section* (labeled *input*). Note that co-immunoprecipitation of Psn with mt Aph-1 is not observed in mt ANPen cells expressing Psn (+), whereas lower level of Psn FL present in wt ANPen cells without (−) transfection of Psn (*left section*) is bound to wt Aph-1 (*right section*). *C*, co-immunoprecipitation analysis in multiple RNAi (*i.e.* Psn, Psn, and Pen-2)-treated ANPen cells. *HA*, hemagglutinin.

iments we prepared lysates that contained equivalent levels of mt Aph-1 (~5-fold total proteins) as compared with those of Aph-1 in wt ANPen cells treated with UTR-targeted RNAi based on an estimation by immunoblot analysis of input samples (Fig. 3*B*, *left*). For co-immunoprecipitation experiments, we used wt ANPen cells treated with UTR-targeted RNAi without (−) transfection of Psn (Fig. 3*B left, input*) instead of those transfected with (+) Psn to keep the cellular Psn at moderate levels. Although the levels of exogenous Psn FL in samples from mt ANPen cells were higher than the levels of endogenous Psn FL in wt ANPen cells, and the levels of the Psn fragments were similar (Fig. 3*B*, *left*, *input*), neither Psn (FL or fragments) nor Pen-2 polypeptides were co-immunoprecipitated with mt Aph-1. In sharp contrast, Nct was again co-precipitated with Aph-1 regardless of the mutations, suggesting the formation of a subcomplex (Fig. 3*B right*). Finally, we performed immunoprecipitation analysis in ANPen cells treated with RNAi for Psn or for Psn and Pen-2 and found that Aph-1 interacts with Nct to form a subcomplex regardless of the presence of Psn or Pen-2 (Fig. 3*C*). These results suggest that the G*XXX*G motif of TMD4 in Aph-1 might play an essential role in the interaction of Aph-1 with Psn and with Pen-2, but not for Nct, in the assembly of a functional γ-secretase complex.

Certain Mutations of the Conserved Glycines of the GXXXG Motif Affect the Transport of the Aph-1·Nct Subcomplex from the ER to the Golgi Depending on the Interaction with Psn through the GXXXG Motif—Subcellular fractionation studies in mammalian cells have shown that the γ-secretase-active protein complexes that contain PS fragments as well as mature Nct are distributed in ER and the Golgi/*trans*-Golgi network (TGN), whereas the γ-inactive complexes harboring the PS holoprotein are distributed exclusively in the ER (8, 36, 47). Subcellular fractionation and localization analyses indicate that overexpressed mammalian Aph-1aL polypeptides are localized predominantly to ER and pre-Golgi compartments (31). Moreover, Aph-1 forms a subcomplex with an immature form of Nct (25). However, complex *N*-glycosylated, mature Nct is preferentially associated with the active γ-secretase complex that harbors the PS fragments, Aph-1 and Pen-2. These data suggest that the intracellular trafficking of Aph-1 is strictly controlled. However, the subcellular localization and trafficking of Psn complex components in *Drosophila* cells have not been extensively examined, although the targeting of Psn to the plasma membrane was reported (48, 49). To determine the subcellular localization of the Psn complex as well as the effect of certain mutations of the conserved glycines on the subcellular localization of Aph-1, we separated the membrane fractions of ANPen cells by discontinuous iodixanol gradients (Fig. 4*A*). Bip/Hsc 70-3, an ER resident protein that is positive for anti-KDEL, was separated in the bottom fraction (fractions 10 and

