Assembly of the γ-Secretase Complex Involves Early Formation of an Intermediate Subcomplex of Aph-1 and Nicastrin*

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The γ-secretase complex is an unusual multimeric protease responsible for the intramembranous cleavage of a variety of type 1 transmembrane proteins, including the β-amyloid precursor protein and Notch. Genetic and biochemical data have revealed that this protease consists of the presenilin heterodimer, a highly glycosylated form of nicastrin, and the recently identified gene products, Aph-1 and Pen-2. Whereas current evidence supports the notion that presenilin comprises the active site of the protease and that the other three components are members of the active complex required for proteolytic activity, the individual roles of the three co-factors remain unclear. Here, we demonstrate that endogenous Aph-1 interacts with an immature species of nicastrin, forming a stable intermediate early in the assembly of the γ-secretase complex, prior to the addition of presenilin and Pen-2. Our data suggest 1) that Aph-1 is involved in the early stages of γ-secretase assembly through the stabilization and perhaps glycosylation of nicastrin and by scaffolding nicastrin to the immature γ-secretase complex, and 2) that presenilin, and later Pen-2, bind to this intermediate during the formation of the mature protease.

The γ-secretase complex is an unusual aspartyl protease responsible for the intramembraneous cleavage of a variety of type 1 integral membrane proteins. This proteolytic activity is essential for the proper functioning of the Notch receptor, a critical signaling factor in metazoan development (1). In fact, essential for the proper functioning of the Notch receptor, a gene product of about 50 kDa, with a half-life of ~1 h (5, 6). Through what appears to be an autoproteolysis, PS holoprotein undergoes cleavage to yield an N-terminal fragment (NTF; ~28 kDa) and a C-terminal fragment (CTF; ~22 kDa) that remain associated (6, 7). This PS heterodimer is significantly more stable than the PS holoprotein, having a half-life of ~30 h, and is thought to be the biologically active form of the protein (7–10). All presenilins contain two conserved intramembranous asparagine residues within all presenilins, and mutation of either asparagine dramatically reduces amyloid β-protein secretion and abrogates PS endoproteolysis (11). This finding, coupled with the demonstration that active-site-directed γ-secretase inhibitors can be covalently linked to PS1 NTF and CTF (12, 13), strongly suggests that the PS NTF/CTF heterodimer comprises the active site of γ-secretase. Because it is the heterodimers, not PS holoprotein, that associate into high molecular weight complexes (9, 10) and bind to an inhibitor affinity resin in an activity-dependent manner (14), the holoprotein is considered an immature PS species, whereas heterodimers are components of the mature, active γ-secretase.

The second member of the γ-secretase complex to be identified was nicastrin (NCT), initially detected through a genetic screen in Caenorhabditis elegans (15) and later biochemically from mammalian cells following partial affinity purification using a PS1-directed antibody (16). NCT is a type 1 membrane protein that possesses many potential glycosylation sites within its large ectodomain. Several studies have established that three principal forms of NCT exist in cells, the unglycosylated, nascent protein (~80 kDa), an “immature” N-linked glycosylated species (NCT; ~110 kDa), and a “mature” N-linked isoform (mNCT; ~150 kDa) that is formed after entering the Golgi apparatus (17–20). It is this mNCT that is associated with active γ-secretase (19), and importantly, PS is required for the full post-translational generation of this mNCT species

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The abbreviations used are: APP, β-amyloid precursor protein; PS, presenilin; NTF, N-terminal fragment; CTF, C-terminal fragment; NCT, nicastrin; mNCT, mature NCT; iNCT, immature NCT; IP, immunoprecipitation; CHO, Chinese hamster ovary; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; HEK, human embryonic kidney; siRNA, small interfering RNA; DDM, n-dodecyl-β-maltoside; Big CHAP, N,N-bis-(3-O-glucosamidopropyl)deoxycholate.

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Assembly of the γ-Secretase Complex

(17–20). Therefore, like PS, NCT undergoes a maturation process that could regulate its association with the γ-secretase complex.

Recent genetic studies in C. elegans and Drosophila melanogaster have revealed that two additional gene products, Aph-1 and Pen-2, are required for γ-secretase function (15, 21). However, these studies did not provide insight into the specific roles of these newly identified members in complex assembly and function. Biochemical studies in mammalian cells have recently shown that both Aph-1 and Pen-2 are physical members of the active protease and not transient interactors merely involved in assisting γ-secretase assembly or protein folding (22–24). However, the specific protein-protein interactions responsible for assembling this polymeric protease complex and their order of assembly remain unknown.

Previously, we have shown that under non-denaturating conditions γ-secretase is associated with a molecular mass of ~250–270 kDa, corresponding to the apparent combined molecular masses of PS, NCT, Aph-1, and Pen-2 (24). Interestingly, we observe an intermediate complex in which NCT and Aph-1 co-migrate at a molecular mass of ~140 kDa, consistent with the predicted size of INCT plus Aph-1. Furthermore, co-immunoprecipitation (co-IP) experiments in multiple cell lines using antibodies against ectopically expressed as well as endogenous Aph-1 reveal a selective association between Aph-1 and iNCT. The interaction between iNCT and Aph-1 was specific, since both the PS heterodimer and Pen-2 preferentially bound mNCT. Furthermore, the partial dissociation of the mature γ-secretase complex using certain detergents demonstrates a persistent association between Aph-1 and NCT, despite disruption of the binding of PS and Pen-2. Taken together, our data support the hypothesis that Aph-1 and NCT directly interact early in γ-secretase assembly, forming a stable intermediate prior to the incorporation of PS and Pen-2, PS endoproteolysis, and the final glycosylation of NCT, all of which are known to correlate with mature, active γ-secretase.

