Detergent-Dependent Dissociation of Active γ-Secretase Reveals an Interaction between Pen-2 and PS1-NTF and Offers a Model for Subunit Organization within the Complex†

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ABSTRACT: γ-Secretase is a member of a new class of proteases with an intramembrane catalytic site and cleaves numerous type I membrane proteins, including the amyloid β-protein precursor (APP) and the Notch receptor. Biochemical and genetic studies have identified four membrane proteins as components of γ-secretase: a heterodimeric form of presenilin (PS), composed of its N- and C-terminal fragments (PS-NTF and PS-CTF, respectively), a highly glycosylated, mature form of nicastrin (NCT), Aph-1, and Pen-2. However, it is unclear how these components interact physically with each other and assemble into functional complexes. We and others recently found that Aph-1 interacts with a less glycosylated, immature form of nicastrin as an intermediate toward full assembly of γ-secretase. Here we show that (1) the detergent dodecyl β-D-maltoside (DDM) mediates the dissociation and inactivation of active γ-secretase in a concentration-dependent manner, (2) DDM-dependent dissociation of the active γ-secretase complex generates two major inactive complexes (Pen-2−PS1-NTF and mNCT−Aph-1) and two minor inactive complexes (mNCT−Aph1−PS1-CTF and PS1-NTF−PS1-CTF), and (3) Pen-2 can also associate with the PS holoprotein in complexes devoid of NCT and Aph-1. Taken together, our results demonstrate that Pen-2 interacts with PS-NTF within active γ-secretase and offer a model for how the components of active γ-secretase interact physically with each other.

γ-Secretase is an unconventional aspartyl protease with an intramembrane catalytic site that represents a new class of intramembrane-cleaving proteases (I-CliPs) (*1*, *2*). This protease is required for the intramembranous cleavage of an expanding variety of type I membrane protein substrates that include the amyloid β-protein precursor (APP)[1] (*3*), the Notch receptor (*4*), and several others (*5*−*9*). Sequential proteolytic cleavage of APP by β-secretase (BACE) and γ-secretase generates amyloid β-proteins (Aβ) of 39−42 amino acids (for a review, see ref *10*). The formation and aggregation of Aβ in the brain are implicated in the pathogenesis of Alzheimer's disease (AD); thus, β- and γ-secretases have emerged as key therapeutic targets (*11*). γ-Secretase is a multiprotein complex that contains presenilin (PS), nicastrin (NCT), Aph-1, and Pen-2. PS holoproteins are cleaved between the sixth and seventh domains of their eight putative TM domains by an unidentified "presenilinase" activity, which converts them into N- and C-terminal fragments (PS-NTF and PS-CTF, respectively) (*12*) that remain associated (*13*). Several lines of evidence suggest that the NTF−CTF heterodimeric form of PS contains the catalytic site of γ-secretase. First, deletion of the PS1 and PS2 genes eliminates γ-secretase activity (*3*, *14*, *15*). Second, γ-secretase exhibits characteristics of aspartyl proteases (*16*, *17*), and mutation of either of two conserved intramembranous aspartates in TM domains 6 and 7 blocks Aβ generation (*18*). Third, aspartyl protease transition state analogue inhibitors of γ-secretase, including some that mimic the TM region of APP that undergoes cleavage, bind directly to PS1-NTF and PS1-CTF (*19*, *20*). Moreover, missense mutations in the human presenilins (PS1 and PS2) alter Aβ production and cause early-onset familial AD (FAD) (for a review, see ref *21*).

NCT, a single-pass transmembrane protein identified via its association with PS1 and Notch (*22*, *23*), is also essential for γ-secretase activity. This protein becomes N-glycosylated in the Golgi/TGN compartments, and the "mature" form of NCT is associated with the active γ-secretase complex (*24*−*27*). Recently, genetic screens in *Caenorhabditis elegans* have revealed two additional PS cofactors, Aph-1 and Pen-

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[1] Abbreviations: Aβ, amyloid β-protein; AD, Alzheimer's disease; APP, amyloid β-protein precursor; AICD, APP intracellular domain; BN, blue native gel analysis; CHO, Chinese hamster ovary; CTF, C-terminal fragment; DDM, dodecyl β-D-maltoside; DC, dissociated complex; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FAD, familial Alzheimer's disease; GST, glutathione *S*-transferase; GSH, glutathione; HMW, high molecular weight; IP, immunoprecipitation; NCT, nicastrin; NTF, N-terminal fragment; PS, presenilin; TM, transmembrane; WT, wild-type; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis.

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2, required for processing of PS into NTF−CTF heterodimers and for γ-secretase cleavage of APP and Notch substrates (*28*, *29*). Several studies demonstrated that Aph1α2, one of the three reported isoforms of Aph1 (Aph1α1, Aph1α2, and Aph1β) arising from alternative transcript splicing, plays an important role in the early maturation and activity of the γ-secretase complex (*29*, *30*). Downregulation of Pen-2 expression in different cell lines by small interfering RNAs (siRNA) abolishes the endoproteolysis of PS1, whereas overexpression of Pen-2 promotes the cleavage of PS1, indicating an important role for Pen-2 in PS1 NTF−CTF heterodimer formation (*31*−*33*). Simultaneous overexpression of the four identified components reconstitutes γ-secretase activity in *Drosophila*, mammalian, and yeast cells and allows for their partial copurification (*33*−*35*).

A model of the stepwise assembly and activation of the γ-secretase complex has recently been proposed, based on Pen-2 depletion (*33*). In this model, the nascent PS holoprotein is stabilized in a high-molecular weight (HMW) inactive γ-secretase complex by binding to Aph1 and NCT; in a second step, Pen-2 binds to this HMW complex, facilitating endoproteolysis of PS and conferring γ-secretase activity. We and others have recently found that Aph1α2 interacts with iNCT in a low-molecular weight subcomplex, apparently as an initial step in the assembly of the active γ-secretase complex (*36*, *37*). Despite this progress, exactly how the four identified components physically interact with each other and assemble into a functional protease is unclear. In this study, we report that (1) a stable mammalian cell line coexpressing human PS1, Aph1α2, Pen-2, and NCT (the S-1 cell line) shows an increase in the amounts of PS1-NTF and -CTF fragments as well as increased γ-secretase activity; (2) both endogenous and overexpressed active γ-secretase complexes can be similarly dissociated in a detergent-dependent manner into two major inactive complexes, one containing Pen-2 and PS1-NTF and the other containing mNCT and Aph1α2, and also into two minor inactive complexes, one containing mNCT, Aph1α2, and PS1-CTF and the other containing PS1-NTF and -CTF; and (3) the PS1 holoprotein can interact with Pen-2 in complexes devoid of NCT and Aph1α2. Taken together, these findings demonstrate that Pen-2 interacts preferentially with PS1-NTF within the active γ-secretase complex. The identification and characterization of the complexes generated by the DDM-mediated dissociation of active γ-secretase offer a model for the organization of the subunits within the complex.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Transfections. Human NCT cDNA without the stop codon was amplified by PCR from the pcDNA3.1-NCT-V5 plasmid (*34*). GST cDNA without the initiation codon was amplified from plasmid pGEX (Amersham Biosciences). An expression construct was generated by subcloning the human NCT cDNA in frame with the GST cDNA at the carboxy terminus of NCT in plasmid pcDNA5.1, thus resulting in the pcDNA5.1-NCT-GST vector. The fused cDNA was fully sequenced.

Cell Lines and Cultures. HeLa S3 cells were cultured as described previously (*38*). Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, penicillin, and streptomycin (Gibco). The cell lines designated P-22 (expressing human PS1 and Pen-2) and γ-30 (expressing human PS1, Pen-2, and Aph1α2) were cultured as previously described (*34*). The cell line designated S-1 was generated by stably transfecting the γ-30 cell line (*34*) with the pcDNA5.1-NCT-GST vector. The S-1 stable cell line was cultured in DMEM supplemented with G418, puromycin, hygromycin, zeocin, and blasticidin.

