A Conserved GXXXG Motif in APH-1 Is Critical for Assembly and Activity of the γ-Secretase Complex

Sheu-Fen Lee‡, Sanjiv Shah‡, Cong Yu‡, W. Christian Wigley§, Harry Li‡, Myungsil Lim‡, Kia Pedersen¶, Weiping Han‡, Philip Thomas§, Johan Lundkvist¶, Yi-Heng Hao‡,†, and Gang Yu‡**

From the ‡Center for Basic Neuroscience and the Departments of ¶Cell Biology and §Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9111 and the ¶Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, Von Ehlers vag 3, SE-171 77 Stockholm, Sweden

The multipass membrane protein APH-1, found in the γ-secretase complex together with presenilin, nicastrin, and PEN-2, is essential for Notch signaling in Caenorhabditis elegans embryos and is required for intramembrane proteolysis of Notch and β-amyloid precursor protein in mammalian and Drosophila cells. In C. elegans, a mutation of the conserved transmembrane Gly123 in APH-1 (mutant or28) leads to a notch/glp-1 loss-of-function phenotype. In this study, we show that the corresponding mutation in mammalian APH-1aL (G122D) disrupts the physical interaction of APH-1aL with hypoglycosylated immature nicastrin and the presenilin holoprotein as well as with mature nicastrin, presenilin, and PEN-2. The G122D mutation also reduced γ-secretase activity in intramembrane proteolysis of membrane-tethered Notch. Moreover, we found that the conserved transmembrane Gly125, Gly126, and Gly130 in the fourth transmembrane region of mammalian APH-1aL are part of the membrane helix-helix interaction GXXXG motif and are essential for the stable association of APH-1aL with presenilin, nicastrin, and PEN-2. These findings suggest that APH-1 plays a GXXXG-dependent scaffolding role in both the initial assembly and subsequent maturation and maintenance of the active γ-secretase complex.

Regulated intramembrane proteolysis of the Notch receptor, the β-amyloid precursor protein, and select type I membrane polypeptides represents a novel mechanism of signal transduction (1). It is now generally believed that the enzyme responsible for the intramembrane proteolysis of these substrates is the high molecular mass, multiprotein γ-secretase complex, which consists of a heterodimer of the presenilin amino- and carboxyl-terminal endoproteolytic fragments (NTF1 and CTF, respectively) as the putative catalytic subunit (2, 3). A single transmembrane glycoprotein (nicastrin) has also been identified as a critical component of the γ-secretase complex (4). Recent genetic studies in Caenorhabditis elegans have uncovered two additional genes, aph-1 and pen-2, both of which encode multipass membrane proteins that are required for γ-secretase activity and notch/glp-1 signal transduction (5, 6). Overexpression of presenilin, nicastrin, APH-1, and PEN-2 together produces or enhances γ-secretase activity in yeast, insect cells, and mammalian cells, further supporting the hypothesis that these four proteins are essential for γ-secretase activity (9–13). Aside from the putative role of presenilin as the catalytic subunit of the γ-secretase complex, the specific functions of nicastrin, APH-1, and PEN-2 remain unclear. Recent studies indicate that PEN-2 may be important in initiating presenilin endoproteolysis, whereas APH-1 may play a role in stabilizing the γ-secretase complex (12–15). Recent studies also suggest that APH-1 and nicastrin may interact to form a subcomplex prior to the assembly of the γ-secretase complex (10, 16). Despite these advances in research, the mechanism of γ-secretase assembly in the lipid bilayers to form and maintain an active membrane protein complex capable of performing intramembrane proteolysis within a hydrophobic environment remains an enigma.