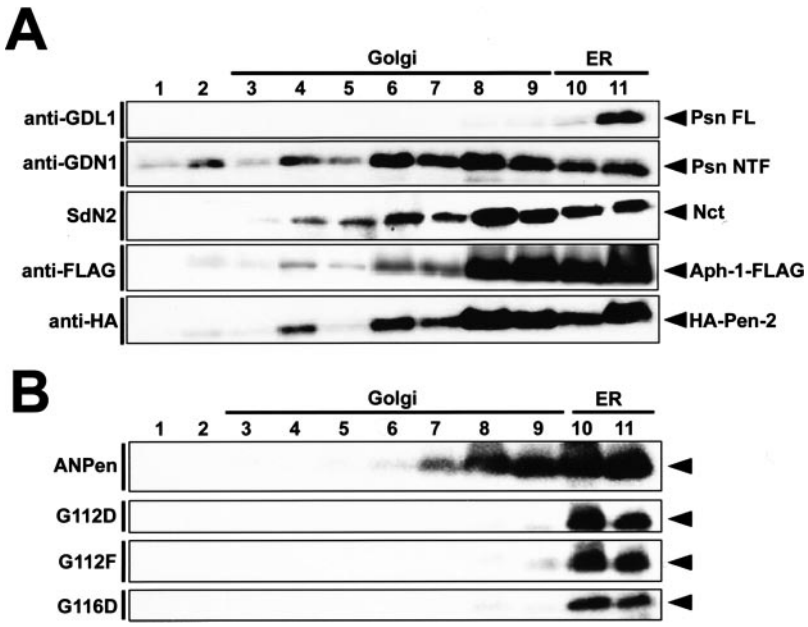

FIG. 4. **Subcellular localization of γ-secretase complex components including mutant Aph-1 in S2 cells.** *A*, analysis of subcellular localization of the γ-secretase complex in ANPen cells. Total membrane proteins from ANPen cells were separated by 2.5–30% discontinuous iodixanol gradients and numbered as fraction 1 (lightest) to fraction 11 (heaviest), as indicated at the *top of the panel*. 20 μl of each fraction was separated by SDS-PAGE and analyzed by immunoblotting. Fractions enriched in ER or Golgi vesicles were revealed by immunoblotting with anti-KDEL, anti-GM130, anti-syntaxin 5, anti-Rab8 or anti-adaptin-γ antibodies, respectively, as *underlined above the panel*. *B*, analysis of subcellular localization of mutant Aph-1 in stable cells. Total membranes from ANPen cells stably expressing Aph-1 (indicated at the *left of the panel*) were fractionated and analyzed as in *panel A*. *HA*, hemagglutinin.

11), whereas Golgi/TGN marker proteins (*i.e.* GM130, syntaxin 5, Rab8, or adaptin-γ) were detected chiefly in the middle fractions (fractions 3–9), which were used as markers for separation of the ER and Golgi proteins, respectively (data not shown). Psn FL was exclusively detected in the ER fraction. In contrast, Psn fragments were distributed widely in ER to Golgi fractions. Nct, wt Aph-1, and Pen-2 in ANPen cells also were detected widely in ER and Golgi fractions, exhibiting fractionation patterns very similar to those of the Psn fragments. These data suggest that endoproteolyzed forms of Psn, which are associated with other Psn components, are able to exit from the ER to the Golgi in a similar manner as that in mammalian cells (37, 47). We next examined the subcellular localization of mt Aph-1 polypeptides (*i.e.* G112D, G112F, or G116D). These mutant proteins were retained exclusively in the bottom fraction, suggesting that mt Aph-1 as well as the subcomplex consisting of mt Aph-1 and Nct are unable to be transported from the ER (Fig. 4*B*). To determine the dependence of the trafficking of Aph-1 on its interaction with other components, we fractionated the membranes from ANPen cells transfected with dsRNAs for Psn, Nct, or Pen-2 by discontinuous iodixanol gradients (Fig. 5*A*). We found that abrogation of the expression of Psn or Nct disrupted the export of Aph-1 from the ER, whereas Pen-2 RNAi had no effect on the subcellular localization of Aph-1 (in the ER and the Golgi). Furthermore, we observed the retention of Aph-1 within the ER fraction in the membranes from cells lacking Psn and Pen-2, suggesting that Aph-1 trafficking requires both Psn and Nct. Finally, we analyzed the subcellular localization of endogenous Nct in cells deficient in other γ-secretase components to determine whether Nct itself has a trafficking activity for other components (Fig. 5*B*). Unlike in mammalian cells, endogenous as well as exogenous Nct polypeptides were detected as a ~100-kDa single band that disappeared upon RNAi treatment against *Drosophila* Nct CDS (data not shown). Moreover, endogenous Nct was clearly detected in Psn knockdown cells similarly as mammalian Nct in PS knock-out cells (50), whereas the migration pattern of Nct was similar to that in mammalian cells. This suggests that Nct stability is independent of Psn (see below) and that the glycosylation pattern of *Drosophila* Nct is different from that in mammalians or, alternatively, that the resolution of SDS-PAGE is not enough to separate the differently glycosylated *Drosophila* Nct. The majority of endogenous