Western Blotting and Antibodies. For Western analysis of PS1-NTF, PS1-CTF, Aph1α2, Pen-2, and NCT, the samples were run on 4 to 20% Tris-glycine PAGE gels, transferred to polyvinylidene difluoride, and probed with Ab14 (for PS1-NTF, 1:2000, a gift of S. Gandy), N19 (for PS1-NTF, 1:200, Santa Cruz), 13A11 (for PS1-CTF, 5 μg/mL, a gift of Elan Pharmaceuticals), 3F10 (for Aph1α2-HA, 50 ng/mL, Roche), H2D2 (for Aph1α2, 1:2000, a gift of G. Yu), O2C2 (for Aph1α2, 1:2500, a gift of P. Fraser), anti-Flag M2 (for Flag-Pen-2, 1:1000, Sigma), anti-Pen-2 (1:1000, a gift of C. Haass), R302 (for NCT, 1:4000, a gift of D. Miller and P. Savam), guinea pig anti-NCT (1:2000, Chemicon), or αGST antibody (for NCT-GST, 1:3000, Sigma). Samples from the γ-secretase activity assays were run on 4 to 20% Tris-glycine gels and transferred to polyvinylidene difluoride membranes for detection of AICD-Flag or to nitrocellulose membranes for detection of Aβ. The Flag-tagged substrate and AICD product generated by γ-secretase proteolysis were both detected by using anti-Flag M2 antibodies; the other product, Met-Aβ, was detected by using the anti-Aβ 6E10 antibody.

Co-Immunoprecipitations (co-IPs), BN−PAGE, and γ-Secretase Assays. For co-IPs shown in Figure 3, equal amounts of HeLa microsomes were solubilized in 1% digitonin or 1% DDM and centrifuged at 100000*g* for 1 h and the supernatants collected. PS1 co-IPs were performed by using antiserum MAB1563 (1:200, Chemicon, raised to the N-terminus of human PS1, residues 21−80), whereas NCT co-IPs were performed by using guinea pig anti-NCT antibodies (1:200, Chemicon). Lysates (adjusted to 0.5% digitonin and 1% DDM) were incubated overnight at 4 °C [MAB1563 with protein G−Sepharose beads (PGS, Roche) and gpNCT with protein A−Sepharose beads (PAS, Roche)], washed three times for 15 min in 0.5% digitonin and 1% DDM, respectively, and collected in 2× Laemmli sample buffer.

For co-IPs shown in Figure 6, whole cell lysates were prepared in 1% CHAPSO-HEPES (pH 7.4) (Calbiochem) containing a protease inhibitor cocktail and centrifuged at 100000*g* for 1 h, and the supernatant was collected. Protein concentrations were determined using a BCA protein assay kit (Pierce), and 800 μg of protein was included in all IPs. Lysates were incubated overnight at 4 °C, washed three times for 15 min in 1% CHAPSO-HEPES lysis buffer, and collected in 2× Laemmli sample buffer. PS1 co-IPs were performed by using antiserum X81 (raised to the first 81 residues of PS1-NTF), antiserum 4624 (raised to residues 343−357 of PS1-CTF), or antiserum 4627 (raised to residues 457−467 of PS1-CTF) (*39*) in 1% CHAPSO-HEPES and were blotted for PS1-NTF, PS1-CTF, Aph1α2, Pen-2, and NCT with N19, 13A11, 3F10, M2, and guinea pig anti-NCT antibodies, respectively.

For experiments in which γ-secretase assays were performed from IPs, X81 antibody (1:200) and PAS beads were added to solubilized γ-secretase preparations that were

adjusted to 1% CHAPSO (Figure 1B,C). For γ-secretase assays performed with HeLa lysates (Figure 5A,B), equal amounts of cells were solubilized in 1% digitonin, 1% DDM, or 0.5% DDM and centrifuged at 100000*g* for 1 h and the supernatants collected. Protein concentrations were determined as described above; 400 μg of proteins was adjusted to 1% CHAPSO-HEPES buffer and incubated overnight with PAS and X81 antibodies as described above. Following overnight IP at 4 °C, beads were washed three times in 1% CHAPSO-HEPES buffer, resuspended in 0.25% CHAPSO-HEPES buffer, and subjected to *in vitro* γ-secretase assays as described previously (*38*). Blue native gel electrophoresis was performed as previously described (*36*), except for the concentration of the gels used in this study (5−19.5%). The molecular masses of the protein complexes investigated under nondenaturing conditions were determined by comparing their electrophoretic mobilities (R_f) with those of protein standards (Amersham Pharmacia Biotech) and plotting using AlphaEase (Alpha Innotech Corp.). *In vitro* γ-secretase assays and Aβ ELISAs using solubilized membrane fractions and the recombinant APP-based substrate, C100Flag, were performed as previously described (*38*, *40*). C100Flag is expressed in *Escherichia coli* as a fusion protein consisting of a Met that serves as the translation start, amino acids 597−695 of the 695-amino acid isoform of APP, and the Flag tag sequence. The processing of C100Flag by γ-secretase generates the Aβ-related product (Met-Aβ) and AICD-Flag.

GSH and Anti-Flag M2 Affinity Chromatography. γ-30 or S-1 cells were resuspended in HEPES lysis buffer containing 1% digitonin or 1% dodecyl β-D-maltoside (DDM, Fluka) and incubated for 1 h at 4 °C. The solubilized γ-secretase preparations were diluted 1:1 in HEPES buffer (final digitonin and DDM concentration of 0.5%) and incubated overnight with M2 resin (for γ-30 samples) and GSH resin (for S-1 samples) at 4 °C with gentle rocking. The resins were washed three times with HEPES buffer containing 0.5% digitonin and 0.5% DDM, respectively, and the bound proteins eluted with the same washing buffers containing 200 μg/mL Flag peptides and 25 mM reduced GSH, respectively.

RESULTS

Stable Overexpression of Human PS1, Aph1α2, Pen-2, and NCT Increases γ-Secretase Activity in CHO Cells. We previously reported that transient coexpression of human PS1, Aph-1α2, Pen-2, and NCT in COS cells and that stable coexpression of human PS1, Aph-1α2, and Pen-2 in CHO cells (the γ-30 line) each lead to substantial increases in the amount of PS heterodimers, full glycosylation of NCT, and enhanced γ-secretase activity (*34*). Similar results have been reported using *Drosophila* S2 cells, in which the coexpression of Pen-2, NCT, and Aph-1 increased the level of accumulation of PS1 fragments and γ-secretase activity (*33*). We engineered a GST tag onto the C-terminus of NCT and, upon transient transfection into the γ-30 cells, found that the exogenous NCT-GST was expressed, glycosylated, and associated with active γ-secretase. Both mature and immature NCT-GST were detected with αGST antibodies in the whole lysate, and all members of the complex were co-IPed with αGST antibodies (data not shown). Moreover, the NCT-GST co-IPed with PS1 antibodies (X81) and preparations co-IPed with GST antibodies generate APP intracellular domain (AICD) and Aβ products in an *in vitro* γ-secretase assay (not shown). On the basis of these preliminary findings using transient GST-NCT expression, we generated a CHO cell line stably coexpressing PS1, Aph1α2-HA, Flag-Pen-2, and NCT-GST by transfecting the γ-30 cells with NCT-GST (yielding the S-1 cell line). As estimated by densitometry, the levels of PS1-CTF, PS1-NTF, Flag-Pen-2, and Aph1 found in S-1 whole lysate prepared in 1% CHAPSO−HEPES buffer were ∼3.2-, 2.1-, 1.4-, and 1.3-fold higher, respectively, than in the parental γ-30 cell line (in Figure 1A, compare lanes 1 and 2). To demonstrate that equal amounts of solubilized proteins were loaded onto the gel, levels of α-tubulin, a control cytoplasmic protein, were assessed. The level of total NCT (endogenous and NCT-GST) was not estimated because we chose to detect these two forms of NCT separately using anti-nicastrin antibody R302 (which does not detect NCT-GST) and αGST antibody (which does not detect endogenous NCT; Figure 1A, lane 2).

