Notch and the Amyloid Precursor Protein Are Cleaved by Similar γ-Secretase(s)†

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ABSTRACT: γ-Secretase is an intramembrane-cleaving protease whose substrates include Notch and the amyloid precursor protein (APP). On the basis of initial genetic and pharmacologic data, the γ-secretase activity responsible for cleavage of both proteins appears to be identical. However, apparent differences in the cleavage site and in sequence specificity raise questions about the degree of similarity between Notch and APP γ-like proteolysis. In an effort to resolve this issue directly, we established an in vitro γ-secretase activity assay that cleaves both APP- and Notch-based substrates, C100Flag and N100Flag. Analysis with specific γ-secretase inhibitors, dominant-negative γ-secretase preparations, and antibody co-immunoprecipitations all demonstrated identical cleavage of these substrates. Most importantly, we found that these substrates prevented cleavage of each other, indicating that the same γ-secretase complex can cleave either protein. Finally, we provide evidence that both substrates are cut at two distinct regions in the transmembrane domain. These data resolve some of the apparent conflicts and strongly indicate that Notch and APP are proteolyzed by the same enzyme(s).

The accumulation of amyloid β-protein (Aβ) in neuritic plaques is one of the principal pathologic features in Alzheimer's disease (AD) (*1*). Because Aβ toxicity and deposition is central to AD pathogenesis, its metabolism is of considerable interest. The 39−43-amino acid peptide is generated by the sequential action of β- and γ-secretase on the β-amyloid precursor protein (APP). APP can also be cut by α-secretase, which precludes the formation of Aβ. Instead, an N-terminally truncated form of Aβ called p3 is produced upon subsequent γ-secretase cleavage. Although the generation of the different isoforms of Aβ and p3 has been intensively studied, the normal biological function of APP proteolysis has remained unclear. Recent evidence suggests that APP acts as a cell surface receptor that mediates signaling via the γ-secretase release of the APP intracellular domain (AICD) (*2*). Surprisingly, the N-terminus of AICD is 8−10 residues beyond the γ-site that delineates the C-terminus of Aβ (*3*−*5*). This cleavage of AICD at position 50 (Aβ numbering) has been dubbed ε-secretase (*6*), although it is not clear whether the cleavage is mediated by γ-secretase.

Another type I transmembrane receptor, Notch, also appears to have a similar signaling mechanism via release of its intracellular domain (NICD) (*7*). Like APP, Notch undergoes sequential cleavages by α- and γ-secretase-like activities (termed S2 and S3, respectively) (*8*). Although nothing is known about the cleavage that generates the Notch p3-like peptide (Np3), the N-terminus of NICD is analogous in location to AICD (*9*). The disruption of γ-secretase activity via ablation or dominant-negative mutagenesis of presenilin 1 (PS1) reduces the extent of S3 cleavage of Notch and the γ-secretase cleavage of APP (*10*−*13*). Combined ablation of PS1 and its homologue, PS2, results in a total absence of S3-mediated and γ-secretase-mediated proteolysis (*14*, *15*). These data argue that γ-secretase and S3 protease are the same.

Despite these striking similarities between Notch and APP proteolysis, some apparent inconsistencies seem to indicate differences in the S3 protease and γ-secretase. Certain mutations in PS appear to separate its function in Aβ production and in Notch proteolysis. Engineered point mutations at position 286 in PS1 increase the level of production of $A\beta_{42}$ but decrease the extent of cleavage of NICD (*16*). Another group has reported the identification of PS-independent Notch signaling (*17*, *18*), which contrasts with the inability of other groups to detect Notch cleavage in PS1 −/−, PS2 −/− cells (*14*, *15*). More recently, it was suggested that γ-secretase was not executing Notch cleavage, based on the ability to detect proteolysis of a Notch-based fluorogenic substrate in PS1 −/−, PS2 −/− cells (*19*).

In an effort to resolve these apparent conflicts and to better understand both APP and Notch processing and function, we established an in vitro assay for APP and Notch cleavage. These cell-free assays permit the analysis of direct effects on the enzyme; i.e., they can be used to evaluate the mechanism of inhibitors and demonstrate competition between substrates. To validate our APP- and Notch-based substrates (C100Flag and N100Flag, respectively), we demonstrated that cleavage generated the expected products.

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Analysis with specific γ-secretase inhibitors, dominant-negative γ-secretase preparations, and antibody co-immunoprecipitations all demonstrated identical cleavage of both substrates. Most importantly, we found that C100Flag and N100Flag directly prevented each other's cleavage, indicating that the same γ-secretase complex can cleave either protein. Finally, we provide evidence that both APP and Notch undergo multiple cleavages within the transmembrane domain and that mutagenesis of the residue corresponding to Val1744 in Notch prevented one of those cleavages. Taken together, these data argue that γ-secretase and S3 are one and the same protease complex.