It has been reported that mutation of Gly123 to aspartic acid within the fourth putative transmembrane region (TMR) of C. elegans APH-1 (mutant or28) inhibits notch/glp-1 signal transduction and leads to the “anterior-pharynx-defective” phenotype associated with the loss of apf-1, aph-2 (nicastrin), or sel-12 plus hop-1(presenilins) (5). This glycine residue in C. elegans APH-1 has been conserved during evolution and corresponds to Gly122 in mammalian APH-1aL and APH-1aS and Gly123 in mammalian APH-1b. APH-1aL and APH-1aS are alternative spliced forms of APH-1a that differ at the carboxyl termini (7). Because it is known that the role of the APH-1 proteins in Notch signaling is conserved during evolution and is associated with γ-secretase function, we set out to investigate the biochemical function of the conserved glycine residue in the assembly and activity of the γ-secretase complex. In this study, we used human APH-1aL as an example to demonstrate that the equivalent mutation in human APH-1aL (G122D) affects the activity of the γ-secretase complex.
fects the ability of APH-1 to associate with the immature as well as the mature γ-secretase complex and inhibits the intramembrane proteolysis of Notch. Moreover, we found that Gly\textsuperscript{122}, Gly\textsuperscript{126}, and Gly\textsuperscript{130} in APH-1\textsubscript{a} belong to a conserved GXXXGXXG motif (where X represents any amino acid) generally accepted as a major determinant in transmembrane helix-helix protein interactions (17–22) and show that these glycine residues are critical for γ-secretase assembly and activity.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Human full-length APH-1\textsubscript{a} and PEN-2 cDNAs were obtained as described (7, 15). Subsequent site-directed mutagenesis studies were performed using the QuikChange kit (Stratagene), and the identities of all clones were confirmed by DNA sequence analyses.

Cell Lines—Cells were maintained in Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum (Invitrogen) with 5% CO\textsubscript{2} at 37 °C. For transient expression, plasmids were transfected into the appropriate cell lines in 10-cm dishes using LipofectAMINE 2000 (Invitrogen), and samples were collected 48 h after transfection. HEK293 cells stably or transiently transfected with Myc- and His-tagged APH-1\textsubscript{a} and its glycine mutants were used to evaluate the interaction of APH-1 with presenilin, nicastrin, and PEN-2.

Antibodies and Chemicals—The antibodies used in this study include anti-Myc monoclonal antibody 9E10 (American Type Culture Collection); anti-Myc polyclonal antibody A14 (Santa Cruz Biotechnology); anti-FLAG antibody M2 (Sigma); antibodies against presenilin-1 (21–80) (Chemicon International, Inc.), presenilin-1 (283–378) (Chemicon International, Inc.), and presenilin-2 (7–24) (Onconecogene); antibody against nicastrin (695–709) (Sigma); anti-PEN-2 antibody PNT2 (15); and antibody against the hemagglutinin (HA) epitope (Santa Cruz Biotechnology). Inhibitors of γ-secretase activity (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (23) and N-[2-naphthyl]-Val-phenylalaninyl (inhibitor IV) (24)) were from Calbiochem.

Coprecipitation Studies—Co-immunoprecipitations were performed as described previously (4, 25, 26). Briefly, proteins extracted using lysis buffer (1% digitonin or 1% CHAPSO in 50 mM sodium phosphate (pH 8.0), protease inhibitors (Roche Applied Sciences) and 300 mM NaCl) were pre-absorbed with preimmune serum and then with protein A-agarose beads for 2 h at 4 °C. The beads were washed four times for 15 min each with lysis buffer. Immunoprecipitated proteins were eluted with 0.1 M glycine HCl (pH 2.5) and 0.25% detergent, neutralized with 1.0 M Tris, and subjected to Western blot analysis. Ni-NTA-agarose affinity pull-down experiments were performed as described previously (7) in 1% digitonin or 1% CHAPSO lysis buffer. Each binding experiment contained equal amounts of proteins with similar expression of Myc/His-tagged proteins.

