Co-expression of Nicastrin and Presenilin Rescues a Loss of Function Mutant of APH-1*

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γ-Secretase is an intramembrane-cleaving aspartyl protease complex that mediates the final cleavage of β-amyloid precursor protein to liberate the neurotoxic amyloid-β peptide implicated in Alzheimer’s disease. The four proteins presenilin (PS), nicastrin (NCT), APH-1, and PEN-2 are sufficient to reconstitute γ-secretase activity in yeast. Although PS seems to contribute the catalytic core of the γ-secretase complex, no distinct function could be attributed to the other components so far. In Caenorhabditis elegans, mutation of a glycin to an aspartic acid within a conserved GXXG motif in the fourth transmembrane domain of APH-1 causes a loss of function phenotype. Surprisingly, we now found that the human homologue APH-1a carrying the equivalent mutation G122D is fully active in yeast co-expressing PS1, NCT, and PEN-2. To address this discrepancy, we expressed APH-1a G122D in HEK293 cells. As reported previously, overexpressed APH-1a G122D was not incorporated into the γ-secretase complex. Separate overexpression of PS1, NCT, or PEN-2 together with APH-1a G122D allowed the formation of heterodimers lacking the other endogenous components. Only the combined overexpression of PS1 and NCT together with APH-1a G122D facilitated the formation of a fully active γ-secretase complex. Under these conditions, APH-1a G122D supported the production of normal amounts of Aβ. We conclude that cooperative effects may stabilize a trimeric complex of APH-1a G122D together with PS1 and NCT. Upon successful complex assembly, the GXXXG motif becomes dispensable for γ-secretase activity.

Aggregated amyloid β-peptide (Aβ)† in cerebral plaques is a major hallmark of Alzheimer’s disease, and soluble oligomeric Aβ disturbs synaptic function even before its deposition into plaques (1). Thus, Aβ is a critical player in Alzheimer’s disease pathology and memory impairment. Aβ is generated from the β-amyloid precursor protein (APP) by the consecutive cleavage of β- and γ-secretase in the APP ectodomain and in the middle of the transmembrane domain (2). The aspartyl protease β-site APP-cleaving enzyme has been identified as the sole β-secretase in humans (3). Several lines of evidence have suggested that the polytopic membrane protein presenilin (PS) functions as γ-secretase. In the absence of both mammalian presenilin homologues PS1 and PS2, no Aβ is produced (4, 5). Moreover, mutations in the PSs associated with familial Alzheimer’s disease shift the ratio from the predominant 40-amino-acid Aβ species to the more aggregation-prone 42-amino-acid variant (2). Furthermore, active site γ-secretase inhibitors can be cross-linked to PSs (6), and mutagenesis of two highly conserved aspartyl residues suggests that PSs belong to a novel class of intramembrane-cleaving aspartyl proteases (7). Indeed, the C-terminal aspartyl residue is part of a conserved GXGD motif that is also contained in other polytopic aspartyl proteases including the signal peptide peptidase and its relatives (8, 9).

Biochemical purification and genetic studies identified nicastrin (NCT), APH-1, and PEN-2 as additional integral membrane proteins crucial for γ-secretase activity (10–13). As for PSs, two homologues of APH-1 have been identified in mammals, APH-1a and APH-1b. PS forms a complex together with NCT, APH-1, and PEN-2 (14–16) that is sufficient to reconstitute γ-secretase activity in Saccharomyces cerevisiae, which lacks endogenous γ-secretase (17). Furthermore, reconstitution of active γ-secretase in yeast was associated with endoproteolysis of PS1 into an N-terminal fragment and a C-terminal fragment, which together are believed to represent the principal cellular form of PS (18). So far, little is known about the distinct functions of the recently identified complex components NCT, APH-1, and PEN-2. Extensive cross-regulation of the expression level of the four complex components severely complicates a separate analysis of the individual components. Both genetic knock-out and RNA interference-mediated down-regulation of PS1, NCT, APH-1, and PEN-2 typically decrease the levels of the other components and prevent maturation of NCT (19–23). Only immature NCT and APH-1 seem to be rather stable even in the absence of other components (20–25). This might be explained by a stable “subcomplex” containing only immature NCT and APH-1 (25–28), although its functional role during physiological γ-secretase assembly has not been demonstrated. During complex assembly, NCT undergoes a conformational change that renders it resistant to trypsin digestion (29). PEN-2 assembly has been implicated in initiating PS endoproteolysis because removal of PEN-2 leads to accumulation of unprocessed PS holoprotein, which is not observed upon knockdown of any other complex component (14, 24, 30). Furthermore, PEN-2 is required to stabilize the N-terminal fragment/C-terminal fragment heterodimer within the γ-secretase complex (24).