EXPERIMENTAL PROCEDURES

Antibodies and Western Blotting. Antibody 66104 (affinity-purified rabbit polyclonal, 5 μg/mL) was raised against the N-terminus of AICD (VMLKKK). Notch Ab1744 antibody (1:1000, Cell Signaling Technologies) is selective for the N-terminus of NICD. 6E10 (1:1000, Signet Laboratories, Inc.) was used to detect Aβ. Nicastrin was blotted with guinea pig anti-nicastrin antibody (1:2000, Chemicon). PS1 was detected with goat α-PS1 N-19 (PS1 NTF, 1:200, Santa Cruz Biotechnologies) and 13A11 (PS1 CTF, 5 μg/mL). For PS2, 2972 (PS2 NTF, 1:2000) and 3711 (1:1000, both gifts of C. Haass) were used. For experiments where both C100Flag and N100Flag were present, polyclonal antibody C7 (*20*) was used for AICD-Flag and Notch Ab1744 was used for NICD-Flag. Samples were separated on 4 to 20% Tris-glycine or 10 to 20% Tris-tricine gels. Unless indicated, Western blotting was performed on polyvinylidene fluoride membranes using standard procedures. M2 Flag antibody (1:1000) was diluted in PBS with 0.3% milk. Blots for Aβ were transferred to nitrocellulose and boiled for 10 min in PBS prior to the blocking step. Visualization for all experiments was with ECL+ reagent (Amersham Pharmacia).

Purification of APP- and Notch-Based Substrates. The recombinant substrate C100Flag was prepared as described by Li et al. (*21*) except that it was purified by M2 Flag chromatography (resin from Sigma). A plasmid encoding N100Flag in vector pET21a(+) (Novagen) was prepared by cloning the Notch sequence from Val1711 to Glu1809 from the mouse (m1) Notch ΔE using PCR (simultaneously incorporating an N-terminal methionine and a C-terminal Flag epitope). For the indicated experiments, a hemagglutanin (HA) tag was fused to the N-terminus of the N100Flag sequence. The V1744L mutant was engineered by site-directed mutagenesis of HAN100Flag using PCR. The BL21-(DE3) expression host (Novagen) was transformed with each sequence-verified construct. After induction for 2 h (37 °C with 1 mM IPTG at an OD_{600} of 1.0), bacteria were lysed in 10 mM Tris (pH 7.0), 200 mM NaCl, and 1% NP-40 by passing twice through a French press. Following centrifugation (3000*g* for 15 min), the supernatant was applied to the M2 Flag column. Bound substrate was eluted with 1% NP-40 and 100 mM glycine (pH 2.7). The N100Flag was purified by anion exchange chromatography (*22*).

Preparation of Solubilized γ-Secretase from Cell Lines. HeLa S3 cells were grown in 3 L batches in suspension and collected at a concentration of 1×10^6 cells/mL. Solubilized γ-secretase was prepared essentially as described by Li et al. (*21*, *22*), except that HeLa cell membranes were washed in 0.1 M sodium carbonate (pH 11.3) to remove nonintegral membrane proteins prior to solubilization in 1% CHAPSO/HEPES buffer [1% CHAPSO, 50 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$]. For select experiments, the indicated stable cell lines were grown in 15 cm dishes, detached by scraping in PBS and 20 mM EDTA, and then prepared as described above.

In Vitro γ-Secretase Assays. To measure γ-secretase activity with the C100Flag and N100Flag substrates, phosphatidylethanolamine (Sigma, 0.025% final concentration from a 5 mg/mL stock in 1% CHAPSO/HEPES buffer) and phosphatidylcholine (Sigma, 0.100% final concentration from a 10 mg/mL stock in 1% CHAPSO/HEPES buffer) were made fresh and added to 50 μL of the solubilized γ-secretase preparation (~0.2 mg/mL). Time 0 reactions were stopped with Laemmli sample buffer (for Western blots) or 0.5% SDS (for ELISAs). Where appropriate, γ-secretase inhibitors were added from a DMSO stock (final DMSO concentration of 1%). DMSO (1%) alone did not affect γ-secretase activity. C100Flag and N100Flag substrates were adjusted to 0.5% SDS, heated at 65 °C for 5 min, and then spun at 14 000 rpm for 1 min to pellet insoluble aggregates. The prepared substrate was added to the reaction mixture to a final concentration of approximately 1 μM (or the indicated amounts). No SDS was added to the substrate preparations intended for the competition reaction. Reaction mixtures were incubated at 37 °C for 0 or 4 h. The substrates and resultant C-terminal cleavage products were detected using Western blotting to the Flag epitope. The Aβ products were detected by a sensitive and specific ELISA (*23*).

Immunoprecipitation of PS1 and PS2 for γ-Secretase Activity. For experiments where γ-secretase assays were performed from immunoprecipitations, X81 antibody (1:400, directed to the N-terminus of PS1) or 2972 (1:200, directed to the N-terminus of PS2, gift of C. Haass) was added to solubilized γ-secretase preparations that were adjusted to 1% CHAPSO. The N-termini of PS1 and PS2 are highly divergent, explaining why X81 does not cross react with PS2 (*24*) and 2972 with PS1 (*25*). Following overnight immunoprecipitation at 4 °C, beads were washed three times in 1% CHAPSO/HEPES buffer. Samples were resuspended in 0.25% CHAPSO/HEPES buffer and were adjusted to 0.025% phosphatidylethanolamine and 0.10% phosphatidylcholine. For unclear reasons, the incubated time 4 h samples had a reproducible increase in the detection of the substrate compared to the time 0 h samples, even though the same amount of substrate was added. This phenomenon consistently occurs with co-immunoprecipitation samples, even in the presence of γ-secretase inhibitors (see Figure 1d in ref *22*). Cleavage reactions were performed as described above.