γ-Secretase Activity Assay—The appropriate cell lines were assayed for γ-cleavage of Notch using the N\textsubscript{AE}-G\textsubscript{V} luciferase reporter as described (7, 27) in an adapted in vitro γ-secretase activity assay (28). The in vitro activity assay involved lysis of membrane proteins with 1% CHAPSO, 50 mM PIPES (pH 7), 5 mM MgCl\textsubscript{2}, 5 mM CaCl\textsubscript{2}, and protease inhibitors (Roche Applied Science) and immunoprecipitation of Myc-tagged proteins with immobilized anti-Myc antibody 9E10. The beads were then washed with CHAPSO lysis buffer and subjected to incubation at 37 °C in the presence of 0.25% CHAPSO, 50 mM PIPES (pH 7), 5 mM MgCl\textsubscript{2}, 5 mM CaCl\textsubscript{2}, 0.125% phosphatidylethanolamine, 0.1% phosphatidylcholine, and N100 substrate. This N100 substrate, which was overexpressed and purified from SF9 cells, harbors Val\textsuperscript{1711}–Glu\textsuperscript{1909} of the mouse Notch-1 receptor and a carboxyl-terminal FLAG/His tag. The γ-secretase-cleaved N100-FLAG/His fragment was detected with anti-FLAG antibody.

RESULTS

Gly\textsuperscript{122} in APH-1\textsubscript{a} Is Critical for γ-Secretase Assembly—To understand the effect of the G122D mutation on the γ-secretase complex, we analyzed the ability of human APH-1\textsubscript{a} (either wild-type (WT) or G122D mutant) to interact with the predominant in vivo active presenilin species, the endogenous presenilin endoproteolytic fragments. To assist in this objective, we generated HEK293 cells stably expressing APH-1\textsubscript{a}-WT/Myc or APH-1\textsubscript{a}-G122D-Myc and examined cell lines that expressed a comparable level of either wild-type or mutant Myc/His-tagged proteins. No obvious difference was observed in the level of endogenous presenilin-1 NTF between the APH-1\textsubscript{a}-WT-Myc/His and APH-1\textsubscript{a}-G122D-Myc/His cells (Fig. 1A, lanes 1–4). In Ni-NTA-agarose pull-down experiments, we observed that endogenous presenilin-1 NTF was able to coprecipitate with APH-1\textsubscript{a}-WT-Myc/His, an observation consistent with a previous report (7). However, the amount of presenilin-1...
Fig. 2. Mutation G122D disrupts the interaction of APH-1α with the presenilin holoprotein and nicastrin. Full-length presenilin-1 was transiently transfected into HEK293 cells stably overexpressing LacZ-Myc/His (lanes 1 and 5), APH-1α WT-Myc/His (lanes 2 and 6), or APH-1α G122D-Myc/His clone 1 (lanes 3 and 7) or clone 2 (lanes 4 and 8), and the cells were solubilized in 1% digitonin. After having determined that the presenilin-1 holoprotein and Myc/His-tagged proteins were expressed at similar levels in each set of cells, we subjected equal amounts of proteins (lanes 1–4) to Ni-NTA pull-down experiments. The resultant Ni-NTA pull-down products (lanes 5–8) were resolved by SDS-PAGE and probed with antibody against amino-terminal presenilin-1 to identify the presenilin-1 holoprotein (PS1-holo) and presenilin-1 NTF (PS1-NTF) and with antibodies against nicastrin (NCT) and the Myc epitope as indicated.

NTF that coprecipitated with APH-1α WT-Myc/His was much higher than the amount that co-isolated with APH-1α G122D-Myc/His (Fig. 1A, lanes 5–8). Using a co-immunoprecipitation approach, we similarly observed that a minute amount of presenilin-1 NTF or presenilin-2 NTF coprecipitated with APH-1α G122D-Myc/His compared with the ample amount of presenilin-1 and -2 fragments that coprecipitated with APH-1α WT-Myc/His (Fig. 1B). Based on these observations, we concluded that the G122D mutation abrogates the association of APH-1α with the mature presenilin species. Next, we tested whether Gly122 in human APH-1α is required for interaction with the immature presenilin holoprotein. Given that the endogenous presenilin holoprotein is usually maintained at a low or undetectable steady-state level, we transiently overexpressed full-length presenilin-1 in cell lines stably expressing APH-1α WT-Myc/His and APH-1α G122D-Myc/His and performed Ni-NTA-agarose pull-down experiments. We found that significantly less full-length presenilin-1, as well as presenilin-1 endoproteolytic fragments, coprecipitated with APH-1α G122D-Myc/His compared with APH-1α WT-Myc/His (Fig. 2, lanes 1–8). These findings suggest that the G122D mutation disrupts the ability of APH-1α to interact with both the mature presenilin endoproteolytic fragments and the immature presenilin holoprotein.