MATERIALS AND METHODS

Cell Culture and Transfection—Chinese hamster ovary (CHO) and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). HEK293 cells stably expressing human NCT (HEK-NCT) (a gift of P. St. George-Hyslop) were cultured in Dulbecco’s modified Eagle’s medium supplemented with G418 and blasticidin as previously described (16). George-Hyslop) were cultured in Dulbecco’s medium supplemented, respectively, with G418, puromycin, zeocin; G418, puro-myacin, hygromycin; or G418, puromycin, zeocin, hygromycin, as previously described (24). All transient transfections were performed using commercially available affinity resins (Roche Applied Science and Sigma, respectively). X81 is a high titer antibody to the N-terminal 81 amino acids of presenilin (8), and A89 is a high titer antibody to the C terminus of Aph-1 (20 amino acids 248–265). All SDS-PAGE analyses were performed using Novex gels (Invitrogen). Western blots were probed for PS holoprotein and NTF with either Ab14 (1:2000; gift of S. Gandy) or N-19 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); for PS CTF with 13A11 (5 μg/ml; gift of Elian Pharmaceuticals); for NCT with R302 (1:4000; gift of D. Miller and P. Savam) or guinea pig anti-NCT (1:2000; Chemicon); for Aph-1-252-NTF with either 12C5 or 3F10 (200 μg/ml and 50 μg/ml, respectively; Roche Applied Science); for FLAG-Pen2 with M2 (1:1000; Sigma); and for endogenous Aph-1 with A89 (1:500; see above). Immunoreactive bands were detected using the ECL-plus chemiluminescent kit (Amersham Biosciences) and exposed onto Blue Sensitive photographic film (Marsh Bio Products, Inc.).

siRNA Treatment—HEK cells stably overexpressing APPsw were plated onto polylysine-coated dishes and treated with siRNA duplexes (Dharmacon) complexed with TKO transfection reagent (Mirus) in Opti-MEM (Invitrogen) at ~50% confluence. Three days later, cells were treated again with siRNA duplexes and collected 3 days later. Cells were lysed in 1% Nonidet P-40, and samples were protein-normalized and probed by Western blot as described above. Scrambled (AAU GCA CAU CAU CAC CAC GAC), NCT (AAG GCG AAG UU CCC GUG CAG), and Pen-2 (AAG GCU AUG UCU GGC GCU CAG) duplexes were used for these experiments.

RESULTS

Association of Aph-1 and Nicastrin in CHO Cell Lines—Previous experiments examining CHO cells stably co-expressing human APP751, PS1, Aph-1, and FLAG-Pen2 (the γ-secretase complex) revealed an immunoreactive γ-secretase complex migrating in native gels at ~250–270 kDa (24). This complex contained mature forms of all of the known γ-secretase components (PS1 NTF, PS1 CTF, NCT, Aph-1, and Pen-2) co-migrating at this molecular mass (Fig. 1a). However, we also observed an apparent intermediate complex on these blue native gels that was only immunoreactive for NCT and Aph-1 and migrated at ~140 kDa (Fig. 1a). Intriguingly, this molecular mass would be appropriate for an assembly intermediate of these two proteins if the isof orm of NCT was iNCT (~110 kDa) and this was coupled with Aph-1 (~30 kDa). We also probed these blots for endogenous Pen-2 and found no evidence of Pen-2 at any molecular weight other than within the active γ-secretase complex (data not shown). To confirm that this putative subcomplex was not an artifact of preferential detergent solubilization or a mild denaturation of the γ-secretase complex, we also treated γ-30 cells with a protein synthesis inhibitor (cycloheximide; 20 μg/ml) and examined for the time-dependent decay of this complex. Data showed that immunoreactivity for both NCT and Aph-1 in this putative subcomplex rapidly diminished within the first 2 h of treatment, whereas the HMW γ-secretase, which has a half-life of ~30 h, did not change (data not shown), demonstrating that this subcomplex was not a detergent artifact.