RESULTS

The C100Flag and N100Flag Substrates Are Valid Surrogates for γ-Secretase-Mediated Cleavage of APP and Notch. The amyloid precursor protein (APP) is first cut by either α- or β-secretase to generate C83 or C89 and C99, respectively. These C-terminal products serve as substrates for the subsequent γ-secretase cleavage. The products of C99 cleavage include the amyloid β-protein (Aβ) and the APP intracellular domain (AICD), whereas C83 generates the p3

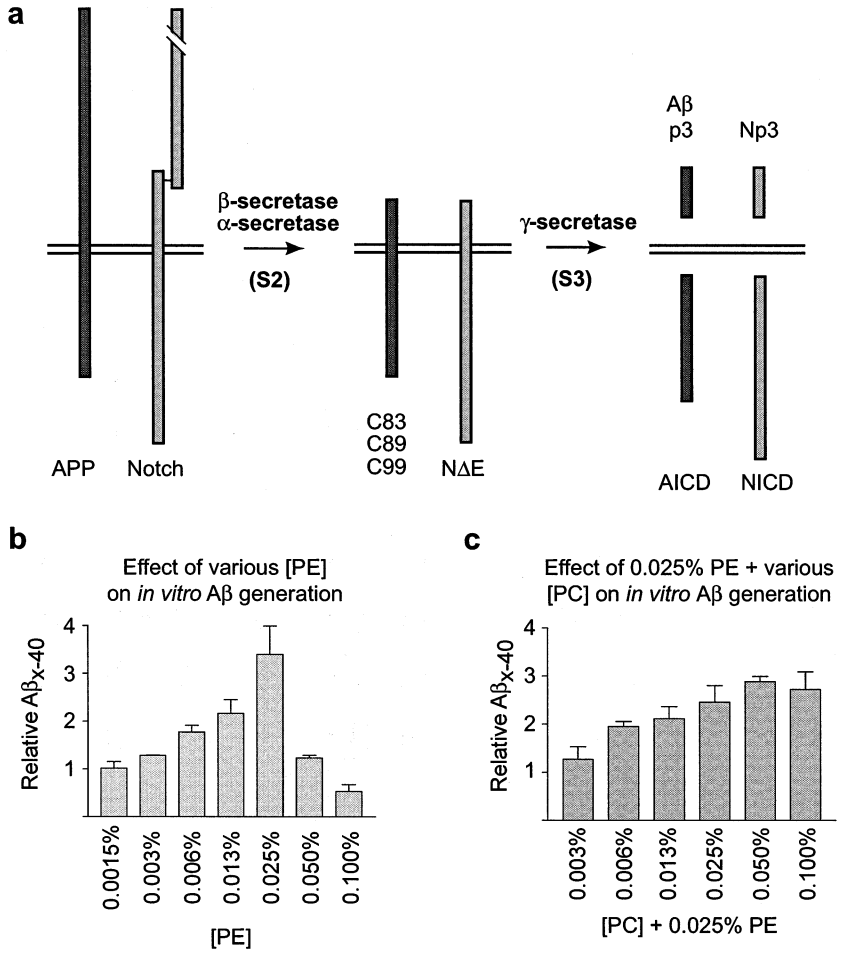

FIGURE 1: Analogous features of APP and Notch proteolysis and optimization of an in vitro γ-secretase activity assay. (a) Both APP and Notch are first cut by a disintegrin metalloprotease that mediates the α-secretase (or S2) cleavage. APP can also be cleaved by β-secretase. The resulting C-terminal stubs (C83, C89, and C99 for APP and NΔE for Notch) are substrates for the γ-secretase cleavage. The cleavage of APP-derived CTFs generates the heterogeneous Aβ and p3 peptides and AICD at the ϵ-site. The cleavage of NΔE is predicted to result in a Notch p3-like peptide (Np3) and NICD at the S3 site, which is analogous to the APP ϵ-site. (b) Effect of various concentrations of phosphatidylethanolamine (PE) on the cleavage efficiency of C100Flag. Error bars represent the standard deviation. (c) Effect of varying concentrations of phosphatidylcholine in the presence of 0.0125 or 0.025% PE. The addition of these two phospholipids does not alter the Aβ 42/40 ratio. Error bars represent the standard deviation.

peptide and AICD (Figure 1a). In a closely similar sequence of cleavages, Notch is first cut by tumor necrosis factor-α converting enzyme (TACE) and then by γ-secretase, generating a Notch p3 peptide (Np3) and the Notch intracellular domain (NICD) (Figure 1a). To study the γ-secretase cleavage of APP and Notch in vitro, we used *Escherichia coli* to generate APP- and Notch-based substrates termed C100Flag and N100Flag, respectively. C100Flag contains an initiating methionine, the 99 C-terminal residues of APP starting at the β-secretase site, and a Flag tag (*21*). N100Flag similarly contains an initiating methionine, 99 amino acids starting at the TACE cleavage site, and a Flag tag.