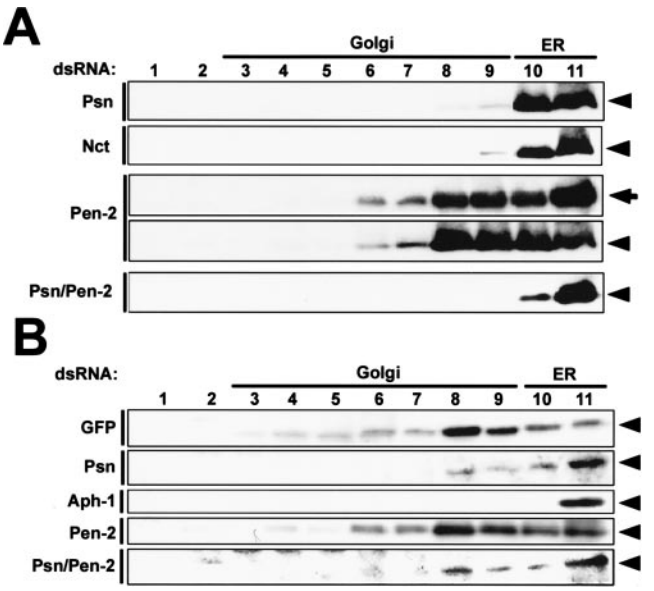

FIG. 5. **Subcellular localization of Aph-1 and Nct in S2 cells treated by multiple RNAi knockdown.** *A*, analysis of subcellular localization of Aph-1 (*arrowheads*) and the Psn holoprotein (*arrow*) in stable cells treated with RNAi. Note that the loss of Psn or Nct expression, but not that of Pen-2, caused the retention of Aph-1 protein in ER fractions. *B*, analysis of subcellular localization of endogenous Nct (*arrowheads*) in S2 cells treated with RNAi, showing that the trafficking of Nct into Golgi/TGN fraction required the expression of Psn and Aph-1. Separation, numbering, and analysis of the fractions were performed as in Fig. 4.

Nct was fractionated in the Golgi/TGN fraction, whereas depletion of Psn caused a marked accumulation of Nct in the ER fraction, similar to that described in mammalian cells (50). Furthermore, Aph-1 RNAi also caused an ER retention of endogenous Nct, whereas RNAi depletion of Pen-2 had no effect on the trafficking of Nct. This suggested that Aph-1, in addition to Psn, is required for the trafficking of Nct. Taken together, these data indicate that Aph-1 forms a subcomplex with Nct in the ER independently of the conserved glycines, the latter being required for the interaction with Psn as well as for the formation of an active γ-secretase complex and for the trafficking of the subcomplex to Golgi/TGN.