To further investigate this putative Aph-1-NCT intermediate, we ran lysates of the γ-30 cells on BN-PAGE gels and then excised the entire lane of natively separated proteins and loaded that gel slab horizontally on to the top of a precast SDS-PAGE gel. The proteins were thus electrophoresed in the second dimension in SDS in order to denature all protein complexes and verify the co-migration of γ-secretase components and also to determine the glycosylation state of the two NCT bands observed in Fig. 1a. This two-dimensional PAGE analysis revealed that the NCT immunoreactive species found at ~250–270 kDa was predominantly composed of mNCT (Fig. 1b). In contrast, the NCT species at ~140 kDa in the BN-PAGE gel migrated slightly faster in the SDS-PAGE dimension than did the NCT species from the 250-kDa band, suggesting that
**FIG. 1.** Aph-1 is selectively associated with iNCT in CHO cell lines. A, BN-PAGE analysis of γ-30 lysates reveals the co-migration of Aph-1 alone (iNCT-Aph-1 complex) and co-migrating with the mature γ-secretase complex (HMW γ-secretase complex). γ-30 cells were solubilized in BN-PAGE lysis buffer, electrophoresed on 5–13.5% BN-PAGE gels, and probed for all known members of the γ-secretase complex. B, two-dimensional PAGE analysis of γ-30 cell lysates demonstrates the glycosylation state of the NCT found co-migrating with Aph-1 alone (iNCT-Aph-1 complex) and co-migrating with the mature γ-secretase complex (HMW γ-secretase complex). γ-30 cells were solubilized in BN-PAGE lysis buffer and electrophoresed on 5–13.5% BN-PAGE gels, and the entire lane was then electrophoresed by SDS-PAGE and probed for NCT and PS holoprotein and PS NTF. #, an R302-nonspecific band that migrates at ~65 kDa on both BN-PAGE and SDS-PAGE gels. *, the iNCT associated with Aph-1 on both BN-PAGE and SDS-PAGE gels. †, the iNCT associated with Aph-1 on both BN-PAGE and SDS-PAGE gels. ‡, the HMW γ-secretase complex, which contains a majority of mNCT. C, IP revealed the selective association of iNCT and not mNCT with Aph-1-HA. Whole cell lysates were prepared from various CHO cell lines in 1% CHAPSO and immunoprecipitated with an antibody against the HA tag present on the C terminus of Aph-1-HA. Crude lysate lanes contain 5% of the material used in the IP. Precipitates were then probed for NCT, PS holoprotein, PS NTF, and PS CTF by Western blot. D, Aph-1-HA preferentially interacts with iNCT in A-5 cells. Whole cell lysates of A-5 cells were prepared in CHAPSO and immunoprecipitated with antibodies against the PS1 NTF antibody, X81 (lane 2), or the α-HA affinity resin, 3F10 (lane 3), and the immunoprecipitates were probed for NCT, E, Pen-2 selectively binds to mNCT and not iNCT. Whole cell lysates were prepared from P22 and γ-30 cells and immunoprecipitated with an antibody recognizing the FLAG tag present on the N terminus of the stably expressed Pen-2. Precipitates were then probed for NCT, PS holoprotein, PS NTF, and PS CTF by Western blot.
this lower NCT form is in fact iNCT.

We also probed these two-dimensional PAGE blots for PS1 and found that the cleaved PS NTF co-migrated with the mNCT, consistent with an active, mature γ-secretase complex (Fig. 1b). However, we observed a small amount of iNCT that migrated somewhat faster in the first (BN-PAGE) dimension and thus was resolved slightly to the left of the mNCT in the second (SDS-PAGE) dimension (Fig. 1b). Interestingly, this band of iNCT from the ~240-kDa complex occurred with PS holoprotein, whereas the mNCT co-migrated with PS NTF, suggesting that both a minor immature and a major mature γ-secretase complex exist at a high molecular weight. The slightly faster migration of the HMW immature complex than the HMW mature complex could be accounted for by the absence of Pen-2 (data not shown), by the difference in molecular weight of iNCT and mNCT, or by a combination of both. The existence of this immature HMW complex is consistent with the hypothetical Aph-1-NCT-PS1 complex that has been proposed from data of several groups that studied the selective disruption of Pen-2 expression.

In order to further investigate the interaction between these two components, we performed a series of co-IP experiments. We chose four experimental conditions for comparison. First, we probed untransfected PS70 cells, which are CHO cells stably expressing human APP751 and PS1. Second, we examined PS70 cells transiently transfected to express a hemagglutinin (HA)-tagged form of Aph-1-2-HA (PS70 + Aph-1). Third, we studied PS70 cells that stably overexpress human Aph-1-2-HA (A-5 cells). Last, we probed A-5 cells that also stably co-express FLAG-tagged human Pen2 (γ-30 cells); this line shows substantially enhanced levels of mature γ-secretase components and protease activity (24). Cell lysates were prepared from all four of these sources and immunoprecipitated with an anti-HA affinity resin. The precipitates were then blotted with antibodies against NCT, PS1 NTF, and PS1 CTF. The results revealed essentially no NCT or PS CTF and very little nonspecific binding of PS NTF and PS holoprotein in the HA precipitates of the untransfected PS70 cells (Fig. 1c, lane 2). In the cells transiently expressing Aph-1, we readily detected iNCT and PS holoprotein in the HA precipitates but very little or no mNCT, PS1 NTF, or PS1 CTF (Fig. 1c, lane 4). Because previous experiments had shown that the transient expression of γ-secretase components results in inefficient incorporation of the exogenous protein into the endogenous complex (24), we next examined stable Aph-1 transfected (the A5 line) and observed that both iNCT and mNCT were associated with the stably expressed Aph-1-2-HA (Fig. 1c, lane 6). Interestingly, only relatively small amounts of PS1 NTF and CTF were found associated with Aph-1-2-HA, even when the latter was stably expressed. On the other hand, substantial levels of PS holoprotein were found in the HA precipitates of the A-5 cells, suggesting that, like iNCT, immature (uncleaved) PS may preferentially associate with Aph-1. However, in the γ-30 cells, which express high levels of all of the γ-secretase components and in which almost all of the NCT has been converted to mNCT (24), we observed significant levels of both iNCT and mNCT brought down with Aph-1 (Fig 1c, lane 8). Interestingly, in these cells, which have more mature γ-secretase and therefore less of its immature components available in excess (24), Aph-1 bound to less PS holoprotein and more abundant amounts of PS heterodimers than the A5 line (compare lanes 6 and 8 of Fig. 1c). These results are consistent with a preferential association between Aph-1 and iNCT that is more apparent when either of these two γ-secretase components is in excess.