Our next investigation focused on the ability of immature and mature nicastrin to coprecipitate with APH-1α. According to published reports, hypoglycosylated immature nicastrin is mainly associated with immature full-length presenilin, whereas fully glycosylated mature nicastrin is associated with presenilin NTF/CTF fragments (29, 30). In our study, we observed that APH-1α WT-Myc/His coprecipitated with more of the immature than the mature species of nicastrin in cells overexpressing APH-1α and the presenilin-1 holoprotein (Fig. 2, lane 6), an observation consistent with earlier studies (10, 14). Moreover, we found that much less immature and mature nicastrin co-isolated with APH-1α WT-Myc/His compared with APH-1α WT-Myc/His (Fig. 2, lanes 5–8). We also observed that cells stably expressing APH-1α WT-Myc/His contained slightly more immature nicastrin than cells stably expressing APH-1α G122D-Myc/His (Fig. 2, lanes 1–4), suggesting that the G122D mutation may also affect the reported ability of wild-type APH-1α to stabilize immature nicastrin (10, 16). Based on these findings, we conclude that Gly122 mediates the association of APH-1α with both immature and mature nicastrin either directly or indirectly during the assembly process of the γ-secretase complex.

To test the effect of the G122D mutation on the interaction between APH-1α and PEN-2, we transiently expressed HA-tagged PEN-2 in either the APH-1α WT-Myc/His or APH-1α G122D-Myc/His cells. Detergent extracts of these cells were then subjected to Ni-NTA pull-down experiments. We observed that the association between APH-1α and overexpressed HA-PEN-2 was not significantly affected by the G122D mutation, even though the interactions between APH-1α and both presenilin-1 and nicastrin were disrupted (Fig. 3A). To examine whether the G122D mutation affects the association of endogenous PEN-2 with APH-1α, we next performed the same Ni-NTA pull-down experiments with detergent-solubilized HEK293 cells stably expressing APH-1α WT-Myc/His or APH-1α G122D-Myc/His. In contrast to overexpressed HA-PEN-2, endogenous PEN-2 did not strongly coprecipitate with mutant APH-1α compared with wild-type APH-1α (Fig. 3B). The reason that the G122D mutation affected binding of APH-1α to endogenous (but not overexpressed) PEN-2 is unclear. One possibility is that overexpressed PEN-2 exists in a state parallel to immature nicastrin and the presenilin holoprotein, whereas endogenous PEN-2 exists mostly in a “mature” state similar to the presenilin endoproteolytic fragments and mature nicastrin. In this context, “immature” overexpressed PEN-2 and APH-1α can directly interact with each other in the absence of both presenilin-1 and nicastrin, perhaps via a site or sites other than Gly122 in APH-1α. Because the active γ-secretase complex is not stable when any one of the four components is missing (2), the failure of mature PEN-2 to interact with G122D mutant APH-1α might be an indirect consequence of the disruption of the association of APH-1 with presenilin and nicastrin (see “Discussion”).
Mutation G122D in APH-1aL Inhibits γ-Secretase Activity—Having established that the G122D mutation in APH-1aL disrupted the structural assembly of the γ-secretase complex, we proceeded to investigate whether this structural defect could lead to deleterious effects on γ-secretase activity. We tested the effect of the G122D mutation in APH-1aL on the production of the Notch intracellular domain (a product of γ-secretase activity) in a cell-based Gal4/VP16-dependent luciferase transactivation assay. This assay, which indirectly measures γ-secretase activity, utilizes a γ-secretase substrate consisting of a chimeric membrane-tethered Notch receptor trimmed in the extracellular domain (N/A) and Gal4 and VP16 domains (GV) inserted at the junction between the transmembrane and intracellular domains of N/A (7,27). In HEK293 cells stably expressing robust amounts of APH-1aL/G122D-Myc/His, we observed a 50–60% reduction in luciferase reporter activity compared with control cells expressing comparable amounts of APH-1aL WT-Myc/His (Fig. 4A). No analysis could be performed on cells expressing higher levels of APH-1aL/G122D-Myc/His because these cells did not grow well (possibly because of toxic effects of the G122D mutation). However, the modest reduction in γ-secretase activity in cells stably expressing APH-1aL/G122D-Myc/His obtained in the transactivation assay contrasts with the dramatic effect of the G122D mutation imposed on the structural assembly of the γ-secretase complex and the loss-of-function notch1glp-1 phenotype observed in the C. elegans or28 mutant. A likely explanation for this discrepancy could be that the effect of the G122D mutation on γ-secretase activity is masked by the presence of endogenous APH-1 proteins in the cells.