We next determined whether the marked increase in the level of PS1 heterodimers is associated with increased γ-secretase activity. First, we co-IPed γ-secretase in 1% CHAPSO-solubilized whole lysates of γ-30 and S-1 cells with antibodies directed to PS1 (X81) (normalized for equal protein amounts as described above). Second, we solubilized γ-30 and S-1 microsomes in 1% CHAPSO-HEPES. Both of these solubilized γ-secretase-rich preparations were incubated with C100Flag substrate in an effort to examine the level of *in vitro* γ-secretase activity (Figure 1B). In accord with the increased amounts of γ-secretase components (Figure 1A), we found a substantial increase in the level of generation of the AICD-Flag and Aβ products in S-1 cells, compared to the γ-30 cells (Figure 1B). When quantified by ELISAs, the relative γ-secretase activity was 1.9-fold (X81 IPs from whole cell lysates) and 1.8-fold (solubilized microsomes) higher in the S-1 cells than in γ-30 cells (Figure 1C). The latter results indicate that cells overexpressing all four components possess more γ-secretase activity than those relying on excess endogenous NCT, consistent with previous reports (*33*, *34*, *41*). This S-1 cell line was used for the study of protein−protein interactions in the protease complex.

The Detergent Dodecyl β-D-Maltoside (DDM) Mediates the Dissociation of γ-Secretase Overexpressed in CHO Cells. To investigate the composition and function of γ-secretase and to help purify the complex, we asked whether certain detergents could alter and/or improve the solubilization of the complex. Lysates of γ-30 cells were prepared in HEPES lysis buffer containing a protease inhibitor cocktail and 1% of one of the following detergents: CHAPSO, digitonin, or dodecyl β-D-maltoside (DDM). The solubilized lysates (Figure 2A, lanes 1−3) were then diluted 1:1 in HEPES buffer (final detergent concentration of 0.5%) and incubated overnight with M2 affinity resin directed at the Flag tag on Pen-2. The beads were washed and directly resuspended in sample buffer (Figure 2A, lanes 4−6). We found that 1% DDM solubilized larger amounts of NCT and/or Flag-Pen-2 (Figure 2A, lane 2), compared with the amounts solubilized with 1% CHAPSO or 1% digitonin (lanes 1 and 3). Surprisingly, after solubilization in 1% DDM, no NCT coprecipitated with Flag-Pen-2 on the anti-Flag M2 affinity beads (Figure 2A, lane 5), whereas NCT coprecipitated with Flag-Pen-2 after solubilization in 1% CHAPSO or digitonin (Figure 2A, lanes 4 and 6). DDM has been used by several

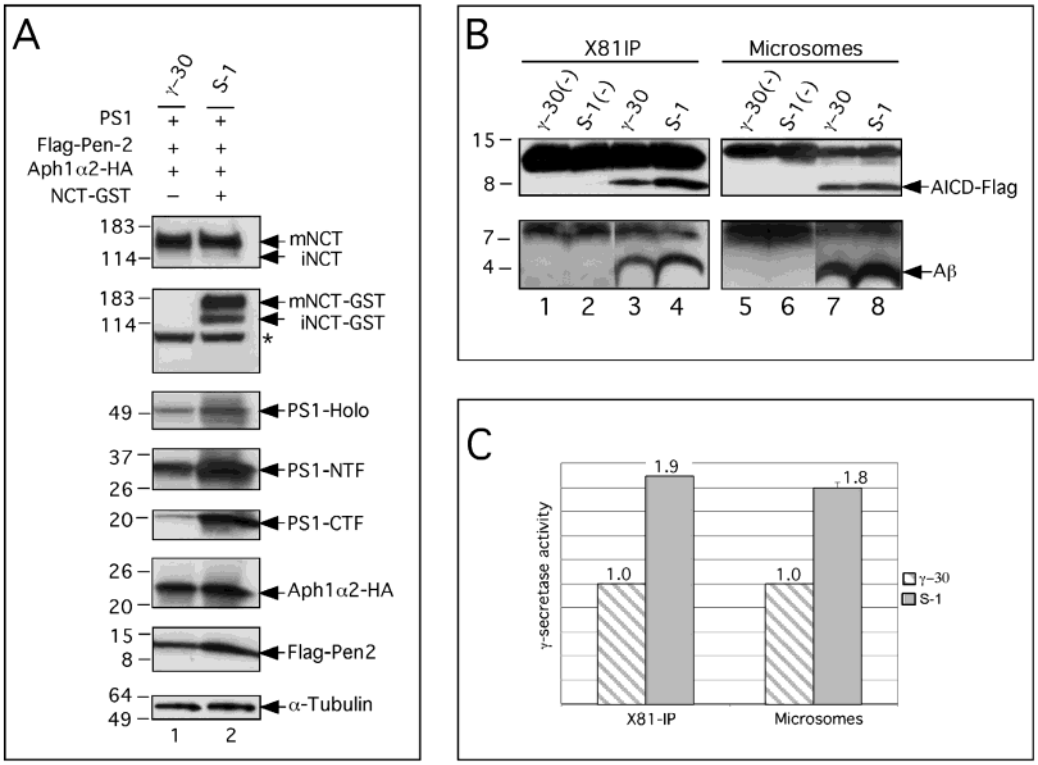

FIGURE 1: Characterization of the S-1 cell line. (A) The Chinese hamster ovary (CHO) S-1 cell line, differing from the previously characterized γ-30 cell line (*34*) by also stably expressing NCT-GST, shows a large increase in the level of mature PS1 heterodimers. Equal amounts of solubilized proteins were blotted for NCT (R302), NCT-GST (αGST), PS1 holoprotein and PS1-NTF (Ab14), PS1-CTF (13A11), Aph1α2 (3F10), and Pen-2 (M2). Levels of α-tubulin are shown as equal loading controls. Note the large increase in the level of mature PS1 heterodimers when all four proteins are coexpressed and the inability of R302 to detect NCT-GST. The asterisk denotes the cross-reactive band recognized by the anti-GST antibodies (αGST). (B) Stable coexpression in the γ-30 cell line of NCT-GST increases γ-secretase activity. For X81 IPs (lanes 1−4), whole cells were solubilized in 1% CHAPSO-HEPES and 400 μg of protein was incubated overnight with X81 antibodies (1:200, raised to the first 81 residues of PS1) and protein A−Sepharose beads; beads were washed three times and γ-secretase C100Flag activity assays performed as previously described (*38*). For the microsomal preparations (lanes 5−8), cell membranes were prepared as previously described (*38*) and solubilized in 1% CHAPSO-HEPES, and equal amounts of proteins were probed for γ-secretase activity as described above. As negative controls for γ-secretase, samples have been treated with SDS (final concentration of 0.5%), prior to the activity assays (lanes 1, 2, 5, and 6). The resulting cleavage products, AICD-Flag and Met-Aβ, were detected with anti-Flag (M2) and anti-Aβ (6E10) antibodies, respectively. (C) The S-1 cell line shows a ∼2-fold increase in γ-secretase activity, when compared to the γ-30 cell lines. Aβ-40 products detected in γ-30 (striped bar) and S-1 (gray bar) after incubation with the C100Flag substrate for 4 h were quantitated with an ELISA. Data that were collected are normalized to activity values measured in γ-30 cells, set equal to 1.