We first wanted to optimize our in vitro cleavage reactions, and we reasoned that phospholipid composition could affect γ-secretase activity. Prior work had demonstrated that phosphatidylethanolamine (PE) was necessary to recover γ-secretase activity following wheat germ lectin chromatography (*26*). Although we were able to obtain activity in CHAPSO detergent alone (as expected), we wanted to determine whether we could further augment γ-secretase activity. We therefore evaluated various concentrations of PE in sodium carbonate-washed, CHAPSO-solubilized HeLa cell membranes, focusing on C100Flag since we had a quantitative ELISA for measuring the Aβ product (Figure 1b). We found that PE augmented Aβ_{x-40} production between 0.003 and 0.025%, but inhibited it at higher concentrations. Peak improvement over baseline occurred at 0.025% PE. Because phosphatidylcholine (PC) and phosphatidylserine also predominate in mammalian cell membranes, we evaluated these phospholipids in the presence of PE. We were unable to improve activity with the addition of phosphatidylserine (data not shown), but we obtained a further increase in activity when PC was added along with either 0.013 or 0.025% PE (Figure 1c). We therefore used 0.025% PE with 0.100% PC for all subsequent γ-secretase reactions.

To ascertain whether the substrates are valid surrogates for their corresponding native molecules in whole cells, we added C100Flag or N100Flag to the solubilized γ-secretase preparations. When C100Flag was incubated at 37 °C for 4 h, we detected a newly generated band that was identified by M2 Flag Western blotting (Figure 2a, lanes 1 and 2) and corresponded to the predicted size of the AICD-Flag product. Because the N-terminus of AICD is located at Val50 (Aβ numbering) (*3*−*5*), we probed the same reactions using an affinity-purified antibody (66104) that we raised to have N-terminal specificity beginning with that residue. We found

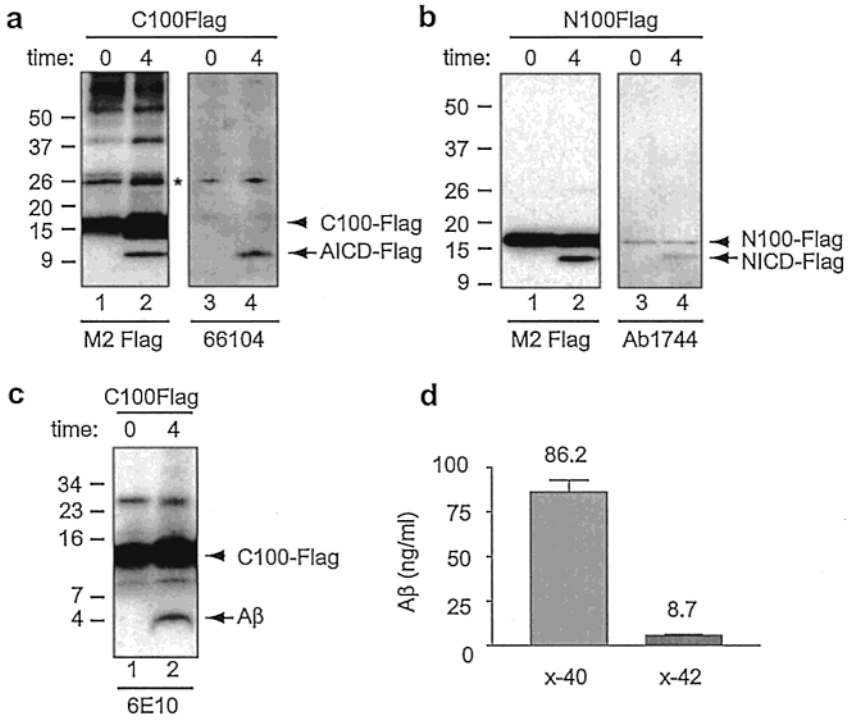

FIGURE 2: C100Flag and N100Flag are cleaved into the physiologically appropriate products. (a) In the left-hand panel, PVDF membranes were detected with the M2 Flag antibody. As expected, the AICD-Flag product (arrow) migrates ~4 kDa below the C100Flag substrate (arrowhead). Note the dimers (asterisk) and higher-order aggregates of C100Flag, likely due to the presence of the Aβ sequence. In the right-hand panel, a similar blot was probed with the affinity-purified antibody 66104, which selectively recognizes the N-terminal VMLKKK sequence of AICD. Note the corresponding selectivity for AICD-Flag as compared to C100Flag. (b) Blots for N100Flag similar to those in panel a. The Notch Ab1744 (right-hand panel) also demonstrates selectivity for NICD-Flag (arrow) as compared to the N100−Flag (arrowhead). For this substrate, there are no SDS-insoluble aggregates. (c) Detection and characterization of Aβ in C100Flag cleavage reactions by Western blotting and (d) with an ELISA. These reaction mixtures contained phospholipids (0.025% PE and 0.100% PC), and the ratio of Aβ 42/40 in their absence was the same (data not shown), suggesting that phospholipids augment activity without altering the cleavage site selectivity.

that the newly generated band detected by M2 Flag Western blotting at 4 h was also identified by antibody 66104, whereas the full-length C100Flag substrate was not (Figure 2a, lanes 3 and 4). This result demonstrates the specificity of 66104 for the neo-epitope of AICD-Flag, and it confirms that the appropriate ϵ-cleavage (*6*) occurs in this system. In analogous reactions, we incubated the N100Flag substrate with the sodium carbonate-washed HeLa membrane lysates. We found the generation of a Flag-tagged product that both was time-dependent (Figure 2b, lanes 1 and 2) and was recognized by the N-terminal specific NICD antibody V1744 (Figure 2b, lanes 3 and 4). These data show that this cell-free system generates the appropriate C-terminal products for both APP and Notch.