To examine the effect of the G122D mutation on γ-secretase activity in the absence of endogenous APH-1, we immunoprecipitated the γ-secretase complex containing either APH-1aL WT-Myc/His or APH-1aL/G122D-Myc/His from the respective CHAPS-solubilized membranes and measured the ability of the immunoprecipitate to cleave a purified Notch substrate containing the transmembrane S3/γ-secretase-like cleavage site (N100) in an in vitro γ-secretase-like activity assay. We observed a much more robust N100 cleavage in the immunoprecipitate containing wild-type APH-1aL compared with mutant APH-1aL. The presence of the N100 proteolytic intracellular fragment was also sensitive to two different γ-secretase inhibitors (Fig. 4B). Taken together, these findings show that the G122D mutation in APH-1aL has an inhibitory effect on γ-secretase activity.

Our studies suggest that the G122D mutation perturbs the ability of APH-1aL to participate in the initial assembly process of the γ-secretase complex and to associate with and stabilize the active γ-secretase complex to directly modulate the intramembrane proteolysis of Notch. However, we currently cannot rule out the possibility that the G122D mutation affects proper trafficking of APH-1aL within cells and thus the formation of the γ-secretase complex. We attempted to address this issue by assessing the cellular distribution of wild-type and G122D mutant APH-1aL. Immunofluorescence data revealed similar intracellular punctate/vesicular staining patterns for both wild-type and G122D mutant APH-1aL (Fig. 5A), suggesting that G122D mutant APH-1aL does not have a gross trafficking defect that could account for its effect on γ-secretase assembly and activity. We also used linear glycerol density centrifugation to examine the native state of wild-type and G122D mutant APH-1aL and found that the G122D mutation did not significantly alter the native state of APH-1aL (Fig. 5B).

Interestingly, G122D mutant APH-1aL, like wild-type APH-1aL, existed in similar high molecular mass glycerol density fractions (Fig. 5B). It is not clear why G122D mutant APH-1aL was found in high molecular mass fractions since it is incapable of tight association with presenilin and nicastrin. One probable explanation is that the APH-1aL mutant recruits additional molecules in the absence of presenilin and nicastrin.

**APH-1aL Contains a Conserved GXXXG Motif Important in Transmembrane Helix-Helix Association**—We have noticed
that the conserved transmembrane Gly\(^{122}\) and Gly\(^{126}\) in APH-1\(^{a}\) are part of a sequence arrangement that resembles a sequence motif important in transmembrane protein interactions (Fig. 6). This highly conserved sequence arrangement known as the GXXXG motif was originally identified as a requirement for the dimerization of a single TMR of glycoporphin A (17, 18, 22, 31) and is recognized as a high affinity transmembrane helix-helix binding motif for many other membrane proteins, including aquaporin-1, ErbB-4, ATP synthase, and the anion-selective membrane channel VacA (19–21, 32–37).