groups to solubilize the γ-secretase complex; BN−PAGE analysis of the endogenous complex solubilized with DDM has revealed a molecular mass of ∼440−550 kDa from mammalian cells and tissues, and co-IPs of DDM-solubilized γ-secretase recovered all four known γ-secretase components under endogenous conditions, as shown in publications from three different laboratories (*25*, *42*, *43*). Because our observation was in apparent contrast to these reports, we decided to investigate the effects of DDM on the structure of the γ-secretase complex. Lysates of γ-30 cells were prepared in 1% digitonin or 1% DDM as described in the legend of Figure 2A and incubated overnight with M2 affinity resin to pull down Pen-2. After washes, the bound proteins were eluted with Flag peptides and probed for all the known components of γ-secretase. We found that after solubilization in 1% digitonin, all four known γ-secretase components were recovered in the eluate (Figure 2B, lane 2). Consistent with our first observation (Figure 2A, lane 5), when solubilized in 1% DDM, PS1-NTF and Pen-2 were recovered in the eluted fraction, but no or very little NCT, PS1-CTF, and Aph1 were recovered (Figure 2B, lane 4). To confirm this effect of 1% DDM on the γ-secretase complex, we prepared lysates from S-1 cells (just as for the γ-30 cells above) and incubated them with GSH affinity resin directed at the GST tag on NCT. The bound proteins were released in batch mode using a buffer containing reduced glutathione. We found again that after solubilization in 1% digitonin, all four known γ-secretase components were recovered in the eluate (Figure 2C, lane 2), whereas when solubilized in 1% DDM, NCT, PS1-CTF, and Aph1 were recovered in the glutathione-eluted fraction; however, no PS1-NTF and very little Pen-2 were recovered (Figure 2C, lane 4). Taken together, these data suggest that 1% DDM mediates the dissociation of the γ-secretase into two complexes: one containing NCT, PS1-CTF, and Aph1 and the other containing PS1-NTF and Pen-2.

DDM Mediates the Dissociation of Endogenous γ-Secretase in HeLa Cells. Because overexpression of proteins could force the association of some members into artificial complexes that may not exist *in vivo*, we decided to repeat the above experiments performed on γ-30 and S-1 cells using HeLa cells and thus investigate the effect of DDM on endogenous γ-secretase. We solubilized HeLa microsomes in 1% digitonin or 1% DDM and performed co-IPs using antiserum MAB1563 raised to N-terminal residues 21−80 of PS1 (Chemicon), or using guinea pig anti-NCT antibodies

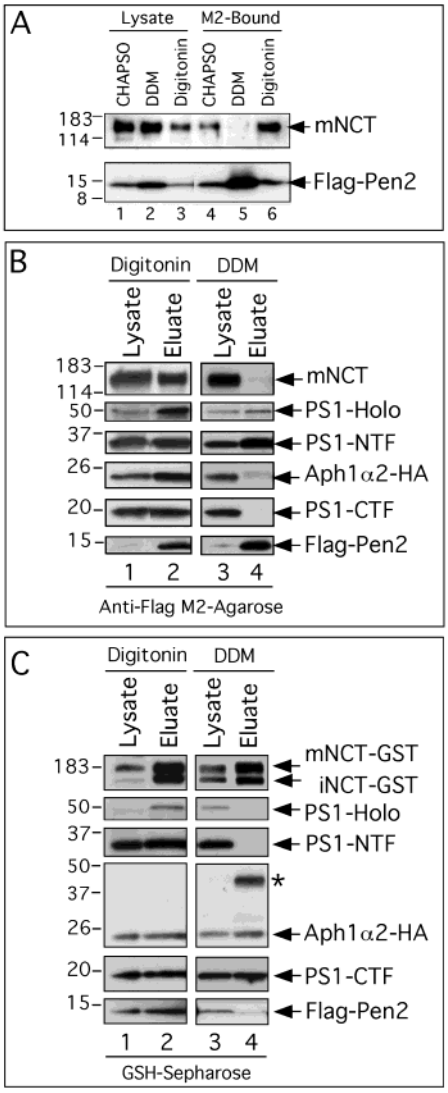

FIGURE 2: DDM, at a concentration of 1% (w/v), mediates the dissociation of the γ-secretase complex. (A) Detergent-dependent affinity co-IP of NCT with Pen-2. Membranes from an equal number of γ-30 cells were solubilized in 1% CHAPSO, 1% DDM, or 1% digitonin, and the resulting lysates (lanes 1−3) were incubated overnight with anti-Flag M2 resin to co-IP Flag-Pen-2. Beads were washed three times in the corresponding solubilization buffers and resuspended in Laemmli sample buffer (M2-bound, lanes 4−6). Lysates and co-IPed proteins were probed for NCT (R302) and Pen-2 (M2). Lysate lanes were loaded with the equivalent of 20% of the material that was co-IPed (M2-bound lanes). Note the absence of NCT co-IPed with Pen-2 specifically in the 1% DDM lysate. (B) Membranes from equal numbers of γ-30 cells were solubilized in 1% digitonin (lanes 1 and 2) or 1% DDM (lanes 3 and 4), and the solubilized proteins (lysates, lanes 1 and 3) were incubated overnight with anti-Flag M2 resin. Beads were washed three times in the corresponding solubilization buffers, and the bound proteins were eluted with 1% digitonin or 1% DDM buffers containing 200 μg/mL Flag peptides (Eluate lanes, lanes 2 and 4). Lysates and eluted proteins were probed for NCT (R302), the PS1 holoprotein and PS1-NTF (Ab14), PS1-CTF (13A11), Aph1α2 (3F10), and Pen-2 (M2). Lysate lanes were loaded with the equivalent of 20% of the affinity-isolated material (Eluate lanes). (C) Membranes from equal numbers of S-1 cells were solubilized as described above for the γ-30 cell line and incubated overnight with GSH affinity resin. The band proteins were eluted as described above, except that the 1% digitonin or 1% DDM elution buffers contained 25 mM reduced glutathione. Lysates (Lysate lanes, lanes 1 and 3) and eluted proteins (Eluate lanes, lanes 2 and 4) were probed as described above, except that αGST was used to detect NCT-GST. The asterisk denotes an Aph1α2 dimer.

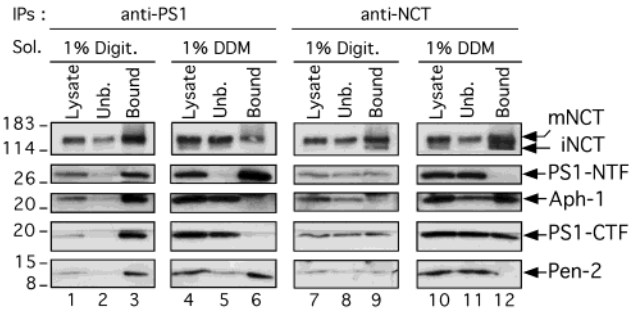

FIGURE 3: DDM, at a concentration of 1% (w/v), mediates the dissociation of the endogenous γ-secretase complex in HeLa cells. Membranes from an equal number of HeLa cells were solubilized in 1% digitonin or 1% DDM, and the resulting solubilized proteins (lysates, lanes 1, 4, 7, and 10) were incubated overnight with monoclonal antibody MAB1563 to co-IP PS1-NTF, or with guinea pig anti-NCT antibody to co-IP NCT. The unbound fractions were recovered (Unb., lanes 2, 5, 8, and 11), and the beads were washed three times in the corresponding solubilization buffers and resuspended in Laemmli sample buffer (Bound, lanes 3, 6, 9, and 12). Lysates and unbound and bound proteins were loaded on a SDS−PAGE gel and were probed for NCT (R302), the PS1 holoprotein and PS1-NTF (Ab14), PS1-CTF (13A11), Aph1α2 (H2D2), and Pen-2 (αPen-2). Lysate and Unb. lanes were loaded with the equivalent of 20% of the material that was co-IPed (Bound) so that the unbound and bound protein levels can directly be compared.