To determine whether this preferential association with iNCT occurred for Aph-1 but not Pen-2, we performed similar co-IP experiments with PS70 cells that stably overexpress human FLAG-Pen2 (P22 cells) and with the above described γ-30 cells. As previously noted (24), transient expression of FLAG-Pen2 provides inefficient entry into the endogenous γ-secretase complex. On the other hand, in lysates from the stable P22 cells, anti-FLAG co-IP revealed efficient binding of FLAG-Pen2 to PS1 NTF, PS1 CTF, and mNCT (Fig. 1d), all surrogates of association with a mature, proteolytically active γ-secretase complex (14, 19). We also observed considerable levels of PS holoprotein co-immunoprecipitated with FLAG-Pen2, consistent with the purported role of Pen-2 in PS endoproteolysis (23, 27). Substantial levels of INCT were not found in the FLAG-Pen2 precipitates; therefore, the binding to INCT appears specific for Aph-1. Because the further glycosylation of iNCT into mNCT has been shown to require PS (18–20, 28), our data suggest that PS and Pen-2 are likely to associate with NCT after the latter has already bound Aph-1. When Pen-2 was immunoprecipitated from γ-30 cell lysates, relative to the levels present in crude lysates, we found similar levels of mNCT and iNCT, PS holoprotein, and PS NTF and CTF associated with FLAG-Pen2 (Fig. 1d, lane 4) as were observed in the P-22 cells (Fig. 1d, lane 2). This result suggests that the association of Pen-2 with components of the γ-secretase complex is unaffected by overexpressed Aph-1, which is present in the γ-30 cells but absent in the P-22 cell line.

Aph-1 and NCT Form a Detergent-resistant Complex—The integrity of the γ-secretase complex is exquisitely sensitive to the detergent in which it has been solubilized (14, 29). Whereas several detergents have been reported to maintain γ-secretase integrity and protease activity, including CHAPS, CHAPS, Big CHAP, and digitonin, other detergents result in either the reversible disruption of γ-secretase activity (Brij-35) or the complete and irreversible dissociation of the complex (Triton X-100 and Nonidet P-40) (14). Therefore, we tested a variety of detergents for their ability to preserve γ-secretase integrity as determined by co-IP of the components. For these experiments, we utilized the γ-30 cell line and solubilized equal numbers of cells in three different detergents (CHAPSO, DDM, Nonidet P-40). We immunoprecipitated equal lystate volumes with an immobilized antibody (3F10 affinity resin) against the HA tag on the C terminus of the stably expressed Aph-1-2-HA. Whole cell lysates prepared from 1% Nonidet P-40-solubilized cells had detectable levels of mNCT, iNCT, PS holoprotein, PS NTF, PS CTF, Aph-1-2-HA, and FLAG-Pen2 (Fig. 2b, lane 1). When the γ-30 cells were solubilized in CHAPSO, a detergent compatible with γ-secretase activity (29), all known components of the γ-secretase complex were efficiently co-immunoprecipitated by 3F10 (Fig. 2b, lane 2). Accordingly, many of the γ-secretase components were reduced in the unbound fraction compared with the starting lysate (Fig. 2b, compare lanes 5 and 1, respectively). When the γ-30 cells were solubilized in DDM, Aph-1-2-HA co-precipitated with robust levels of mNCT and iNCT, but there was essentially no PS holoprotein, PS NTF, PS CTF, or FLAG-Pen2 (Fig. 2b, lane 3). Accordingly, NCT and Aph-1-2-HA were the only γ-secretase components that were less abundant in the unbound than bound fractions from the DDM-solubilized lysates (Fig. 2b, compare lanes 6 and 3, respectively). When the γ-30 cells were solubilized in Nonidet P-40, Aph-1-2-HA was the only γ-secretase member efficiently bound by the anti-HA resin (Fig. 2b, lane 4), with all other components remaining in the unbound fraction (lane 7). These data demonstrate that whereas all members of the γ-secretase complex are tightly bound to Aph-1 in the presence of CHAPSO and are completely dissociated from it in Nonidet P-40, DDM solubilization imparts a partial dissociation of the mature complex that removes FLAG-Pen2 and the PS heterodimers from...
**A**

FIG. 2. A, the γ-secretase complex shows similar integrity by co-IP following lysis in either 1% CHAPSO or in the BN-PAGE lysis buffer (containing 1% digitonin). Whole cell lysates from γ-30 cells were prepared in the respective lysis buffers and co-immunoprecipitated overnight with anti-HA affinity resin, and bound and unbound fractions were probed for members of the γ-secretase complex. Crude lysate lanes contain 5% of the material used in the immunoprecipitation.

**B**

B, Aph-1 and NCT form a detergent-stable complex after dissociation of the γ-secretase complex. Whole cell lysates were prepared from γ-30 cells in either 1% CHAPSO, 1% DDM, or 1% Nonidet P-40. All three lysates were immunoprecipitated with an anti-HA affinity resin to bind the HA tag present on the C terminus of Aph-1a2-HA. The bound and unbound fractions were probed for all components of the γ-secretase complex by Western blot. Crude lysate lanes contain 5% of the material used in the IP.
Aph-1α2 but preserves a subcomplex of Aph-1α2 and NCT. This finding is consistent with the BN-PAGE observation of a stable Aph-1α2-NCT complex and the preferential association between these two proteins in CHO cells (Fig. 1).