To determine whether the \(^{122}\)GXXXG\(^{126}\) sequence is indeed part of a \textit{bona fide} transmembrane interaction motif, we evaluated the possibility that the disruptive effect of G122D APH-1\(^{a}\) on the \(\gamma\)-secretase complex is not dependent exclusively on changing the glycine to a residue with a charged side chain such as aspartic acid. We replaced Gly122 with either the non-polar residue alanine or proline and tested their ability to interact with endogenous presenilin, nicastrin, and PEN-2. In our Ni-NTA-agarose pull-down experiments, transiently transfected APH-1\(^{a}\)G122A-Myc/His coprecipitated with similar amounts of presenilin-1 NTF, nicastrin, and PEN-2 as APH-1\(^{a}\)WT-Myc/His (Fig. 7A, lanes 12 and 14), whereas APH-1\(^{a}\)G122P-Myc/His failed to interact with presenilin-1 NTF, nicastrin, or PEN-2 (lane 15), suggesting that the effect of the Gly\(^{122}\) mutation is not solely dependent on the aspartate. The subtle/no difference in the binding of G122A mutant APH-1\(^{a}\) to presenilin-1 NTF, nicastrin, or PEN-2 compared with wild-type APH-1\(^{a}\) is likely due to the conservative substitution of alanine for glycine. In accordance with previous observations (18, 22), in some (not all) cases, less steric clashes may occur upon replacing the glycine with alanine compared with other residues and thus could be less disruptive for the \(\gamma\)-secretase complex.

To further establish that the APH-1\(^{a}\)\(^{122}\)GXXXG\(^{126}\) sequence is part of an authentic transmembrane interaction motif, we replaced the conserved transmembrane glycine residue of APH-1\(^{a}\) at position 126 with either alanine or leucine. Compared with APH-1\(^{a}\)WT-Myc/His, the G126A mutation diminished the interaction of APH-1\(^{a}\)Myc/His with endogenous presenilin-1 NTF, nicastrin, and PEN-2 (Fig. 7A, compare lanes 12 and 16). Moreover, the G126L mutation in APH-1\(^{a}\) ablated the interaction to the same extent as the G122P or G122D mutation (Fig. 7A, compare lanes 13, 15, and 17). Mutations of other transmembrane or cytosolic glycine residues to alanine in APH-1\(^{a}\) such as at position 15 (in TMR-1), position 130 (in TMR-4), or position 145 (in the cytosolic region after TMR-4) did not significantly affect the interaction of presenilin-1 NTF, nicastrin, or PEN-2 with APH-1\(^{a}\) (Fig. 7A, lanes 18–20).

We next tested the effects of some of these glycine mutations
FIG. 6. Conserved GXXXG motif in the APH-1 family of proteins. The primary structure alignment of the fourth putative transmembrane helix of APH-1 family members and the TMR of human glycophorin A (GpA) is shown. Residue numbers correspond to human APH-1a. The glycine residues in the GXXXG motif believed to be critical for helix-helix interaction are shown in boldface, and those examined in this study are shown in black boxes. Note that the APH-1 GXXXG motif could be extended to potentially include (A/S)XXXGXXXGXXXG/G(A). The GXXXG motif in glycophorin A is shown in a gray box, and the important residues in the motif are underlined. Species and GenBank™/EBI accession numbers are as follows: Homo sapiens (Hs), AAH01230 and AAH20905; Mus musculus (Mm), AAH24111 and AAH12406; Danio rerio (Dr), AAM83225; Anopheles gambiae (Ag), EAA14158; Drosophila melanogaster (Dm), AAF51212; C. elegans (Ce), CAA16282; and A. thaliana (At), AAL36063.