Because we also had antibodies that recognize the N-terminal products of C100Flag cleavage (Aβ), we probed for the presence of these products. As for the appearance of AICD-Flag, Aβ was generated in a time-dependent manner (Figure 2c). Moreover, the ratio of Aβ_{42} to Aβ_{40} species agreed with previously reported ratios in intact cells and animal fluids (10.1 $\pm$ 2.6%, Figure 2d). Therefore, by all available measures, C100Flag and N100Flag both appear to generate the physiologically relevant products in this cell-free assay.

Because these substrates appeared to be valid for in vitro study, we next confirmed that the cleavage of both substrates could be equally inhibited by a variety of potent and selective γ-secretase inhibitors. Three compounds representing structurally different classes of γ-inhibitors [III-31C (*22*), DAPT (*27*), and compound E (*28*)] each inhibited the production of NICD-Flag or AICD-Flag, whereas the inactive control compounds [III-112 (*22*) and DAT (*27*)] did not (Figure 3a). The similar degree of inhibition between N100Flag and C100Flag is consistent with closely similar IC_{50}'s of such inhibitors reported for Notch and APP cleavage in whole cells (*11*).

CHO cells overexpressing aspartate mutant PS1 and PS2 reduce γ-secretase activity in a dose-dependent manner (*12*, *29*, *30*). We therefore prepared CHAPSO-solubilized membrane fractions from cells expressing wild-type (wt) PS1, PS1 D257A, and PS1 D257A and PS2 D366A and incubated them with our substrates. We found a small reduction in the extent of cleavage of N100Flag and C100Flag in membranes containing PS1 D257A, consistent with the partial loss of γ-secretase activity in these cells (Figure 3b, lanes 1−4) (cell line 2-1 in ref *12*). Cleavage was essentially absent when high levels of both PS1 D257A and PS2 D366A were stably expressed (cell line 2A-2), consistent with the near absence of γ-secretase activity in these cells (Figure 3b, lanes 5 and 6). The highly similar results obtained with N100Flag and C100Flag demonstrate that both undergo PS-dependent transmembrane cleavage in a parallel manner.

C100Flag and N100Flag Can Be Cleaved by PS1/γ-Secretase or PS2/γ-Secretase, and They Compete with Each Other for Cleavage. PS1 is intimately associated with γ-secretase activity and most likely serves as the active site of the protease (*12*, *31*, *32*). PS2 is highly homologous to PS1 (*33*, *34*) and is therefore likely to perform the same

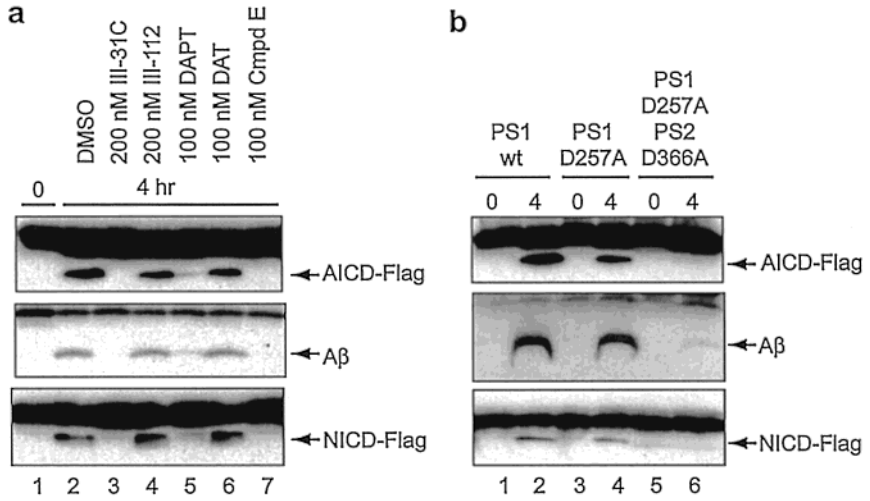

FIGURE 3: C100Flag and N100Flag cleavage is inhibited by γ-secretase inhibitors and aspartate mutant presenilins. (a) Parallel reaction mixtures were incubated in the presence of the indicated inhibitors and probed by Western blotting for AICD-Flag (top), Aβ (middle), and NICD-Flag (bottom). (b) Solubilized γ-secretase was prepared from CHO cells stably expressing wild-type PS1 (PS70 line), PS1 D257A (2-1 line), or PS1 D257A and PS2 D366A (2A-2 line). The very faint band present in lanes 5 and 6 in the NICD-Flag Western blot (bottom) presumably corresponds to the cross reactivity to C83, which is present in a very high abundance in this line.