(Chemicon). If 1% DDM also dissociates the endogenous complex, then MAB1563, like the anti-Flag M2 resin, should pull down specifically the complex of PS1-NTF and Pen-2, whereas anti-NCT, like the GSH resin, should pull down specifically the complex of NCT, PS1-CTF, and Aph1. As expected, when endogenous γ-secretase from HeLa cells was solubilized in 1% digitonin, all four known γ-secretase components were co-IPed by both MAB1563 and gpNCT antibodies (Figure 3A, lanes 3 and 9), suggesting that under these conditions, the integrity of the complex was conserved. When solubilized in 1% DDM, PS1-NTF and Pen-2 were efficiently co-IPed with MAB1563, whereas NCT, PS1-CTF, and Aph1 were not clearly associated (Figure 3A, lane 6). In accord with these observations, NCT, PS1-CTF, and Aph1 were found in the MAB1563 unbound fraction in equal levels, when compared with the starting material (Figure 3A, lanes 4 and 5), suggesting that none of these proteins was clearly associated with the co-IPed proteins. We also found that, when solubilized in 1% DDM, NCT, PS1-CTF, and Aph1 were efficiently co-IPed with anti-NCT, whereas PS1-NTF and Pen-2 were not (Figure 3A, lane 12). PS1-NTF and Pen-2 were found in the anti-NCT unbound fraction at unchanged levels when compared with the starting material (Figure 3A, lanes 10 and 11), suggesting again that these proteins were not associated with the co-IPed proteins. Taken together, the analysis by co-IP performed on the endogenous γ-secretase complex of HeLa cells confirms the results observed with γ-30 and S-1 cells and suggests that 1% DDM dissociates the complex into two complexes: one containing NCT, PS1-CTF, and Aph1 and the other containing PS1-NTF and Pen-2.

Characterization of the Complexes Generated by the DDM-Mediated Dissociation of γ-Secretase. To characterize the composition of the complexes generated by 1% DDM and observed by co-IPs, we subjected the same γ-secretase preparations (solubilized HeLa microsomes) to blue native (BN)−PAGE analysis. Because several groups have suc-

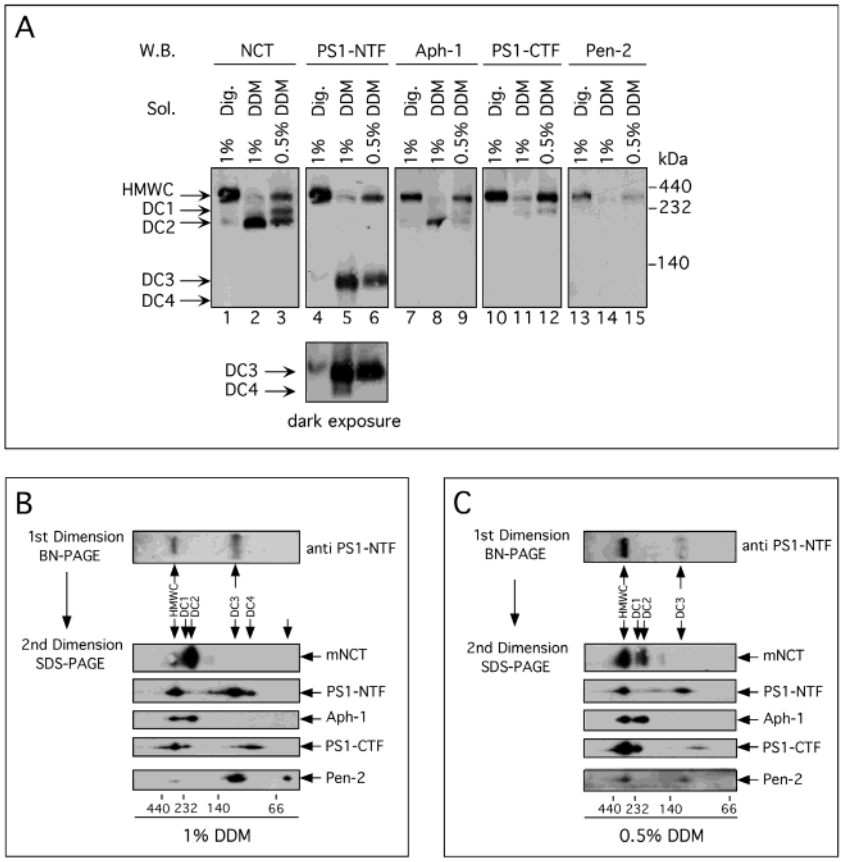

FIGURE 4: DDM-mediated dissociation of endogenous γ-secretase reveals four stable complexes generated in a concentration-dependent manner: DC1 (NCT, Aph1, and PS1-CTF), DC2 (NCT and Aph1), DC3 (PS1-NTF and Pen-2), and DC4 (PS1-NTF and PS1-CTF). (A) BN−PAGE analysis of endogenous γ-secretase after solubilization in 1% digitonin, 0.5% DDM, and 1% DDM. Membranes from an equal amount of HeLa cells were solubilized in 1% digitonin, 0.5% DDM, or 1% DDM. Five equal fractions of each preparation (proteins solubilized in 1% digitonin, 0.5% DDM, and 1% DDM) were electophoresed on 5−19.5% BN−PAGE gels and the lanes probed for NCT (R302), the PS1 holoprotein and PS1-NTF (Ab14), PS1-CTF (13A11), Aph1α2 (O2C2), and Pen-2 (αPen-2). The same 0.5% DDM and 1% DDM γ-secretase preparations were then analyzed by 2D-PAGE (B and C); they were electrophoresed on 5−19.5% BN−PAGE gels, and the entire lanes were then electrophoresed by SDS−PAGE and probed for all known members of the γ-secretase complex as described above, except that H2D2 was used to probe Aph1α2.

cessfully used DDM to solubilize γ-secretase, specifically at a final concentration of 0.5% (*25*, *43*), we decided to analyze the composition of the endogenous complex prepared in this specific condition, side by side with the complexes solubilized in 1% digitonin or 1% DDM (Figure 4A). As expected, we found that all components of the endogenous γ-secretase protease complex solubilized in 1% digitonin can be detected by BN−PAGE analysis and comigrate in a HMW complex with an apparent molecular mass of ~250−350 kDa (Figure 4A, lanes 1, 4, 7, 10, and 13), consistent with previously reported molecular masses for the active γ-secretase complex (*27*, *34*, *36*). It should be noted that some other prior studies using BN−PAGE analysis have reported molecular masses of ~440−550 kDa for the native complex (*25*, *31*, *42*, *43*), approximating the size of a dimer of all four known components. When the mixture was solubilized in 0.5% DDM, our BN−PAGE analysis revealed four distinct complexes called HMWC, DC1, DC2, and DC3 (DC, for dissociated complex; Figure 4A, lanes 3, 6, 9, 12, and 15), with apparent molecular masses of ~250−350, ~200−220, ~180−200, and ~100−120 kDa, respectively. When the mixture is solubilized in 1% DDM, two major complexes, DC2 and DC3, as well as three minor complexes, HMWC, DC1, and DC4, are detected (Figure 4A, lanes 2, 5, 8, 11, and 14), suggesting that the HMWC and DC1 complexes described above were detergent-sensitive and dissociated at this higher concentration of DDM. DC4 was found migrating with an apparent molecular mass of ~80−100 kDa (Figure 4A, lane 5). Whereas the HMWC contained immunoreactive species for all components of γ-secretase, we found that DC1 was immunoreactive for NCT, Aph1, and PS1-CTF, DC2 for NCT and Aph1, and DC3 and DC4 for PS1-NTF (Figure 4A, lanes 1−15). To rule out the possibility that DDM-mediated dissociation of γ-secretase may due to the long overnight incubation (as can be argued for analyses done by IP, in which overnight incubation with the antibodies occurs), we analyzed side-by-side solubilized γ-secretase (prepared in 1% digitonin, 0.5% DDM, or 1% DDM-HEPES buffers), preincubated for 16 h at 4 °C, prior to the BN−PAGE. We found similar BN−PAGE profiles (as in Figure 4A) with or without preincubation for 16 h, suggesting that the DDM-mediated dissociation of the complex was not time-dependent (data not shown). Taken together, these observations suggest that the endogenous γ-secretase is dissociated by DDM in a concentration-dependent manner. When the DDM concentration is increased from 0.5 to 1%, HMWC (NCT, Aph1, PS1-NTF, PS1-CTF, and Pen-2) and DC1 (NCT, Aph1, and PS1-CTF) become more detergent-sensitive and are apparently dissociated into DC2 (NCT and Aph1) and DC3 (PS1-NTF).