**Identification of the Aph-1-NCT Subcomplex in Human 293 Cell Lines**—To confirm these observations in an independent cell line, we repeated these experiments using HEK293 cells stably transfected with APPsw and NCT-V5 (HEK-NCT cells). These cells were transiently transfected with Aph-1α2-HA, and the Aph-1α2-HA-transfected and untransfected membrane fractions were compared. Samples were normalized for total protein, run on BN-PAGE gels, and probed for NCT and Aph-1α2-HA. Both endogenous and V5-tagged (exogenous) NCT were detected at three different molecular weights on the native gels (Fig. 3a, lanes 1–4). The middle of these three bands migrated at the approximate relative position of the iNCT-Aph-1 subcomplex we had identified in the CHO cells (Fig. 1a), and the upper band represents the mature γ-secretase complex described previously at ~250–270 kDa (Fig. 1a) (24). We found that the transiently transfected Aph-1α2-HA protein was detected solely in the middle NCT-reactive band and spared the iNCT-only band observed by BN-PAGE. Membrane fractions were prepared from HEK293 cells and were probed for endogenous NCT and endogenous Aph-1. D, two independent pools of endogenous Aph-1 and NCT are separated by two-dimensional PAGE analysis, whereas Pen-2 and PS NTF are only found within one of these complexes (i.e., the HMW active γ-secretase complex).

![Fig. 3. Aph-1 and NCT from a stable γ-secretase intermediate in HEK cells.](image)

A, HEK-NCT cells either mock-transfected or transfected to express Aph-1α2-HA and membrane preparations were probed by BN-PAGE analysis. Aph-1 and NCT co-migrated at molecular weights that correspond to the active, HMW γ-secretase complex, to the Aph-1-NCT intermediate, and to iNCT alone. B, the addition of 1% Nonidet P-40 caused selective disruption of the HMW γ-secretase band and the Aph-1-NCT band and spared the NCT-only band observed by BN-PAGE. C, endogenous NCT and endogenous Aph-1 co-migrate following native gel electrophoresis. Membrane fractions were prepared from HEK293 cells and were probed for endogenous NCT and endogenous Aph-1. D, two independent pools of endogenous Aph-1 and NCT are separated by two-dimensional PAGE analysis, whereas Pen-2 and PS NTF are only found within one of these complexes (i.e., the HMW active γ-secretase complex).
complex (Fig. 3a, lane 5). The observation of endogenous Aph-1 in the high molecular weight γ-secretase complex (lane 5) confirms previous observations that when expressed at endogenous levels, Aph-1 is a bona fide member of the active γ-secretase complex (30). Consistent with previous evidence that transient expression of Aph-1 results in poor incorporation into the endogenous γ-secretase complex (24), BN-PAGE analysis of cell lysates following transient expression of Aph-12-HA showed that the exogenous HA-tagged Aph-1 co-migrated with the Aph-1-NCT subcomplex but not the mature four-component complex (Fig. 3a, lane 8).

To verify that the intermediate NCT band present on the BN-PAGE blots was in fact a subcomplex of Aph-1 and NCT and that the lowest NCT band was unassociated iNCT, we relied on the disruption of the Aph-1-NCT complex by Nonidet P-40 demonstrated in Fig. 2. The addition of 1% Nonidet P-40 into the digitonin-solubilized HEK-NCT cell lysates caused a selective decrease of the HMW γ-secretase complex and the intermediate NCT-reactive bands but not the lowest NCT-reactive band on BN-PAGE gels (Fig. 3b), indicating that the slower migration of these two bands is due to their association with another protein(s) that is disrupted by Nonidet P-40. This disruption of the subcomplex in the HEK-NCT cells is consistent with its disruption in our CHO co-IP experiments (Fig. 2b).

In the HEK-NCT cells, the Aph-1-NCT complex migrated at a molecular mass slightly larger than the ~140 kDa that was found in the CHO cells. The explanation for the difference in apparent size of the intermediate complexes in these two cell types is unknown, but we have found some variability in the migration of this intermediate depending on the detergent conditions, number of centrifugations, etc. Therefore, it may well be due to differences in the preparation of HEK membranes necessary to concentrate and enrich the samples for γ-secretase components, a step that was not needed for the γ-30 whole cell lysates.

To validate the observation of a stable subcomplex of Aph-1 and NCT from solely endogenous proteins, we analyzed parental (untransfected) HEK293 cells by BN-PAGE for endogenous NCT and observed two bands (Fig. 3c, lane 1), in contrast to the three bands detected in the HEK-NCT transfectants (Fig. 3a, lanes 1 and 3). These two bands migrated at the positions of the HMW γ-secretase complex and the Aph-1-NCT subcomplex we had observed in the HEK-NCT cells, with no band migrating at the position of the iNCT band in the latter cells. Importantly, we found two bands immunopositive for endogenous Aph-1 from the HEK293 cells that correspond to 1) the Aph-1-NCT subcomplex and 2) the mature γ-secretase complex (Fig. 3c, lane 2). We further characterized the γ-secretase complex composed entirely of endogenous proteins by two-dimensional PAGE analysis and confirmed that both Aph-1 bands detected by BN-PAGE with the polyclonal antisera corresponded to bona fide Aph-1 (Fig. 3d). Likewise, we found NCT to co-migrate with both pools of Aph-1, whereas Pen-2 and PS NTF were only found in the HMW, mature γ-secretase complex. These data, consistent with the result seen in the CHO γ-30 cells, also demonstrate that the slightly higher molecular weight of the Aph-1-NCT subcomplex observed in HEK293 microsomes cannot be accounted for by the presence of another γ-secretase complex member, supporting the notion that this might be an artifact of microsomal membrane preparation.