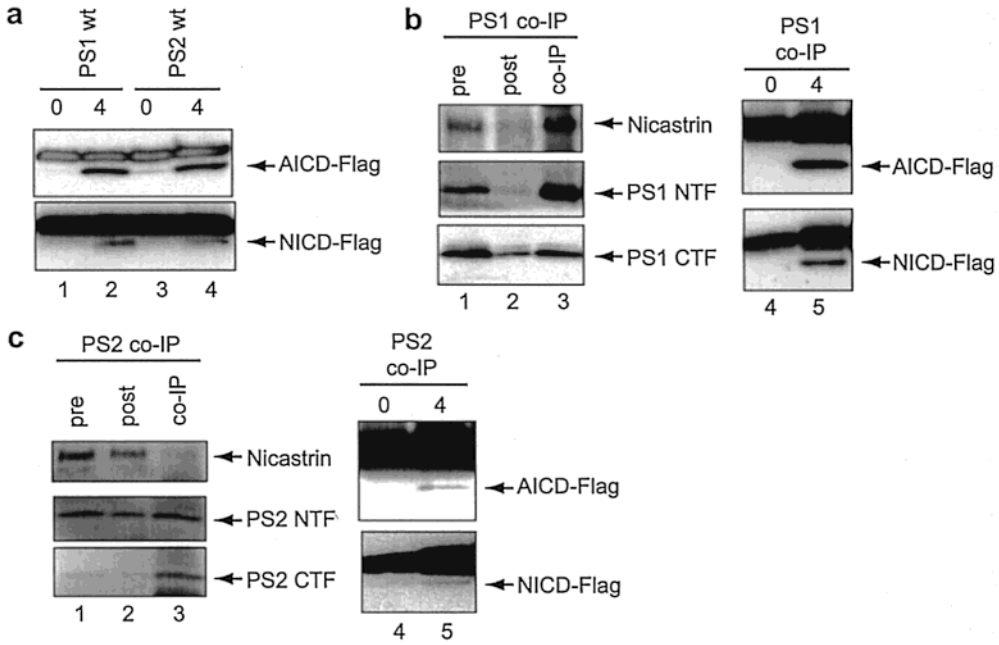

FIGURE 4: PS1 and PS2 can mediate the γ-secretase cleavage of C100Flag and N100Flag equivalently. (a) Solubilized γ-secretase was prepared from CHO cells stably overexpressing wild-type PS1 (PS70) and wild-type PS2 (72106). In these cells, a majority of the endogenous PS is replaced with the exogenous protein (*45*). (b) Solubilized HeLa membranes were co-immunoprecipitationed with antibody X81, and beads were incubated with C100Flag or N100Flag. The left-hand panel demonstrates the presence of nicastrin, PS1 NTF, and PS1 CTF in the co-immunoprecipitationed beads, consistent with the predicted role of these proteins in γ-secretase (*22*). (c) Analogous co-immunoprecipitations were performed with antibody 2972. Analysis as described for panel b.

function. To directly test whether PS1/γ-secretase and PS2/γ-secretase were able to cleave N100Flag and C100Flag similarly, we prepared CHAPSO-solubilized membrane lysates from CHO cells that stably overexpress PS1 or PS2. In each of these cell lines, essentially all endogenous PS is replaced with either wild-type human PS1 (PS70 line) or wild-type human PS2 (72106 line) (*35*). We found that bicarbonate-washed, CHAPSO-solubilized membranes containing PS1/γ-secretase or PS2/γ-secretase were capable of cleaving N100Flag and C100Flag (Figure 4a). To assess whether *endogenous* PS1/γ-secretase and PS2/γ-secretase behaved similarly, we used antibodies to the N-terminus of either PS1 or PS2 to immunoprecipitate activity. PS1 antibodies were previously shown to precipitate γ-secretase activity capable of cleaving C100Flag (*21*, *22*). When we co-immunoprecipitated HeLa lysates with antibody X81, we were able to precipitate PS1 heterodimers with nicastrin (Figure 4b, lanes 1−3), another putative member of the γ-secretase complex (*22*, *36*). When we added either N100Flag or C100Flag to the beads, we found robust generation of NICD-Flag and AICD-Flag (Figure 4b, lanes 4 and 5).

We similarly performed co-immunoprecipitations from HeLa lysates with antibody 2972 directed to the N-terminus of PS2. The expression level of PS2 is significantly lower than that of PS1 in these cells; nevertheless, 2972 precipitated

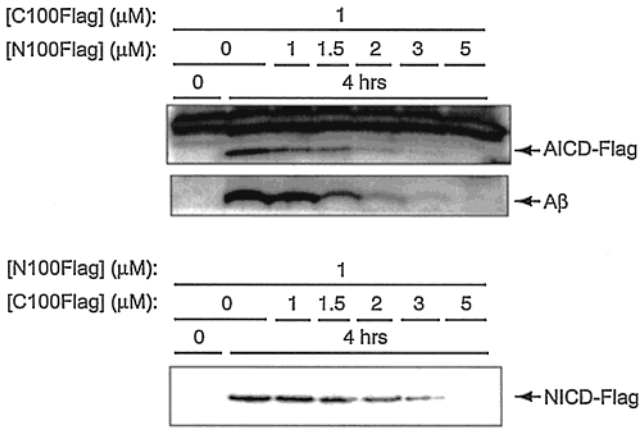

FIGURE 5: C100Flag and N100Flag prevent the γ-secretase cleavage of each other. In the top panel, the amount of C100Flag was kept constant and N100Flag was added in increasing amounts. AICD-Flag was probed with antibody C7, and Aβ was detected with 6E10. In the bottom panel, the level of N100Flag was constant and increasing amounts of C100Flag were added. NICD-Flag was detected with antibody Ab1744.