Table 1: Summary of All the Various Complexes Identified by BN−PAGE and 2D PAGE in HeLa Membrane Preparations Solubilized in 0.5 and 1% DDM[a]

	HMWC (250−350 kDa)	DC1 (200−220 kDa)	DC2 (180−200 kDa)	DC3 (100−120 kDa)	DC4 (80−100 kDa)
NCT	+	+	+		
Aph-1	+	+	+		
PS1-CTF	+	+			+
PS1-NTF	+			+	+
Pen-2	+			+	

[a] The apparent molecular mass of each complex, estimated by BN−PAGE, as well as the constituent proteins of each complex, identified by BN−PAGE and 2D PAGE.

To more carefully determine the composition of the complexes observed by BN−PAGE in 0.5 and 1% DDM and to ensure that the failure to detect certain components in Figure 4A was not due to epitope masking under the native conditions (the IP results in Figures 2B,C and 3 suggest Pen-2 is a part of DC3), we analyzed the BN−PAGE-separated complexes in a second dimension using SDS−PAGE (*36*). Our results confirmed that (1) when solubilized in 1% DDM the endogenous HeLa γ-secretase was dissociated into two major complexes identified as DC2 (NCT and Aph1) and DC3 (PS1-NTF and Pen-2) (Figure 4B) and (2) when solubilized in 0.5% DDM γ-secretase remains in large part in the nondissociated HMWC form, and becomes dissociated into minor complexes identified as DC1 (NCT, Aph1, and PS1-CTF), DC2 (NCT and Aph1), and DC3 (PS1-NTF and Pen-2) (Figure 4C). Interestingly, the second-dimension analysis of the complexes generated by the solubilization of γ-secretase in 1% DDM revealed Pen-2 as a part of the DC3 complex, confirming the association of Pen-2 with PS1-NTF in a complex suggested by the IP results (Figures 2B,C and 3). The same analysis also revealed the identity of the DC4 complex, immunoreactive solely for PS1-NTF and PS1-CTF as shown by two-dimensional (2D) analysis (Figure 4B).

Taken together, the above results support the identity of the complexes generated by the DDM concentration-dependent dissociation of the HMW γ-secretase complex into detergent-stable complexes called DC1 (NCT, Aph1, and PS1-CTF), DC2 (NCT and Aph1), DC3 (PS1-NTF and Pen-2), and DC4 (PS1-NTF and PS1-CTF) (summarized in Table 1).

The Structural Changes Mediated by DDM on the HMW Complex Also Affect γ-Secretase Activity. We next determined whether the dissociation of endogenous γ-secretase reported above affects γ-secretase activity. We solubilized equal amounts of HeLa cells in equal volumes of 1% digitonin, 0.5% DDM, and 1% DDM buffers. After centrifugation, equal volumes were incubated with C100Flag substrate to examine the level of γ-secretase activity (Figure 5A,B). The resulting cleavage product AICD-Flag was detected with anti-Flag M2 antibodies (Figure 5A), and the Met-Aβ products were quantified by ELISA analysis (Figure 5B). The ELISA quantification revealed that (1) when compared to the activity found in the 1% digitonin preparation, ~20 and ~85% decreases in γ-secretase activity were found in the 0.5 and 1% DDM preparations, respectively (Figure 5B), and (2) when compared to the activity found in the 0.5% DDM preparation, a further ~80% decrease in γ-secretase activity was found in the 1% DDM preparation

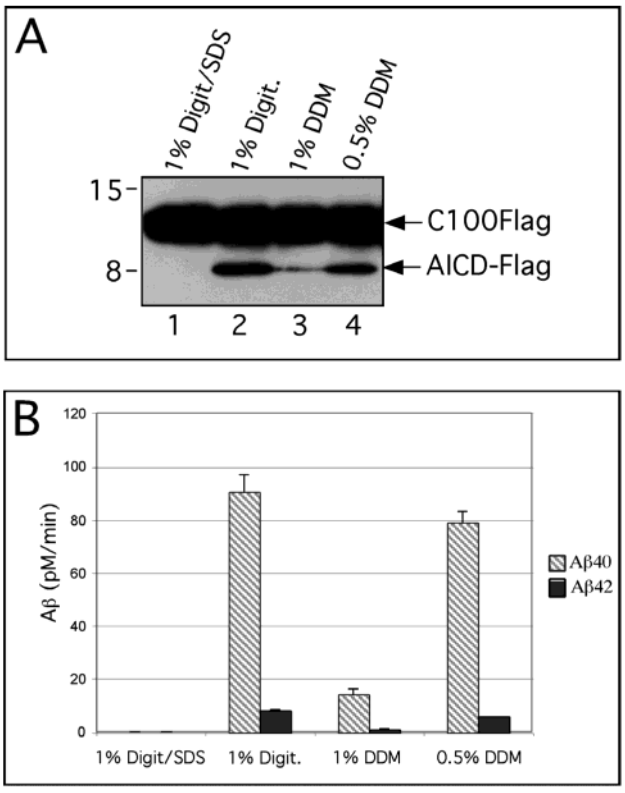

FIGURE 5: DDM-mediated dissociation of endogenous γ-secretase affects γ-secretase activity. Equal amounts of HeLa cells were solubilized in HEPES buffer containing 1% digitonin, 0.5% DDM, or 1% DDM, and 400 μg of protein was adjusted to 1% CHAPSO-HEPES and then incubated overnight with X81 antibodies (1:200) and protein A−Sepharose beads. Beads were washed three times in 1% CHAPSO-HEPES and resuspended in 0.25% CHAPSO-HEPES, and γ-secretase C100Flag activity assays were performed as previously described (*38*). As a negative control for γ-secretase activity, a 1% digitonin lysate IPed with X81 under the same conditions as described above has been treated with SDS (final concentration of 0.5%), prior to the activity assays. The resulting cleavage product, AICD-Flag, was detected with anti-Flag (M2) antibodies (A), and Met-Aβ-40 and Met-Aβ-42 were quantitated with an ELISA (B).

(Figure 5B). These results are in good accord with the AICD-Flag levels detected in the same activity assays (Figure 5A) and suggest that the 1% DDM-mediated dissociation of the HMWC is associated with a substantial decrease in activity. The latter observation suggests that the complexes generated by the 1% DDM-mediated dissociation of the HMWC are not active, supporting a relation between the composition and the proteolytic activity of the γ-secretase complex.