A loss of function allele of APH-1 in Caenorhabditis elegans has drawn the attention to a highly conserved GXXXG motif in the fourth transmembrane domain (12, 13, 31). Such motifs are

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The abbreviations used are: Aβ, amyloid β-peptide; APP, β-amyloid precursor protein; PS, presenilin; NCT, nicastrin, APH-1 anterior pharynx defective; PEN-2, presenilin enhancer 2; HA, hemagglutinin; WT, wild type; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-hydroxy-1-propanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine.
known to mediate helix-helix interactions within transmembrane domains (32). A point mutation of the first glycine residue of the GXXGX motif in APH-1 was lethal in C. elegans (12) and disrupted γ-secretase complex formation of the human homologue APH-1a (31), demonstrating the general importance of this motif. We now reconstituted γ-secretase in yeast to further dissect the role of the conserved GXXG in APH-1a for γ-secretase complex assembly and activity. Surprisingly, we found that the mutation causing a loss of function in C. elegans was fully active when γ-secretase was reconstituted in yeast. Moreover, by overexpressing PS1 and NCT together with APH-1a G122D in human cells, we were able to fully rescue the loss of function APH-1 allele.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies against PEN-2 (1638), the large cytoplasmic loop of PS1 (to PS1), NCT (to NCT), the HA epitope (polyclonal H6908, monoclonal HA-7 antibody, and 1638 (to PEN-2). APH-1a-HA WT formed a robust complex together with NCT, PS1, and PEN-2. In contrast, APH-1a G122D did not bind significant amounts of endogenous PS1, NCT, or PEN-2 in HEK293/Sw cells. NCTmat, mature NCT; NCTimm, immature NCT.

**S. cerevisiae**

**Fig. 1.** APH-1a G122D is active in yeast but is not incorporated into the γ-secretase complex in mammalian cells. A, γ-secretase was reconstituted in yeast cells by expressing the indicated mutants of APH-1a together with wild type PS1, NCT, and PEN-2. γ-Secretase cleavage of the APP-based C155-GAL4 substrate strongly activates β-galactosidase (β-gal) expression when APH-1a WT, G122A, or G122D are expressed together with PS1 WT, NCT, and PEN-2 (upper panel). Western blotting of membrane fractions using antibodies N1660 (to NCT), PS1N (to PS1), HA-7 (to APH-1a-HA), and M2 (to FLAG-PEN-2) revealed robust expression of all γ-secretase components including mutant APH-1a. Note that both APH-1a G122A and G122D allow PS endoproteolysis as APH-1a WT. NTF, N-terminal fragment. B, membrane fractions of HEK293/Sw cells stably expressing APH-1a WT or G122D were lysed in 1% CHAPSO. Co-immunoprecipitations (IP) of γ-secretase components together with PS1 (antibody 3027) and overexpressed APH-1a-HA (anti-HA antibody H6908) were analyzed by immunoblotting using antibodies N1660, PS1N, HA-7, and 1638 (to PEN-2). APH-1a-HA WT formed a robust complex together with NCT, PS1, and PEN-2. In contrast, APH-1a G122D did not bind significant amounts of endogenous PS1, NCT, or PEN-2 in HERK293/Sw cells. NCTmat, mature NCT; NCTimm, immature NCT.
RESULTS AND DISCUSSION

To analyze the role of the conserved GXXXG motif in APH-1 for γ-secretase complex assembly and activity in the absence of endogenous APH-1, we used S. cerevisiae, which allows reconstitution of γ-secretase activity upon expression of APH-1 WT together with PS1, NCT, and PEN-2 (17). To monitor γ-secretase activity, we used the APP-based reporter substrate C 1–55-GAL4 (17). Upon γ-secretase cleavage, the membrane-tethered transcriptional activator GAL4 is liberated and activates LacZ transcription via the GAL1 promoter. Consistent with previous results (17, 31), both APH-1a WT and G122A were active in the γ-secretase reporter assay in yeast (Fig. 1A, upper panel). Surprisingly, the loss of function mutation G122D, which was lethal in C. elegans (12), allowed robust γ-secretase activity in yeast (Fig. 1A, upper panel). Consistent with that finding, APH-1a G122D also facilitated endoproteolysis of PS1 similar to APH-1a WT and G122A (Fig. 1A). In contrast, no γ-secretase activity and PS1 endoproteolysis were detectable upon co-expression of inactive PS1 D385A as expected (7, 17).