small amounts of nicastrin and PS2 heterodimers (Figure 4c, lanes 1−3). Moreover, when the 2972-precipitated beads were incubated with either substrate, we detected modest cleavage of C100Flag and N100Flag (Figure 4c, lanes 4 and 5). Importantly, the amounts of N100Flag and C100Flag cleavage products relative to each other were similar whether PS1/γ-secretase or PS2/γ-secretase served as the source of the enzyme. This result suggests that either form of γ-secretase is capable of cleaving each substrate.

Since the cleavages of N100Flag and C100Flag were remarkably similar, we next tested whether these substrates could directly prevent cleavage of each other. Prior experiments performed in whole cells provided conflicting results regarding competition between Notch and APP (*37*, *38*). Our cell-free in vitro assay has an advantage in that the amounts of N100Flag and C100Flag can be carefully controlled. Under these simplified in vitro conditions, we can test the effects of the two substrates more rigorously. When we kept the C100Flag concentration constant at its K_m of 1 μM (*21*) and varied the N100Flag concentration, we found a dose-dependent decrease in the extent of C100Flag cleavage, as measured by both Aβ and AICD levels (Figure 5, top panel). Conversely, when we maintained a constant N100Flag concentration and varied the C100Flag concentration, we found a highly similar dose dependence (Figure 5, bottom panel). We cannot readily differentiate between competitive and noncompetitive interactions because the γ-secretase complex apparently has two substrate binding sites (*22*) and demonstrates complex enzyme reaction kinetics (*39*). We nevertheless surmise that the substrates directly compete with each other since both molecules must access the active site for cleavage. These results imply that the two substrates are equally accessible to the same γ-secretase complex, and that the cleavages of APP and Notch are essentially indistinguishable.

Although the p3 and Aβ peptides derived from APP have been extensively characterized, the peptide counterparts in Notch have not been described. We therefore engineered an N-terminal hemagglutinin (HA) tag in N100Flag to generate HAN100Flag. Using this construct, we monitored the production of both the Np3 and NICD-Flag fragments.

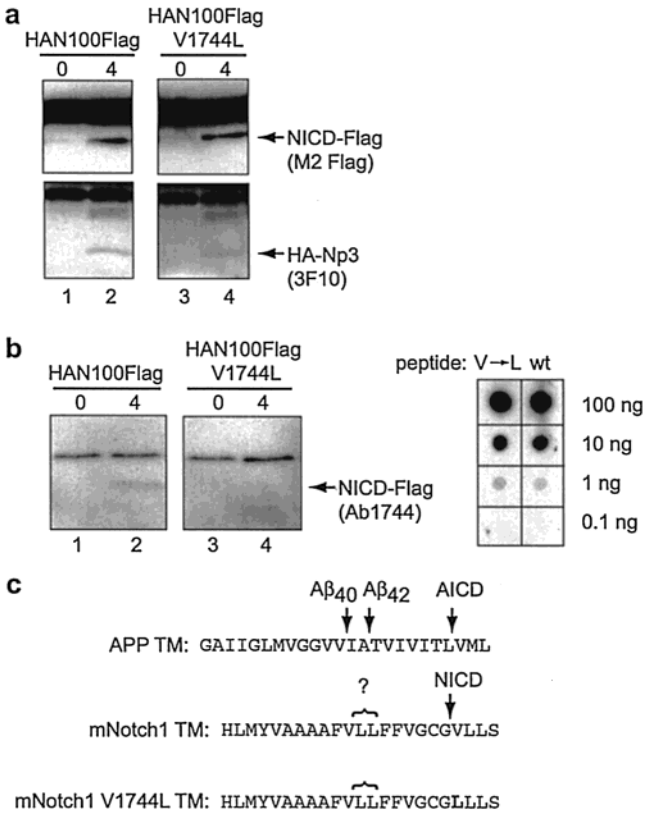

FIGURE 6: Detection of the Notch p3 peptide and analysis of the Notch V1744L mutation. (a) Wild-type or V1744L mutant HAN100Flag was incubated with solubilized γ-secretase. The NICD-Flag products were detected with M2 Flag antibody, and the HA-Np3 products were detected with 3F10 antibody. (b) The same reactions were probed with Ab1744 antibody that recognizes the NICD neoepitope. On the right, dot blot analysis demonstrates the equivalent sensitivity of the antibody for peptide containing V or L at the N-terminus. (c) Summary of known and inferred cleavages of APP and Notch within the transmembrane domain.

Following incubation with the solubilized γ-secretase preparation, we observed time-dependent production of both cleavage products (Figure 6a, lanes 1 and 2). We next introduced a point mutation corresponding to Val1744 in the Notch1 sequence. Mutagenesis of this residue abolishes Notch function by interfering with S3 cleavage (*9*, *40*), which is in apparent contradiction with the lack of sequence specificity for APP (*41*). In an effort to shed light on this discrepancy, we incubated the HAN100Flag V → L mutant substrate with solubilized γ-secretase. Surprisingly, we found that this substrate was cleaved into Np3 and NICD-Flag products (Figure 6a, lanes 3 and 4). However, when we probed the NICD-Flag product with the N-terminal specific antibody Ab1744, it was not detectable (Figure 6b, lanes 3 and 4) whereas the NICD-Flag derived from HAN100Flag was (Figure 6b, lanes 1 and 2).