Characterization of the Aph-1-associated NCT Isoform in Human 293 Cells—Mature, fully glycosylated NCT is readily detectable in all cell lines that we have examined for endogenous γ-secretase activity: mouse primary neurons and fibroblasts and CHO, COS, HEK293, HeLa, and Jurkat cell lines.2 Because the mature form has been shown by several laboratories to be a necessary component of γ-secretase, this result is expected. However, not all cell types also contain high steady-state levels of immature NCT. In particular, HEK293 cells and COS cells do not show substantial levels of iNCT protein. We therefore examined whole cell lysates of the HEK-NCT transfectants, because iNCT and mNCT are detected at similar levels in the HEK293 cells, by BN-PAGE blots and the polyclonal antibody to the PS1 NTF. We then probed both immunoprecipitates for NCT, PS1 NTF, and PS1 CTF. In both mock-transfected and Aph-12-HA-transfected cells, X81 selectively brought down mNCT with significant amounts of PS1 NTF and CTF (Fig. 4a, lanes 3 and 6). In contrast, IP of Aph-12-HA revealed a selective co-IP of substantial amounts of iNCT, with little or no mNCT (Fig. 4a, lane 10). Furthermore, very little PS1 heterodimer was brought down with the exogenous Aph-12-2-HA. The co-precipitation of iNCT with Aph-12-2-HA was specific, since no NCT bound to the anti-HA resin in the mock-transfected cell lysates (Fig. 4a, lane 8).

To confirm these results using just endogenous NCT, we repeated these experiments in parental HEK293 cells. Whereas the levels of iNCT were dramatically lower in these cells (compare Fig. 4b, lane 1, with Fig. 4a, lane 1), transient transfection with Aph-12-2-HA and IP with the 3F10 anti-HA resin again revealed the preferential association with iNCT, despite the presence of far more mNCT in the starting lysate (Fig. 4b, lane 5). Indeed, Aph-12-2-HA associated weakly with the mature components of γ-secretase: mNCT, PS1 NTF, and PS1 CTF (Fig. 4b, lane 5). Taken together with our BN-PAGE experiments, these results support the existence of an intermediate subcomplex of Aph-12-2-HA and iNCT. However, these experiments still relied on transient transfection of a tagged Aph-1 construct. We therefore performed co-IP experiments on untransfected HEK293 cells, demonstrating that unlike X81, the Aph-1 antibody to endogenous Aph-1 resulted principally in the marked enrichment of iNCT plus some mNCT, PS1 NTF, and PS1 CTF (Fig. 4c, compare lanes 3 and 6). These findings are consistent with our BN-PAGE results (Figs. 1a and 3) and confirm that one pool of Aph-1 is associated (perhaps solely) with iNCT and another is associated with mNCT and PS heterodimers in the active γ-secretase complex. Importantly, these two Aph-1 pools are detectable at endogenous expression levels and at steady state.

Depletion of Pen-2 Results in the Stabilization of both iNCT and PS Holoprotein—Our results thus far suggest that not only do Aph-1 and iNCT form a subcomplex prior to the assembly of the complete and active γ-secretase but also that the PS holoprotein may interact with this subcomplex before the incorporation of Pen-2. To investigate the functional evidence for this apparent trimeric complex (Aph-1-NCT-PS), we treated HEK cells stably overexpressing Swedish mutant APP (HEKsw) with siRNA directed against NCT, Pen-2, or a scrambled control. The data showed that the Pen-2 siRNA resulted in a reduction of mNCT but a paradoxical increase in iNCT (Fig. 5a, lanes 5 and 6), whereas the NCT siRNA reduced both forms, as expected (Fig. 5a, lanes 3 and 4). Both siRNA duplexes increased APP CTFs (indicative of decreased γ-secretase activi-
ity), but with different efficacies (Fig. 5b). The fact that Pen-2 depletion increased the levels of both iNCT and PS holoprotein is consistent with our earlier observation that these proteins interact with Aph-1 in an additional, stable γ-secretase subcomplex containing these three co-factors (i.e. the addition of Pen-2 as the final member of the complex allows NCT maturation and PS endoproteolysis) (23, 27).

**DISCUSSION**

Here, we demonstrate three novel principles regarding the biochemical composition and assembly of the γ-secretase complex. First, we find a stable, intermediate complex between NCT and Aph-1 in an additional, stable γ-secretase subcomplex containing these three co-factors (i.e. the addition of Pen-2 as the final member of the complex allows NCT maturation and PS endoproteolysis) (23, 27).

**Assembly of the γ-Secretase Complex**

**A**

| Transfection: | Mock | Aph1α2-HA | Mock Aph1α2-HA |
|---------------|------|-----------|----------------|
| IP:           |      |           |                |
|               | Lysate | post | Lysate | post | Lysate | post | Lysate | post |

**B**

| Transfection: | Aph1α2-HA |
|---------------|-----------|
| IP:           |           |
|               | Lysate | post | Lysate | post | 3F10 |

**C**

| IP: | X81 | A89 |
|-----|-----|-----|
|     | Lysate | post | Lysate | post | Lysate | post |