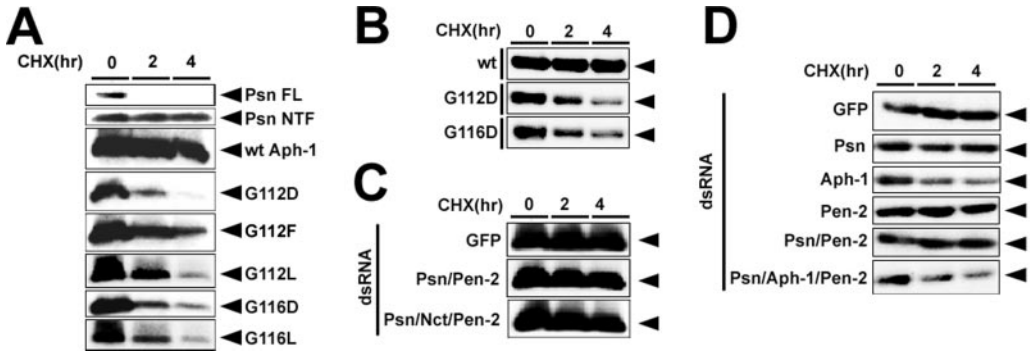

FIG. 6. **Stability of wild-type or glycine mutant Aph-1 and Nct polypeptides in ANPen cells.** *A*, analysis of the half-lives of Psn and wt or mt Aph-1 in stable ANPen cells incubated in culture media containing CHX (30 μg/ml). Lysates prepared after various incubation periods (0–4 h) were analyzed by immunoblotting with anti-GDN1 (for Psn) or anti-FLAG (for Aph-1) antibodies. Note that all of the glycine mutant Aph-1 polypeptides were degraded rapidly compared with wt during the CHX chase period. *B*, Nct stability in wt or mt ANPen cells treated with CHX, showing the destabilization of Nct polypeptides upon the overexpression of mt Aph-1. *C*, lack of decremental effects on the stability of Aph-1 by the depletion of other γ-secretase components. Cell lysates of ANPen cells transfected with dsRNA for GFP or Psn, Nct, and Pen-2 were analyzed by immunoblotting for Aph-1 (*arrowheads*) as in *panel A*. *D*, Nct stability in knockdown cells. ANPen cells transfected with dsRNAs (indicated at the *left of the panel*) were analyzed by immunoblotting for Nct (*arrowheads*) as in *panel A*. Note that the stability of Nct was dependent on Aph-1 expression, whereas Aph-1 was highly stable in the absence of any other component.

The Stability of Aph-1 Is Dependent on the Conserved Glycines but Not on Association with Other Components—Stabilization of the components of the Psn complex is the prerequisite for γ-secretase activity (5, 7, 19). Pen-2 RNAi caused the accumulation of a stable Psn·Nct·Aph-1 HMW complex, and the persistence of the Pen-2 protein depends on the presence of other components, suggesting that Pen-2 is a highly unstable protein and is not involved in the stabilization of the γ-secretase complex (19, 23, 51). Furthermore, the accumulation of PS fragments as well as holoproteins was abolished by RNAi for Nct or Aph-1, suggesting that Nct and Aph-1 are required for the stability of PS (19, 32, 50–53). To investigate the effect of glycine mutations on the stability of Aph-1 as a polypeptide, we treated wt and mt ANPen cells by cycloheximide (CHX) (Fig. 6*A*). Immunoblot analysis revealed that all three mt Aph-1 polypeptides were rapidly degraded during 4 h of chase in contrast to the persistence of wt Aph-1 ($t_{1/2}$ = >12 h for wt, 2.1 h for G112D, 3.5 h for G112F, 4.3 h for G112L, 2.3 h for G116D, and 2.7 h for G116L, respectively, calculated by densitometric analysis), suggesting that the conserved glycines are required for the stability of Aph-1 itself. Because mt Aph-1 is capable of interacting with Nct but not with Psn, we investigated the stability of Nct in cells stably expressing mt Aph-1. Consistent with the result that the steady-state levels of Nct were decreased in mt ANPen cells (Fig. 3*A*), the stability of Nct was lower than that in wt ANPen cells (Fig, 6*B*). Thus, the stability of Nct appears to be dependent on that of Aph-1. Furthermore, we investigated the dependence of the stability of Aph-1 polypeptides on other components in ANPen cells using RNAi. The levels of Aph-1 were constant during CHX chase in the absence of any other component proteins (*i.e.* Psn, Nct, and Pen-2) (Fig. 6*C*). These data suggest that Aph-1 *per se* is a highly stable membrane protein. We next analyzed the stability of Nct, a binding partner of Aph-1 within the subcomplex. As expected from the results in mt ANPen cells, Nct was short-lived in Aph-1 depleted cells ($t_{1/2}$ = 6.8 h), whereas RNAi for Psn and/or Pen-2 had no effect on the stability of Nct ($t_{1/2}$ = <12 h) (Fig. 6*D*). Notably, stable Psn fragments were completely lost, and the Psn holoprotein was rapidly degraded ($t_{1/2}$ < 2 h) in Aph-1 depleted cells, whereas a fraction of Nct proteins persisted, suggesting that Nct is unable to stabilize Psn in the absence of Aph-1. Taken together, we conclude that the interaction with Aph-1, which itself is a highly stable protein, confers stability to all the other components of the Psn complex.