To investigate this surprising finding on a biochemical level, we stably expressed HA-tagged APH-1a WT and G122D in HEK293 cells. We performed co-immunoprecipitation studies with antibody 3926 because CHAPSO disturbed the separation of Aβo and Aβc on Tris-Bicine-urea gels (36).

Functional Rescue of Inactive APH-1

Fig. 2. Overexpression of PS1 and NCT restores assembly of mutant APH-1 into the γ-secretase complex in HEK293 cells. HEK293/Sw cells stably expressing APH-1a-HA G122D were transiently transfected with combinations of PS1, NCT, and PEN-2 as indicated and subjected to co-immunoprecipitation (IP) using anti-HA antibody H6908 as in Fig. 1B. NCT, PS1, APH-1a-HA, and PEN-2 were detected by immunoblotting as described in the legend for Fig. 1B. Note that the minor amounts of mature NCT (NCTmat) bound to APH-1a G122D without co-transfection of PS1 most likely represent premature export from the endoplasmic reticulum due to transient overexpression. The asterisk denotes the IgG heavy chain. NCTimmat, immature NCT; NTF, N-terminal fragment.

APH-1a G122D was not co-immunoprecipitated with PS1 (Fig. 1B). Similarly, anti-HA immunoprecipitation of APH-1a WT did co-precipitate endogenous PS1, NCT, and PEN-2, whereas APH-1a G122D did not interact with endogenous γ-secretase components (Fig. 1B) as reported previously (21, 31). This suggests that APH1a G122D cannot compete with endogenous APH-1a for the other complex components present in HEK293 cells at endogenous levels. Thus, the GXXXG motif in APH-1 may be required for γ-secretase complex assembly by mediating the interaction with one or more other γ-secretase components, as suggested previously (31).

In yeast, all γ-secretase components were expressed at fairly high levels, which might overcome a weaker binding affinity of APH-1a G122D to other complex components. We therefore expressed APH-1a G122D in all double and triple combinations with PS1, NCT, and PEN-2 in HEK293 cells to analyze whether excess amounts of other γ-secretase components would facilitate the incorporation of APH-1a G122D into a functional γ-secretase complex as observed when expressed in yeast. In addition, this approach might also reveal which protein-protein interaction requires the GXXXG motif. We transiently transfected the cell line stably expressing APH-1a-HA G122D additionally with PS1, NCT, and combinations thereof (Fig. 2). By anti-HA immunoprecipitation, we analyzed whether APH-1a-HA G122D formed a stable complex with PS1, NCT, and PEN-2 (Fig. 2). Indeed, excess amounts of PS1 holoprotein (Fig. 2, lanes 2 and 9), immature NCT (lanes 3 and 10), and PEN-2 (lanes 4 and 11) allowed the interaction with APH-1a G122D. Thus, GXXXG-independent interactions can occur between APH-1a and PS1, NCT, and PEN-2. How-
ever, overexpression of APH-1a G122D with only one additional component did not facilitate the full assembly of a γ-secretase complex containing APH-1a G122D with the remaining γ-secretase components present at endogenous levels. In accordance with that finding, APH-1a G122D almost exclusively bound to unprocessed PS1 holoprotein and immature NCT, respectively (lanes 9–11). In contrast, when overexpressed together with PS1 and NCT, the mutant APH-1a G122D associated with large amounts of PS1 endoproteolytic fragments and mature NCT and also with endogenous PEN-2 (Fig. 2, lanes 5 and 12). Therefore, excess amounts of PS1 and NCT fully rescued the complex assembly defect in APH-1a G122D. In contrast, overexpression of APH-1a G122D in the other triple combinations (Fig. 2, compare lanes 6 and 13 and lanes 7 and 14, respectively) was not sufficient to rescue complex assembly of APH-1a G122D. This may support a model of ordered assembly of γ-secretase components. Interestingly, such a trimeric complex of PS1, NCT, and APH-1a has been proposed as the final intermediate during γ-secretase complex assembly before PEN-2 binding initiates endoproteolysis of PS1 (14). The fact that excess PS1 and NCT are necessary to allow the complex incorporation of APH-1a G122D indicates that the GXXXG motif is required for stabilizing such a putative trimeric complex of PS1, NCT, and APH-1a. Most likely, the direct interaction of PS1 and NCT is able to stabilize such a trimer as long as PS1 and NCT are in excess. Upon full assembly through the binding of PEN-2, the complex containing APH-1a G122D may be further stabilized through additional interactions via cooperative binding among the components.