GpA ITLIIFGVMAGVIGTILLISYG

FIG. 7. Mutations of the critical glycine residues in the 122GXXXG138 sequence disrupt the assembly and activity of the γ-secretase complex. A, HEK293 cells transiently transfected with LacZ-Myc/His (lanes 1 and 11), APH-1a WT-Myc/His (lanes 2 and 12), or APH-1a G12A-Myc/His harboring a single mutation of the glycine residue indicated (lanes 3–10 and 13–20) were lysed in 1% digitonin. After determining that similar amounts of the Myc/His-tagged proteins, endogenous presenilin-1, nicastrin, and PEN-2 were expressed in each set of cells, we subjected equal amounts of proteins (lanes 1–10) to Ni-NTA pull-down experiments, and the resultant products (lanes 11–20) were resolved by SDS-PAGE and probed with antibodies to presenilin-1 NTF (PS1-NTF), nicastrin (NCT), and PEN-2, and the Myc epitope as indicated. B, cell lines stably expressing LacZ-Myc/His (lane 1), APH-1a WT-Myc/His (lanes 2 and 6), or APH-1a G12A-Myc/His harboring a single mutation of the glycine residue indicated (lanes 3–5 and 7–8) were processed as described in the legend to Fig. 4B and subjected to incubation with N100-FLAG/His at 37 °C for 0 and 6 h. Samples were electrophoresed on SDS-polyacrylamide gel and probed with anti-FLAG antibody (upper and middle panels) or with anti-Myc antibody (lower panel). IP, Immunoprecipitation.
The assembly and activity of the multimeric proteins that make up a highly specific GXXXG-dependent scaffolding role for APH-1 in γ-secretase is elucidated by the exact sequence and structural requirements for this critical motif in the assembly and activity of the γ-secretase complex.

DISCUSSION

As the γ-secretase complex is largely buried in the cellular membrane, and γ-secretase activity is carried out within the hydrophobic lipid bilayer, it is conceivable that molecular interactions among the four components of the γ-secretase complex occur mainly through their TMRs and that helix-helix interactions are essential for the assembly and molecular actions of γ-secretase. To date, little is known about the transmembrane interactions among the four components of the γ-secretase complex. In this study, we have identified the conserved transmembrane Gly122, Gly126, and Gly130 in TMR-4 of mammalian APH-1L as part of the GXXXG motif, which is widely accepted as an important determinant in transmembrane helix-helix interactions. Indeed, mutations of Gly122, Gly126, and Gly130 in the GXXXG motif prevent the stable and specific association of APH-1L with the γ-secretase complex. The disruption in the assembly of the γ-secretase complex is the most probable explanation for the loss of γ-secretase activity associated with G122D mutant APH-1L shown in this study and for the recessive loss-of-function notch1glp-1 phenotype associated with the C. elegans or28 mutant, which harbors an equivalent mutation (5). We propose that the GXXXG motif of APH-1 is a major and highly specific docking or packing site for the assembly of the γ-secretase complex.