DISCUSSION

The RNAi technique made it possible to analyze the functions of proteins, including mammalian Nct and Aph-1 (32, 54), under null or partial loss-of-function backgrounds in cultured cells. However, it is necessary to establish stable knockdown cell lines for the analysis of proteins that have long half-lives, *e.g.* the γ-secretase complex components. Moreover, the low transfection efficiency as well as the technical limitations in introducing long dsRNAs into mammalian cells renders simultaneous RNAi knockdown of multiple molecules highly difficult. In contrast, the high transfection efficiency and the propagation of RNAi in *Drosophila* cells enable the simultaneous suppression of multiple gene expression. The lack of transitive RNAi in *Drosophila* cells makes it possible to target RNAi against the UTR sequences also (41, 42). Taking advantage of these merits we utilized two novel RNAi strategies; the first was UTR-targeted RNAi followed by the rescue by transfection of CDS, the second was suppression of up to three different genes by multiple RNAi, and we then examined the role of Aph-1 on Psn complex formation and its metabolism in *Drosophila* S2 cell lines in the absence of endogenous Aph-1 and other components. We have shown the following. (i) Aph-1 is a highly stable protein that is required for the assembly and stabilization of the Psn complex. (ii) Mutations in the G*XXX*G motif of Aph-1 do not affect the formation of the Aph-1·Nct subcomplex. (iii) Mutations in the G*XXX*G motif decrease the stability of the Aph-1 polypeptide itself (Fig. 7). Recent reports suggest that the γ-secretase components, including Pen-2, are assembled within the ER and that subsequently the complex is transported to the Golgi in mammalian cells (55, 56). Although the process of assembly and the localization of *Drosophila* Pen-2 await further analysis, our results from the *Drosophila* S2 cells described above are in agreement with the previous findings indicating that the G*XXX*G motif in Aph-1a is required for the assembly and activity of γ-secretase in mammalian cells and yeast (28, 29), and they extend considerably the previous data in such a way as to provide further insight into the mechanistic roles of Aph-1 in the trafficking and stability of the γ-secretase complex.

Stabilization of the PS complex is the prerequisite for γ-secretase activity (5, 7, 8). The high stability of Aph-1 *per se*, as well as its association with other components, may render the γ-secretase complex highly resistant to degradation and able to perform proteolytic activity. The molecular basis for the

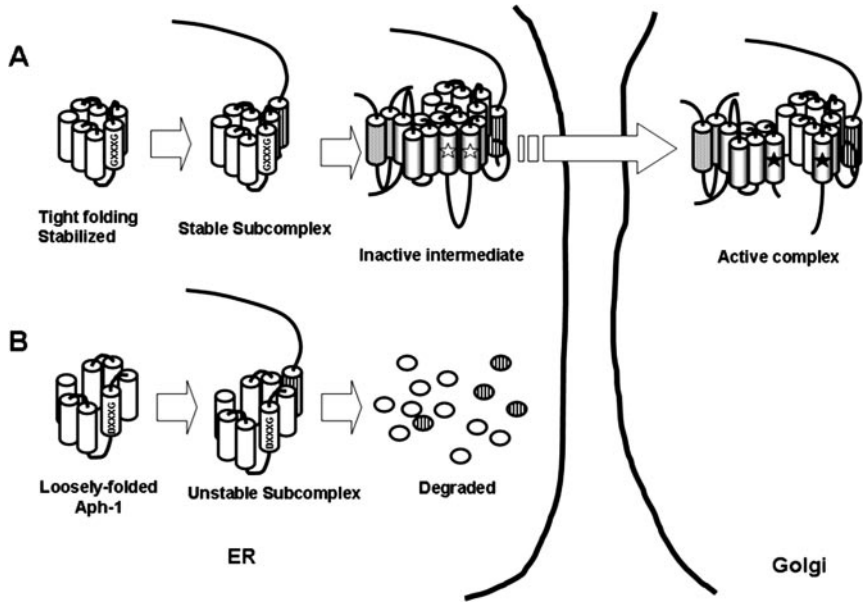

FIG. 7. **Schematic representations on the possible role of Aph-1 in the trafficking and maturation of γ-secretase complex.** *A*, Aph-1 polypeptides (*open cylinders*; *cylinders* represent TMDs) are tightly folded in a manner dependent on the G*XXX*G motif and form a stable subcomplex with Nct (*striped cylinder*) within the ER. This stable subcomplex interacts with nascent Psn (*cylinders with gradation*) through the hydrophobic interface formed by the TMDs of Aph-1 and Nct. This trimeric intermediate, further assembled with Pen-2 (*dotted cylinders*), is sorted out from the ER and activated. *B*, mt Aph-1 proteins are folded loosely and degraded rapidly within ER, although they are able to interact with Nct.