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**Fig. 4.** Aph-1 preferentially binds to iNCT and not mNCT in HEK cell lines. A, exogenous Aph-1α2-HA binds to iNCT in HEK-NCT cells, whereas PS is found associated with mNCT. HEK-NCT cells were either mock-transfected or transfected with Aph-1α2-HA. Whole cell lysates were immunoprecipitated with either X81, an antibody directed against the N terminus of PS1, or with an affinity resin against the HA tag present on the C terminus of Aph-1α2-HA. Precipitates were probed for NCT, PS holoprotein, PS NTF, and PS CTF by Western blot. Crude lysate lanes contain 5% of the material used in the IP. B, Aph-1α2-HA selectively interacts with iNCT and not mNCT in HEK293 cells. HEK293 cells were transfected with Aph-1α2-HA and immunoprecipitated with an anti-HA affinity resin. Precipitates were probed for NCT, PS holoprotein, PS NTF, and PS CTF by Western blot. Crude lysate lanes contain 5% of the material used in the IP. C, endogenous Aph-1 interacts with both iNCT and mNCT. Untransfected HEK293 cells were prepared and immunoprecipitated with either X81 or A89, a high titer antibody directed against human Aph-1. Precipitates were probed for the association of PS with either NCT or PS CTF. Crude lysate lanes contain 5% of the material used in the IP.
does not require additional γ-secretase members. Taken together, these data provide new insights about the nature and order of assembly among the multiple components of the γ-secretase complex.

Native gel electrophoresis and subsequent Western blotting of the γ-secretase complex revealed important information regarding the features of γ-secretase assembly. Some previous studies that used BN-PAGE analysis of γ-secretase reported the complex at a molecular mass of 500 kDa (22), which would approximate the size of a dimer of all the four known components. In contrast, we have observed only an apparent monomeric complex of the four components in numerous experiments performed in two distinct cell lines (CHO and 293). In this work, we electrophoresed as standards two different tissue sources of both catalase (232 kDa) and ferritin (440 kDa) to establish the positions of native molecular weight markers (data not shown), and we employed the BN-PAGE-compatible detergent, 0.5% DDM, in an effort to reconcile these contrasting findings. However, under the various conditions tested, the migration of the four-component γ-secretase complex always occurred between catalase and ferritin, not above ferritin, suggesting that only a monomeric complex is present under our experimental conditions. Whereas we have only observed a monomeric γ-secretase complex following native electrophoresis, this does not exclude the possibility that γ-secretase may also function in vivo as a dimeric complex or be biochemically visualized as such following different preparations. Additional work will be required to resolve this complex issue.

The mature, proteolytically active γ-secretase complex is currently thought to consist of the PS-NTF-CTF heterodimer, a highly glycosylated form of NCT, Aph-1, and Pen-2 (22–24). Two forms of glycosylated NCT have been documented by several laboratories. It is not known what factors contribute to the modification of unglycosylated NCT (80 kDa) into the iNCT form (110 kDa), but it has been well established that the presence of the PS heterodimer is required for the further maturation of INCT to mNCT (150 kDa). Moreover, it has been shown that the active complex is selectively associated with mNCT isoform. Here, we show for the first time that an intermediate subcomplex appears to form early in γ-secretase assembly and is composed of Aph-1 and iNCT. The existence of this stable intermediate can be shown with both endogenous and exogenously expressed components using native gel electrophoresis and co-IP, and we have observed the interaction in multiple cell lines. Consistent with published evidence that PS is required for the formation and stabilization of mNCT, we did not find PS associated with the 140-kDa iNCT-Aph-1 subcomplex. This result suggests that PS interacts with Aph-1 and NCT following initial association of the latter components. There are at least two possible circumstances for the initial Aph-1-NCT association. First, Aph-1 might act to stabilize nascent NCT and allow its N-linked glycosylation in the ER and then remain associated with NCT throughout γ-secretase assembly. Second, Aph-1 might not be able to bind nascent NCT until the latter has been post-translationally modified to iNCT and then encounters Aph-1 in the Golgi. Discrimination between these possibilities will require further study.

The co-IP experiments we performed (Figs. 1, 2, and 4) provide evidence about when Pen-2 enters the γ-secretase complex as well. Whereas Aph-1 can associate with both iNCT and mNCT with a preference for iNCT, Pen-2 appears to interact preferentially with mNCT. This finding suggests that Pen-2 interacts with the Aph-1-NCT subcomplex relatively late in the process of assembly, perhaps just prior to or following the full glycosylation of NCT (which is indicative of a mature, active γ-secretase complex). These data are consistent with the recent observation that siRNA-mediated depletion of Pen-2 results in the unusual stabilization of the PS holoprotein (23, 27). This stabilization apparently results from an increased half-life of the protein (23, 27) along with its entry into a stable high molecular weight complex (23). Taken together, these studies suggest that the increased stability of PS holoprotein is due to its association with the remaining members of the γ-secretase complex (Aph-1 and NCT), and consistent with this hypothesis, siRNA depletion of endogenous Aph-1 or NCT blocked the stabilizing effects of Pen-2 depletion on the PS holoprotein (23, 27). A model was proposed by Takasugi et al. (23), suggesting that PS binds to NCT and Aph-1, forms a stable high molecular weight complex, and then subsequently binds Pen-2, allowing for PS endoproteolysis and the final glycosylation of NCT. Our data are consistent with this model; however, the present observations suggest that PS associates with a precomplexed Aph-1-NCT intermediate and that PS does not independently bind to Aph-1 and NCT. Whereas a direct interaction between Aph-1 and PS has not been formally ruled out, our data from two-dimensional PAGE analysis of both γ-30 and HEK293 cells demonstrate that PS holoprotein migrates in a HMW complex with both NCT and Aph-1. This modification of the Takasugi model is supported by the observation that all of the endogenous iNCT in HEK293 cells was found complexed with endogenous Aph-1 (Fig. 3c), such that there would be no free NCT to associate with PS other than in this observed intermediate. Furthermore, we could dissociate the Aph-1-NCT intermediate from the rest of the γ-secretase complex in the presence of a mildly denaturing detergent (DDM), demonstrating that the interaction between Aph-1 and NCT in the mature complex does not require other co-factors for binding and stability. Our data are also consistent with recent observations in fly cells that suggested a unique protein-protein interaction between Aph-1 and NCT, where it was suggested that these two proteins might form a stable subcomplex, which we have now shown (31). However, our present data do support the conclusions of Takasugi et al. (23), who suggested that following depletion of Pen-2, a stable, high molecular weight complex of
Aph-1, NCT, and PS forms. Here, we report evidence of this trimeric intermediate at steady-state levels and under conditions that do not require the disruption of Pen-2 expression (Fig. 1b), but we also provide Pen-2 siRNA data that support the existence of this intermediate (Fig. 5). Therefore, the stabilization of PS holoprotein through its interaction with Aph-1 and NCT does not appear to be an artifact of Pen-2 siRNA treatment (23, 27) but rather appears to represent part of the physiological process of γ-secretase assembly. Since PS is thought to contain the catalytic site of γ-secretase, most hypothetical models of γ-secretase have focused on the binding of co-factors to PS itself. Therefore, it is particularly interesting that two γ-secretase co-factors (Aph-1 and NCT) can assemble in the absence of PS. The later association of PS with two of its three co-factors during assembly may serve a mechanistic function, in that the catalytic component is not called into play until conditions are appropriate (i.e. until there has been stabilization of an earlier intermediate). Considering the vast number of γ-secretase substrates and the promiscuous nature and constitutive activity of the mature γ-secretase complex, such temporal and perhaps spatial regulation may be beneficial.