Stable Coexpression of Pen-2 and PS1 in CHO Cells Promotes the Formation of a Complex of Pen-2 with PS1 Holoprotein. To confirm the interactions among members of the γ-secretase complex, we performed co-IPs with stable cell lines P-22 [overexpressing PS1 and Pen-2 (*34*)], γ-30, and S-1 (characterized in Figure 1A) using antibodies raised against three distinct domains of PS1 (Figure 6A). First, as a positive control, we examined X81 antiserum (to the N-terminus), which we previously found to co-IP active γ-secretase complexes (*34*). Consistent with our whole cell lysate analysis (Figure 1A), larger amounts of PS1-NTF, PS1-CTF, Aph1, and Pen-2 were co-IPed with X81 antibodies from S-1 than from P-22 and γ-30 cells (Figure 6B, lanes 1−3). These results confirm that X81 antibodies are able to pull down all the mature members of the γ-secretase

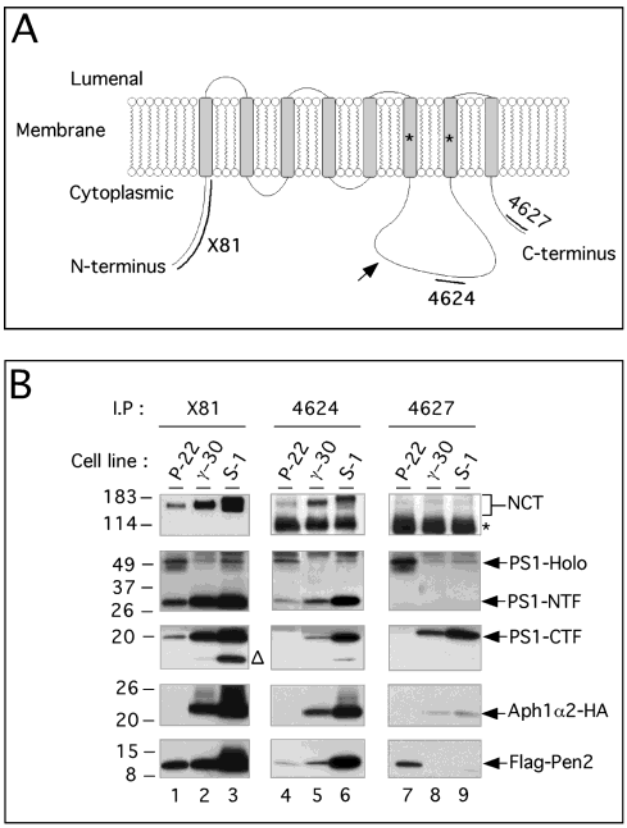

FIGURE 6: Pen-2 is associated with immature and mature PS1. (A) Schematic representation of the PS1 topology. The X81 antibody was raised against the N-terminal cytoplasmic domain of PS1-NTF (residues 1−81), 4624 against the N-terminal domain of PS1-CTF (residues 343−357), and 4627 against the C-terminal domain of PS1-CTF (residues 457−467). The arrow denotes the endoproteolytic site. (B) Stable coexpression of Pen-2 and PS1 in CHO cells promotes the formation of a complex of Pen-2 with the PS1 holoprotein. Membranes from the indicated cell lines were solubilized in 1% CHAPSO-HEPES and subjected to X81, 4624, and 4627 co-IPs as described in the legend of Figure 1B. Beads were washed three times in 1% CHAPSO-HEPES buffer, resuspended in Laemmli sample buffer, and co-IPed proteins were blotted for NCT (αgpNCT), the PS1 holoprotein and PS1-NTF (N19), PS1-CTF (13A11), Aph1α2 (3F10), and Pen-2 (M2). Note that active γ-secretase is not co-IPed with 4627 antibodies, whereas PS1-CTFs are. By using the same antibody, Pen-2 is co-IPed with the PS1 holoprotein and in a manner independent of NCT, Aph1, and PS1-NTF and -CTF, indicating a specific association of both proteins. The asterisk denotes the cross-reactive band associated with 4624 and 4627 antibodies. The triangle denotes the PS1-CTF cleavage product.

complex, suggesting that the N-terminal domain of PS1 is not involved in any physical interaction in the active γ-secretase complex that prevents access to it.

Next, we repeated the IPs using 4624 and 4627, antibodies raised against the N-terminal (4624, amino acids 343−357) and C-terminal (4627, amino acids 457−467) regions, respectively, of the PS1-CTF fragment (Figure 6A). Importantly, 4624 was able to co-IP mNCT (Figure 6B, top box, lanes 4−6), whereas 4627 was not (Figure 6B, top box, lanes 7−9). This result suggests that after its assembly into the active γ-secretase complex, the C-terminal domain of PS1-CTF becomes inaccessible to the antibodies. Not only is mature NCT absent from co-IPs using 4627 but so are PS1-NTF, Aph1α2, and Pen-2, as illustrated in the S-1 cells (Figure 6B, lane 9 vs lane 6). Large amounts of PS1-CTF fragments were IPed by 4627 (Figure 6B, lanes 8 and 9), suggesting that either some of the processed PS1-CTFs are not associated with any member of the γ-secretase complex or 4627 somehow breaks down the complex. Interestingly, we noticed that Pen-2 could be co-IPed with the PS1 holoprotein using 4627, specifically in the P-22 stable cell line lysate, and without co-IP of mNCT, Aph1, PS1-NTF, and PS1-CTF (Figure 6B, lane 7). Relative to the PS1 holoprotein IPed from P-22 cells (Figure 6B, lanes 4 and 7, second box from top), similar amounts of Pen-2 co-IPed with both 4624 and 4627 (Figure 6B, lanes 4 and 7, bottom box), suggesting that 4624 and 4627 epitopes are still accessible, even after the association of Pen-2 with the PS1 holoprotein. Taken together, these observations suggest that Pen-2 can interact with the PS1 holoprotein independently of NCT, Aph1, PS1-NTF, and PS1-CTF, and may likely reflect the physical interaction between PS1-NTF and Pen-2 described above under endogenous conditions. However, we cannot exclude the possibility that the interaction between the PS1 holoprotein and Pen-2 may have been artificially created by the overexpression in the P-22 cell line of just these two subunits.

DISCUSSION

Here we provide further information about the structural organization of the active γ-secretase complex, an important issue in dissecting the biochemistry of γ-secretase, involved in the proteolysis of APP, Notch, E-Cadherin, and several other type I membrane proteins. These kinds of studies are important because it may be difficult to derive information about the complex from structural biology approaches, given its ≥18 transmembrane domains. Our experiments suggest that DDM can dissociate active γ-secretase complexes in a concentration-dependent manner into two major complexes we call DC2 and DC3 and two minor complexes we call DC1 and DC4. BN−PAGE, combined with 2D PAGE analysis, revealed that the two major complexes are composed of mNCT and Aph1 (DC2) and Pen-2 and PS1-NTF (DC3), respectively. The same analysis also revealed that the two minor complexes generated by DDM dissociation are composed of mNCT, Aph1, and PS1-CTF (DC1) and PS1-NTF and PS1-CTF (DC4). These DDM-resistant interactions between Pen-2 and PS1-NTF, mNCT and Aph1, PS1-CTF and PS1-NTF, and PS1-CTF and NCT and Aph1 likely reflect the physical interactions that exist in the active γ-secretase complex. On the basis of the characterization of these various complexes, we propose a model for the DDM-mediated dissociation of γ-secretase as well as a model for the organization of the active HMWC (see Figure 7). The interactions between PS1-CTF and PS1-NTF, and between PS1-CTF and NCT and Aph1, are apparently more DDM-sensitive than those between Pen-2 and PS1-NTF and between mNCT and Aph1. We speculate that the weaker interactions associating PS1-CTF with γ-secretase may reflect flexibility in this subunit that is needed for substrate accessibility from the lipid-associated surface of the complex into the hydrophilic active site.