stability of Aph-1, however, remains unclear. Substitutions of conserved glycines in the G*XXX*G motif, which is known as an interaction motif for TMDs (44), disrupted the stability as well as the interaction of Aph-1 with Psn and Pen-2, although the stability of Aph-1 was independent of the intermolecular interactions (Fig. 6). We cannot completely exclude the possibility that impairment in the proper structure of the TMD4 caused by amino acid substitutions led to a loss-of-function effect by disrupting the stability of the entire molecule as well as the assembly of TMDs. However, the substitution to leucine, but not to alanine, of these conserved glycines abolished the functions (*e.g.* molecular assembly and stability) of Aph-1. This result is consistent with the recent finding that substitution to leucine, the most frequent residue in TMD, at the glycine residue disrupts G*XXX*G-mediated interaction (46). Thus, in terms of the stabilization of the Aph-1 polypeptide, we suggest that the G*XXX*G motif itself may play a role in the intramolecular interaction via TMDs to correctly fold and stabilize Aph-1 (Fig. 7*B*). The G*XXX*G motif forms helix-helix interactions with an extended Cα–H···O hydrogen bond within the lipid bilayer (57). Intriguingly, a recent report indicates that substitution to alanine at either of the glycines in the G*XXX*G motif disrupts TMD homodimerization, whereas the alanine mutant is still capable of facilitating heterodimerization with a TMD containing a (small)*XXX*(small) motif (*e.g.* G*XXX*S, G*XXX*S) (58). Considering our result that Aph-1/G112A or Aph-1/G116A retain its functions, it is possible that the G*XXX*G motif of TMD4 of Aph-1 is involved in the intramolecular heterodimeric TMD association. The additional, G*XXX*G-like, (small)*XXX*(small) motifs within TMD1 (^{15}GPPFA^{19}), TMD2 (^{45}SLLIS^{49}), and TMD3 (^{65}GVVFS^{69}) of Aph-1, which contain relatively conserved glycines or small residues and have similar topology within the TMDs, might be candidates for being intramolecular interaction domains for the G*XXX*G sequence in TMD4.

The molecular stoichiometry and interaction domains of individual components in the γ-secretase complex, especially in its active state *in situ*, still remain unknown. Aph-1 forms a subcomplex with Nct in the absence of Psn and Pen-2 in *Drosophila* cells in a similar manner to that observed in mammalian cells (Fig. 3*C*) (59). G*XXX*G-mutant Aph-1 retained the capacity to bind Nct but failed to associate with Psn and Pen-2. Indeed, the interaction of Nct/Aph-1 regardless of the mutations in TMD4 is consistent with the recent data showing that Nct interacts with the Aph-1 "lower" carboxyl-terminal fragment outside the amino-terminal fragment region of Aph-1 that harbors the TMD4 (60). Thus, the G*XXX*G motif may be important for the direct interaction of Aph-1 with Psn. This was somewhat different from the previous observations indicating that GXXXG mutations disrupt the co-immunoprecipitation of Aph-1 and Nct in mammalian cells (28, 29). The reason for this discrepancy is unknown, but one possible explanation would be that the use of 1% CHAPSO as a detergent in our study had a lesser effect on the sensitive but specific interaction with mt Aph-1 and Nct (24, 26, 61). Another factor might be the species difference between mammalian and *Drosophila* cells; it is noteworthy that two PS (PS1 and PS2) and two (human) or three (rodent) Aph-1 family genes, as well as their splice variants, have been documented in mammals (15). It has been shown that these distinct mammalian Aph-1 proteins form different complexes that contain either PS1 or PS2, suggesting that each mammalian Aph-1 homologue/isoform has different biological activity, although only the carboxyl-terminal 20 amino acids are different between Aph-1aS and Aph-1aL (32). The observation that Aph-1aS or Aph-1b transfected in mammalian cultured cells replaced the endogenous Aph-1aL suggests that overexpression of a single Aph-1 homologue/isoform in mammalian cells may compromose the function and metabolism of the others (31), although the effect of overexpression of a single homologue on the assembly of distinct γ-secretase complexes awaits further determination. Moreover, co-expression of other components in mammalian cells and yeast restored the complex formation and activity; G122D mutant Aph-1aL (an equivalent of G112D in *Drosophila*) rescued γ-secretase complex formation in yeast (29). Taken together, the knockdown/rescue paradigm used in this study in *Drosophila* cells (with the latter harboring single *Psn* and *Aph-1* genes) may provide the most clear-cut results in the analysis of the role of each component in the assembly of the γ-secretase complex.