To rule out the possibility that mutagenesis to Leu interfered with antibody recognition, we performed dot blots of peptides starting with Val or Leu. There was no difference in the sensitivity of the antibody for either peptide (Figure 6b, right panel), indicating that this antibody would recognize the NICD-Flag product if it started at L1744. Both AICD and NICD have N-terminal start sites just inside the membrane (the ϵ-cleavage site and S3 site, respectively). Mutagenesis of V1744 in Notch apparently abolishes this cleavage (refs *9* and *40* and panels a and b of Figure 6).

However, the existence of the NICD-Flag and HA-Np3 products indicates that a cleavage still occurs, albeit at another site. The most parsimonious explanation is that a cleavage within the middle of the TM domain occurs in Notch, equivalent to the Aβ sites in APP (Figure 6c). Direct sequencing of these fragments would definitively answer this question, which is not yet possible due to the low cleavage efficiency of the HAN100Flag substrates (as compared to C100Flag and N100Flag).

DISCUSSION

In this study, we provide evidence that Notch and APP are proteolyzed in a similar manner. Considerable evidence suggests that the same PS-dependent enzyme that is responsible for γ-secretase cleavage of APP also cuts the transmembrane region of Notch. Deletion of both PS1 and PS2 in mice is lethal in utero and leads to a phenotype similar to the knockout phenotype of Notch1 (*14*, *42*). As was observed for APP (*10*), Notch shows no S3 proteolysis in cells cultured from PS1/PS2 deficient mouse embryos (*14*, *15*). Structurally diverse γ-secretase inhibitors or mutation of either conserved PS aspartate residue blocks Aβ production (*43*) and also blocks proteolysis of Notch (*11*, *29*, *44*). Moreover, APP and Notch molecules can compete for each other for γ-secretase processing in primary neuronal cultures from APP transgenic mice (*37*). Nevertheless, conflicting data from several labs (*5*, *16*−*19*) have generated confusion over the degree of similarity between Notch and APP processing.

The advantage of our analysis is that a cell-free assay avoids indirect, whole cell-related effects on Notch and APP proteolysis. It excludes potential confounding factors that complicated the conclusions of previous studies. Indeed, some studies assayed Notch cleavage indirectly via Notch-sensitive reporter assays (*17*, *18*), whereas others did not assay for both APP products (Aβ and AICD) and Notch products (Np3 and NICD) in parallel (*16*). We carefully validate our in vitro assay using Flag-tagged substrates by recapitulating all known features of Notch and APP cleavage in whole cells. Our detection of both products rules out nonspecific cleavage and/or degradation of the substrate, which potentially confounds prior in vitro Notch-based assays (*19*). In our system, not only can we study γ-secretase cleavage independent of α- and β-secretase activity, but the cleavage of our substrates is completely specific to γ-secretase.

We found that the cleavages of Notch and APP TM domains possess identical inhibitory profiles, exhibit similar sensitivities to aspartate mutant PS, and have equal dependence on PS1 or PS2 for cleavage. Furthermore, we found that our APP- and Notch-based substrates prevent each other's cleavage by γ-secretase. Although we cannot readily demonstrate the mechanism of competition in a classical sense, these data argue that the same γ-secretase complex is capable of proteolyzing either protein. Finally, we provide evidence that Notch still undergoes cleavage when Val1744 is mutagenized to Leu. Interestingly, antibody Ab1744 (which recognizes the normal N-terminus of NICD) does not recognize the C-terminal product of HAN100Flag V1744L. This raises the intriguing possibility that a second cleavage occurs in the middle of the TM domain (as it does for APP) (Figure 6c). The V1744L mutation generates a Notch knockout-like phenotype when introduced into the genome of mice (*40*). Our data provide an explanation for this phenomenon. Indeed, it suggests that cleavage can still occur within the middle of the TM domain of Notch, but that the resulting NICD fragment cannot be efficiently released from the membrane. We therefore hypothesize that the reason for two cleavages (one in the middle and one near the edge of the TM domain) is to permit the proper release of both proteolytic products from the membrane.

Although γ-secretase is required for both TM domain cleavages to occur, we cannot rule out the possibility that two proteases or two active sites in the PS complex mediate each cleavage. Biochemical purification of γ-secretase to homogeneity (a technically challenging endeavor) could eventually resolve this question. Nevertheless, our current data provide evidence that γ-secretase, S3 protease, and ϵ-secretase are all intimately related and that the same proteases act indistinguishably on APP and Notch.

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NOTE ADDED IN PROOF

While this paper was under review, a report has been published demonstrating in transfected cells that Notch, like APP, is cleaved twice within its transmembrane domain (*46*). The secreted Np3 peptides were analyzed by mass spectrometry and found to be generated from proteolysis within the middle of the Notch transmembrane domain at sites analogous to those in APP that produce Aβ.

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