Reconstitution of γ-secretase activity in yeast with APH-1a G122D suggests that the GXXXG motif is not required for proteolytic activity once APH-1a is incorporated into the γ-secretase complex. To recapitulate this in mammalian cells, we analyzed γ-secretase function in a cell-free assay using recombinant C100-His6 substrate (17). We used transient transfection to overexpress all combinations of PS1, NCT, and PEN-2 together with APH-1a G122D (as in Fig. 2). As expected,
APH-1a G122D immunoprecipitated from HEK293 cells stably overexpressing mutant APH-1a alone is not associated with γ-secretase activity (Fig. 3A). Only upon co-transfection of both PS1 and NCT was the defective γ-secretase activity restored. In contrast, all other combinations of γ-secretase components failed to recruit APH-1a G122D into an active γ-secretase complex. Apparently, only the combined overexpression of PS1 and NCT overcomes the deficient complex assembly of APH-1a G122D and restores γ-secretase activity.

For further analysis, we generated stable HEK293 cell lines co-expressing PS1 and NCT with either APH-1a WT or G122D. Without additional overexpression of PS1 and NCT, only APH-1a WT was associated with γ-secretase activity (Fig. 3B). In contrast, when stably overexpressed together with PS1 and NCT, both APH-1a WT and G122D interacted with PS1, NCT, and PEN-2 (compare Fig. 2) and generated similar amounts of γ-secretase activity from the recombinant substrate. This is genuine γ-secretase activity since γ-secretase activity was strongly blocked by L-685,458, a γ-secretase inhibitor known to directly interact with PSs (6). The increased γ-secretase activity production in triple-transfected cells resulted from increased levels of APH-1a and enhanced amounts of mature γ-secretase complexes (data not shown) (14–16, 27).

We next used a Tris-Bicine-urea gel system (36) that allows the specific identification of γ-secretase components to analyze the proteins produced in vitro from active γ-secretase containing APH-1a G122D. The ratio of AB10 and AB12 was largely unaffected in the APH-1a mutant (Fig. 3C). This shows that the mature γ-secretase complex no longer vitally depends on the GXXG motif for its stabilization. Once the assembly defect of APH-1a G122D is overcome, it allows γ-secretase production in amounts similar to APH-1 WT. The mutation G122D does not affect the ratio of AB10/AB12 in vitro, suggesting that glycine 122 is only required during γ-secretase complex assembly but is not directly required for the proteolytic function of γ-secretase.

Taken together, we demonstrate that the combined overexpression of PS1 and NCT with the loss of function APH-1a G122D mutant fully restores γ-secretase complex assembly and activity. A similar rescue of inactive APH-1 G122D was not observed upon transfection of other combinations of PS1, NCT, and PEN-2, suggesting a putative trimeric complex containing APH-1, PS1, and NCT as an intermediate during ordered complex assembly (14, 26). Most likely, the GXXG motif in APH-1 facilitates the interaction of APH-1 with PS1 or NCT and not both PS1 and NCT. Strikingly, PS1 contains a well conserved 372GXXG382 motif, which may mediate the interaction with APH-1, although most GXXG motifs analyzed so far mediate homodimerization (32, 37). Interestingly, the signal peptide peptide, which does not require additional components (9), shares the GXGD active site motif with PSs but lacks this GXXG motif (9). Up to now, APH-1a G122D is the only loss of function allele in a γ-secretase complex that can be rescued by overexpression of other γ-secretase components.

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