Because mutations of the GXXXG motif disrupt the interaction of APH-1L with both the immature and mature forms of presenilin and nicastrin and subsequently affect γ-secretase activity, it is likely that the GXXXG-mediated docking or packing of APH-1 is an early event in the formation of the active complex and that the GXXXG motif in APH-1 also exerts its functional effect to maintain the stability of the active mature γ-secretase complex (Fig. 9). This model is in agreement with the proposed stabilizing role for APH-1 in the assembly of the γ-secretase complex (10–15). However, the exact mechanism of how the APH-1 GXXXG motif regulates the assembly and maturation process of the γ-secretase complex awaits further investigation. Among the key unresolved issues are the identities of the direct APH-1-binding partner and the sequence of the opposing transmembrane helix that associates with the APH-1 GXXXG motif. In several reported cases, the homo- and hetero-oligomerization of two transmembrane helices require the packing of the GXXXG sequences in conjunction with their surrounding residues contributed by the respective transmembrane helices (20, 31). In this regard, it is possible that the opposing sequence that associates with the GXXXG sequence in APH-1L is also a transmembrane GXXXG motif or a variation of the motif. It is thus interesting to speculate that two or more copies of APH-1 could associate via their GXXXG sequences to serve as a molecular scaffold for oligomerization of the other γ-secretase subunits, such as the putative presenilin dimer (38). Another possibility is that the APH-1 GXXXG sequence may directly interact with presenilin. The presenilin family proteins harbor several conserved transmembrane glycine residues that could potentially constitute the GXXXG motif. On the other hand, recent biochemical reports suggest that APH-1 and nicastrin interact to form a stable intermediate subcomplex at the early stage of γ-secretase assembly (16). It is possible that the inability of the presenilin proteins to be co-isolated with the GXXXG mutants of APH-1L is caused in some way by a prior defect in the direct binding of the TMRs of APH-1L and nicastrin. However, the single TMR of nicastrin does not possess a typical GXXXG motif. It remains to be determined whether APH-1 interacts
Fig. 9. Model of the GXXXG-dependent helix-helix association in modulating the assembly and activity of the γ-secretase complex. A, in this simplified model, APH-1, PEN-2, presenilin, and nicastrin (NCT) exist in at least three major states during the initial assembly and subsequent maturation of the active γ-secretase complex: 1) unassembled immature components, 2) immature complex (probably labile in most cells), and 3) active/mature complex. Our data and results from other groups (10–16) suggest that APH-1 functions as a molecular scaffold for the γ-secretase complex. GXXXG-dependent helix-helix association plays an essential role in the initial assembly of the immature complex (Step I) and is also required for stabilizing the active mature complex (Step II). Transition of the immature complex to the mature complex is probably associated with a presenilin (PS) endoproteolysis-triggered conformational change (Step II). Note that multiple substeps likely exist within the two major steps described here. B, mutations of the GXXXG motif prevent APH-1 from performing its scaffolding role in the initial assembly of the immature complex (and consequently in maturation of the active complex). GXXXG mutant APH-1 may recruit additional molecule(s) (marked X), self-oligomerize, or adopt an alternative folding. In agreement with this view, we found that mutant APH-1 could still associate with overexpressed (presumably unassembled/immature) PEN-2 and that mutant APH-1 existed in a high molecular weight complex without associating with presenilin and nicastrin. It should be noted that the cognate site of the APH-1 GXXXG motif is unknown and that APH-1, PEN-2, presenilin, and nicastrin could have multiple contacts with one another. It should also be noted that the stoichiometry of the γ-secretase complex is unclear and that γ-secretase subunits could potentially form homodimers or homo-oligomers. For simplicity, only one copy of each subunit was depicted. Im, immature; m, mature; PS-holo, presenilin holoprotein.

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with nicastrin via a GXXXG dependent or GXXXG-independent mechanism. Finally, it is possible that mutations of the GXXXG motif in putative TMR-4 of APH-1 could disrupt interaction with another TMH in APH-1 itself or with another APH-1 molecule.

In summary, our findings have revealed a specific role for APH-1 in the scaffolding of the γ-secretase complex. We have demonstrated that the conserved membrane helix-helix interaction GXXXG motif in APH-1 is critical determinant for the specific interactions among the constituents of the γ-secretase complex. Although the GXXXG motif is unlikely to be the sole determinant in this process, this study, which focused on the packing/docking of APH-1α to the other γ-secretase components, should provide a framework for understanding the molecular mechanism underlying the assembly, folding, and other regulatory processes of the γ-secretase complex in intramembrane proteolysis. Further investigation into the role of the GXXXG motif in the assembly and activity of the γ-secretase complex should shed new light on transmembrane protein–protein interactions within the lipid bilayers, a largely uncharted challenge that extends beyond the boundaries of the γ-secretase complex. Finally, our findings suggest that targeting the GXXXG motif in APH-1 could be an alternative therapeutic strategy for Alzheimer’s disease and related disorders because compounds that block the docking or packing sites in the γ-secretase complex might control the assembly and activity of the active enzyme.
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