Previous studies have shown that the levels of γ-secretase activity are tightly regulated, presumably by limited availability of other cellular factors (6, 32). Recently, it has become apparent that high expression of four proteins (the PS heterodimer, NCT, Aph-1, and Pen-2) is sufficient to overcome the regulation of γ-secretase activity and increase the levels of mature PS and NCT (24, 27) and that expression of these four components in yeast can reconstitute γ-secretase activity (33).

In the current work, we show that these tenets are also true of the Aph-1-NCT intermediate. When we compared HEK293 cells (which only express endogenous NCT) with the HEK-NCT cells (which express excess exogenous NCT), we found that only when NCT was ectopically overexpressed was there a pool of NCT that was not complexed with Aph-1, suggesting that like the assembly of the mature γ-secretase complex, levels of the Aph-1-NCT intermediate subcomplex are also regulated. In conclusion, our findings demonstrate that Aph-1 and NCT form a stable intermediate that later binds to PS holoprotein and Pen-2 during the assembly of the active γ-secretase complex, which mediates the intramembranous proteolysis of diverse signaling proteins. However, many details remain to be uncovered, such as determining which γ-secretase co-factors physically interact with one another and ultimately identifying the protein-protein binding domains that govern these interactions. The current data regarding the order of γ-secretase assembly provide important insights into designing these further studies.

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REFERENCES
1. Munem, J. S., and Kopan, R. (2000) Dev. Biol. 228, 151–165
2. Ebihara, D. J., and Yankner, B. A. (2002) Neuron 34, 499–502
3. Brown, M. S., Ye, J., Lawson, R. B., and Goldstein, J. L. (2000) Cell 100, 391–398
4. Wolfe, M. S., and Selkoe, D. J. (2002) Science 296, 2156–2157
5. Zhang, J., Kang, D. E., Xia, W., Okochi, M., Mori, H., Selkoe, D. J., and Koo, E. H. (1998) J. Biol. Chem. 273, 12436–12442
6. Rottkowsk, T., Slunt, H. H., Thakarak, G., Price, D. L., Sisodia, S. S., and Borchelt, D. R. (1997) J. Biol. Chem. 272, 24536–24541
7. Thakarak, G., Borchelt, D. R., Lee, M. X., Slunt, H. H., Spitzer, L., Kim, G., Rottkowsk, T., Slunt, H., Cao, A., Li, Y., Lai, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1996) Neuron 17, 181–190
8. Podlisn, M. B., Citron, M., Amara, P., Sherrington, R., Xia, W., Zhang, J., Diehl, T., Levesque, G., Fraser, P., Haas, C., Koo, E. H., and Seubert, P., St. George-Hyslop, P., Teplew, D. B., and Selkoe, D. J. (1997) Neurobiol. Dis. 3, 325–337
9. George-Hyslop, P., Borchelt, D. R., Diehl, T. S., Moore, C. L., Tsai, J.-Y., Rahmati, T., Xia, W., Selkoe, D. J., and Wolfe, M. S. (2000) Nat. Cell Biol. 2, 429–434
10. Hyslop for providing the HEK-NCT cell line and the Aph-1 antibody, Ab14, D. Miller and P. Savam for the NCT antibody, R302, and R.302, and Pen-2 during the assembly of the active Aph-1-iNCT intermediate subcomplex are also regulated. In conclusion, our findings demonstrate that Aph-1 and NCT form a stable intermediate that later binds to PS holoprotein and Pen-2 during the assembly of the active γ-secretase complex, which mediates the intramembranous proteolysis of diverse signaling proteins. However, many details remain to be uncovered, such as determining which γ-secretase co-factors physically interact with one another and ultimately identifying the protein-protein binding domains that govern these interactions. The current data regarding the order of γ-secretase assembly provide important insights into designing these further studies.

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