When estimated by BN−PAGE analysis, the apparent molecular masses of the complexes generated by the DDM dissociation of the γ-secretase HMWC were ∼200−220 (DC1), ∼180−200 (DC2), ∼100−120 (DC3), and ∼80−100 kDa (DC4). The apparent molecular masses of DC1 (∼200−220 kDa) and DC2 (∼180−200 kDa) are in good

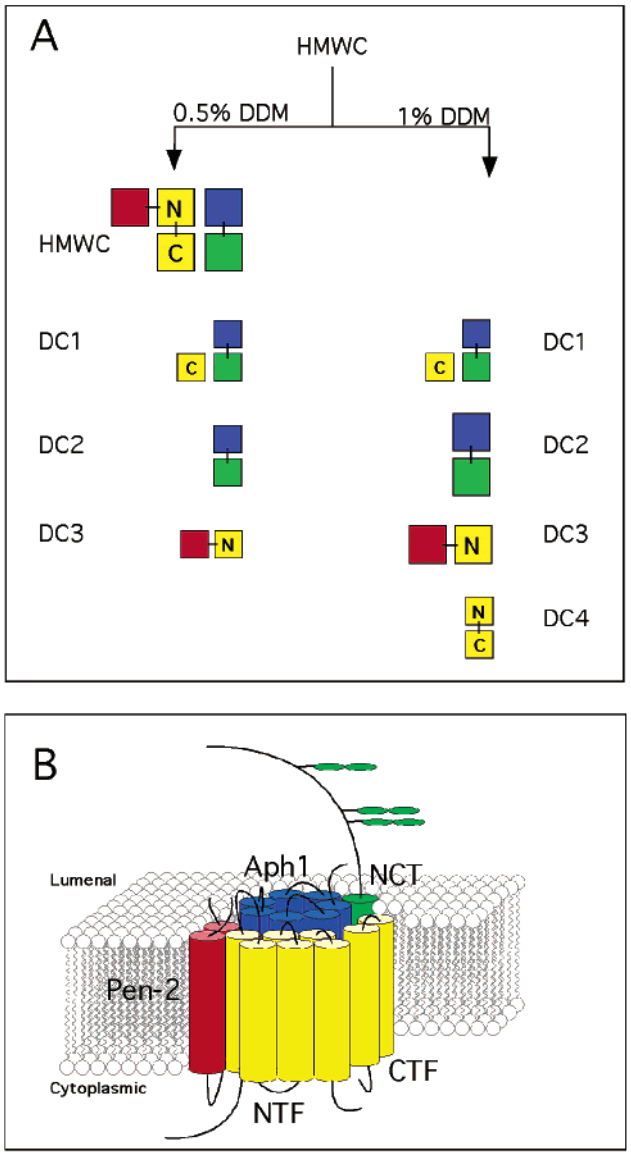

FIGURE 7: Dissection and characterization of the complexes generated by the DDM-mediated dissociation of the HMWC reveals a physical interaction between Pen-2 and PS1-NTF and offers a model for the subunit organization within the complex. (A) Schematic representation of the DDM-mediated dissociation of active γ-secretase into various stable complexes. The constituent proteins, for each complex, are represented in different colors: NCT (green), Aph1 (blue), Pen-2 (red), PS1-NTF (yellow with the letter N), and PS1-CTF (yellow with the letter C). The known protein−protein interactions within the complexes are represented by solid bars (the major complexes are represented with larger symbols than the minor complexes). The mobility of DC3 and DC4 in the BN gel leaves open the possibility that more than one copy of the identified subunits is present in these complexes. (B) Schematic representation of the subunit organization within the active γ-secretase complex. This model is based on the protein−protein interactions identified in the complexes generated by the DDM-mediated dissociation of γ-secretase: (1) Pen-2 interacting directly with PS1-NTF, (2) mNCT interacting directly with Aph1, (3) PS1-CTF interacting directly with PS1-NTF, and (4) PS1-CTF interacting with NCT and Aph1. The estimated sizes of two complexes (DC3 and DC4) generated by the DDM-mediated dissociation of the HMWC leaves open the possibility that more than one copy of PS1-NTF, PS1-CTF, or Pen-2 is present in the complex.

accord with the calculated molecular masses for the corresponding complexes, when made up of one of each of the identified subunits. The calculated molecular masses are ~194 (150 + 24 + 20) kDa for DC1 (mNCT, Aph1, and PS1-CTF) and ~174 (150 + 24) kDa for DC2 (mNCT and Aph1). The expected molecular masses for the DC3 and DC4 complexes, when made up of one of each of the identified subunits, are ~42 and ~50 kDa, respectively. The apparent molecular masses for these two complexes, when estimated by BN−PAGE, are ~100−120 kDa (DC3) and ~80−100 kDa (DC4). The differences observed between the expected and apparent molecular masses suggest that more than one of the identified subunits is present in both DC3 and DC4 complexes. This latter possibility is supported by a growing body of evidence which shows that PS1 may exist as homo-oligomers (*44*, *45*). However, we cannot exclude the possibility that putative complexes generated after the dissociation of the γ-secretase complex and made up of one of each of the identified subunits may interact artificially with other proteins present in our solubilized preparations.

DDM has successfully been used by several groups to solubilize γ-secretase under endogenous conditions (from mammalian cells and tissues) to perform BN−PAGE and co-IP analysis. Under the reported conditions, the co-IPs recovered all four known γ-secretase components, suggesting that the integrity of the complex was conserved. Because in the majority of these reported studies, DDM has been used at a final concentration of 0.5%, the corresponding data are consistent with and supported by our results. When solubilized in 0.5% DDM, the major part of the endogenous γ-secretase complex remains intact and active. However, a minor part of the active complexes becomes dissociated into smaller and presumably inactive complexes that are not detected in preparations corresponding to γ-secretase solubilized in 1% digitonin. When the DDM concentration is increased to 1%, most of the γ-secretase complex becomes dissociated into smaller complexes, with only residual activity, as quantified in the *in vitro* C100Flag assay.

Our co-IP results demonstrate that Pen-2 can associate with full-length PS1 in a manner independent of NCT, Aph1, and PS1-NTF. This result suggests that Pen-2 and PS1 are capable of interacting physically with each other, and this may reflect the physical interation between PS1-NTF and Pen-2 within the active γ-secretase complex. Our co-IP results also reveal that the C-terminal domain of PS1-CTF is inaccessible to antibodies when assembled into the active γ-secretase complex. This domain may be folded into the structure of the complex, perhaps involved directly in a protein−protein interaction with one or more component(s) of γ-secretase. This finding suggests that the C-terminus of PS may play a role in the stabilization and activation of the γ-secretase complex, consistent with results from mutagenesis of this domain (*46*, *47*). The N-terminal domain of PS1-CTF remains accessible to the 4624 antibody in the active complex, suggesting that the epitope for this antibody (in the PS1 loop) may not be involved in any physical interaction with a member of the γ-secretase complex.

Taken together, our results support a model for the organization of the active γ-secretase complex in which Pen-2 interacts directly with PS1-NTF. Our results also provide further support for a physical association in active γ-secretase between mNCT and Aph1, fully consistent with the recent demonstration in different cell lines of a preferential association between NCT and Aph1 during the assembly and maturation of the γ-secretase complex (*36*,

37, *48*). Our model for the structural organization of the γ-secretase complex summarized and displayed in Figure 7B is supported by (1) the interactions between Pen-2 and PS1-NTF, (2) the interactions between mNCT and Aph1, and (3) the common interactions between PS1-CTF and NCT and Aph1, and between PS1-CTF and PS1-NTF, as deduced here from the characterization of the complexes generated by the detergent-dependent dissociation of γ-secretase. This model is also supported by previously reported data demonstrating that PS1-NTF and -CTF can both be cross-linked with active site-directed inhibitors (*19*, *20*). Moreover, an active site-directed inhibitor containing two reactive moieties cross-links PS1-NTF to PS1-CTF (*44*).

Additional structural studies, preferentially performed on purified and active γ-secretase complexes, will be necessary to estimate and confirm the number of each subunit in both the dissociated PS1-NTF−Pen-2 and PS1-NTF−PS1-CTF complexes, and also in the active HMW γ-secretase complex.

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