The subdomain in Aph-1 that is required for its interaction with Nct remains unknown, although the carboxyl-terminal half of Aph-1 has been shown to be associated with Nct in mammalian cells (60). It may be noteworthy that Aph-1 harbors a highly conserved serine cluster within the TMD2, as well as a couple of hydrophilic residues located in the middle of TMDs that have been reported as being determinants of the stability and specificity of TMD association (62–65). Glycine mutations of Aph-1 disrupted the interaction of the Aph-1·Nct

subcomplex with Psn, raising the possibility that Aph-1 may interact directly with Psn through the G*XXX*G motif in TMD4. In agreement with this notion, Aph-1 depletion completely diminished the stability of Psn, whereas Nct was relatively stable in the absence of Aph-1 (Fig. 6). However, the abrogation of Nct expression also resulted in a complete depletion of stable Psn polypeptides, whereas the expression levels and stability of Aph-1 were unchanged. These discrepancies in the stability of Psn compared with those of Nct or Aph-1 in knockdown cells may suggest that Nct and Aph-1 are both required for the stability of Psn. Recent reports suggest that the TMD of Nct plays an important role in the interaction with PS and the assembly of the PS complex, implicating the Nct TMD also in its interaction with PS (27, 66, 67). Furthermore, the aforementioned observation that overexpression of G*XXX*G mutant Aph-1 together with PS and Nct restored the loss-of-function phenotype in yeast may suggest that the loss of stability of Aph-1 is overcome by a high level expression of mutant Aph-1 protein together with other components, especially Nct (Fig. 6; 29). Thus, it would be possible that the helix-helix interface formed by TMDs of the Aph-1·Nct subcomplex, including TMD4 of Aph-1 and Nct TMD, may provide the interaction surface with Psn, leading to the subsequent stabilization and eventual activation of the complex (Fig. 7).

The intracellular compartment(s) wherein the individual components assemble to form the active γ-secretase complex is an open question. The mutant Aph-1 polypeptides were retained in the ER fraction, which was similar to the localization of wt Aph-1 in cells depleted of Psn or Nct. In agreement with this observation, subcellular localization of Aph-2·Nct was altered in the embryos mutated in *sel-12* or *hop-1* as well as in *aph-1* in *C. elegans* (16), and it has been shown that the proper trafficking of Nct requires the expression of PS in mammalian cells (15). Of particular interest is the observation that the carboxyl-terminal aromatic/hydrophobic amino acids of PS that are essential to the formation of a γ-secretase-active HMW PS complex through the interaction with Nct (7, 67), are required for the efficient export of PS from the ER to the intermediate compartment (ERGIC) (68). Moreover, recent report indicates that the PALP motif at the carboxyl terminus of PS harbors an ER retention signal (67). These findings altogether suggest that PS might act as a regulator for trafficking of the early γ-secretase subcomplex harboring Nct and Aph-1 from the ER. In contrast, it has been documented that Pen-2 primarily localizes to the Golgi (50) and that stabilized Psn holoproteins induced by Pen-2 depletion in S2 cells are chiefly fractionated in the Golgi fraction together with Aph-1 (Fig. 5). In sum, the proper intracellular trafficking of the Aph-1·Nct subcomplex requires an interaction with Psn, which in turn facilitates the sorting of the γ-secretase to the Golgi.

Our present study underscores the importance of the helix-helix interactions through TMDs of γ-secretase components in the assembly and activation of the complex. Further genetic, biochemical, and cell biological studies on the roles of γ-secretase components will facilitate clarification of the remaining important problems (*e.g.* the molecular mechanism of intramembrane cleavage, the binding domains mediating interactions of individual components, and the three-dimensional structure of the active complex) in γ-secretase research.

Acknowledgments—We thank Drs. K. Maruyama for SC100 cDNA, Y. Ihara for the anti-C4 antibody, M. Lowe for the MLO7 and JSEE1 antibodies, and the Takeda Pharmaceutical Company for Aβ ELISA. We also thank Y. Takahashi, S. Tanaka, I. Hayashi, C. Sato, M. Shinohara, and N. Watanabe for helpful discussions and technical assistance and Dr. D. M. A. Mann for invaluable comments on the manuscript.

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