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Nicastrin Functions as a γ-Secretase-Substrate Receptor

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Summary

γ-secretase catalyzes the intramembrane cleavage of amyloid precursor protein (APP) and Notch after their extracellular domains are shed by site-specific proteolysis. Nicastrin is an essential glycoprotein component of the γ-secretase complex but has no known function. We now show that the ectodomain of nicastrin binds the new amino terminus that is generated upon proteolysis of the extracellular APP and Notch domains, thereby recruiting the APP and Notch substrates into the γ-secretase complex. Chemical- or antibody-mediated blocking of the free amino terminus, addition of purified nicastrin ectodomain, or mutations in the ectodomain markedly reduce the binding and cleavage of substrate by γ-secretase. These results indicate that nicastrin is a receptor for the amino-terminal stubs that are generated by ectodomain shedding of type I transmembrane proteins. Our data are consistent with a model where nicastrin presents these substrates to γ-secretase and thereby facilitates their cleavage via intramembrane proteolysis.

Introduction

Regulated intramembrane proteolysis (RIP) has been uncovered in diverse biological processes such as cholesterol metabolism, immune surveillance, intercellular communication, and Alzheimer's disease. Several classes of intramembrane proteases have been identified, including the site 2 protease (S2P) family of metalloproteases, the Rhomboid family of serine proteases, and the γ-secretase and signal peptide peptidase (SPP) family of aspartyl proteases. Known substrates of these unusual enzymes include the sterol regulatory element binding protein (SREBP) for S2P (reviewed in Brown et al., 2000), TGFα-like growth factor Spitz for Rhomboid (Urban et al., 2001), signal peptides for SPP (Weihofen et al., 2002), and, for γ-secretase, the amyloid precursor protein APP (De Strooper et al., 1998) and Notch (De Strooper et al., 1999). In many recognized substrates for S2P, Rhomboid, and SPP, helix-breaking residues are critical determinants for substrate specificity (Lemberg and Martoglio, 2002; Urban and Freeman, 2003; Ye et al., 2000). This, however, does not appear to be the case for γ-secretase substrates (Lichtenthaler et al., 1999). Indeed, γ-secretase appears to have a very broad substrate specificity (reviewed in Kopan and Ilagan, 2004). The only known prerequisites for γ-secretase substrates are (1) type I membrane proteins and (2) shedding the bulk of the extracellular domains from the full-length precursor proteins (Struhl and Adachi, 2000). Thus, one critical unanswered question concerning γ-secretase-mediated RIP is how γ-secretase specifically recognizes its many substrates that share no overt sequence similarity.

It is now clear that γ-secretase activity resides in a multimeric membrane-protein complex with presenilin (PS1 or PS2), nicastrin (Nct), APH-1 (APH-1aL, APH-1aS, or APH-1b), and PEN-2 as four essential components. Presenilin exists in the active γ-secretase complex as a heterodimer of the N- and C-terminal fragments (NTF and CTF) resulting from endoproteolysis. Current evidence is compatible with presenilin being the catalytic subunit of γ-secretase (Wolfe et al., 1999). The precise biochemical functions of the other γ-secretase subunits, particularly nicastrin, remain unclear. Consistent with their largely membrane-embedded topologies, APH-1 and PEN-2 may play structural roles in the assembly and maturation of the γ-secretase complex (Lee et al., 2004). Unlike the multipass membrane proteins APH-1, PEN-2, and presenilin, nicastrin is a type I membrane protein with a large ectodomain that accounts for approximately 45% of the calculated protein molecular mass of the four γ-secretase components combined. Extensive glycosylation further increases the size of the nicastrin ectodomain in relation to the other proteins. Presenilin-dependent nicastrin hyperglycosylation is important for γ-secretase maturation and trafficking to the cell surface but is not required for γ-secretase activity (Herreman et al., 2003). Examination of residues 261–502 in the nicastrin ectodomain reveals sequence similarity to a peptidase family that includes aminopeptidases, carboxypeptidases, and transferrin receptor proteins (pfam04839; Marchler-Bauer et al., 2005; Fagan et al., 2001), although no peptidase activity has been detected to date for nicastrin. This region also spans the most conserved sequence amongst nicastrin orthologs (near residues 306–360)—the DYIGS motif. Mutation of the DYIGS motif in previous studies has shown significant effects on γ-secretase maturation and/or activity (Chen et al., 2001; Shirotani et al., 2004; Yu et al., 2000), but the mechanistic role of the DYIGS motif in γ-secretase-mediated RIP is not clear. For ease of presentation, we refer to the *D*YIGS *a*nd *p*eptidase homologous region as the DAP domain.

In the current study, we address two interrelated questions central to the molecular mechanism of γ-secretase-mediated RIP. First, what is the biochemical function of nicastrin in the γ-secretase complex? Second, how is the recognition of a broad range of substrates determined in the γ-secretase complex? We show that

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the APP- and Notch-derived γ-secretase substrates stoichiometrically, directly, and functionally interact with nicastrin. We demonstrate that this interaction is mediated by the nicastrin ectodomain and the extracellular N-terminal stub of the substrate. Using both in vitro and in vivo assays, we find that the extracellular DAP domain of nicastrin is essential for γ-secretase-substrate recognition but not catalysis. These results thus define a biochemical function for nicastrin as a receptor for γ-secretase substrates.

Results

The Nicastrin Ectodomain Interacts with γ-Secretase Substrates

Previous studies have shown that γ-secretase substrates could be coimmunoprecipitated with both presenilin and nicastrin in total-cell extracts (Xia et al., 2000; Yu et al., 2000). To study whether nicastrin binds γ-secretase substrates directly or indirectly via presenilin, we examined whether nicastrin copurifies with the C-terminal APP fragment C99, a cleavage product of β-secretase, from baculovirus-infected Sf9 cells (Figure 1A). For ease of detection, nicastrin was fused with a His_6 tag at the C terminus (Nct-His) and C99 was C-terminally Flag tagged (C99-Flag). Triton X-100 extracts of membranes from Sf9 cells infected with *Nct-His*, *Nct-His* plus *C99-Flag*, or *C99-Flag* baculoviruses were subjected to immunoprecipitation (IP) with an anti-Flag antibody. His-tagged nicastrin from Sf9 cells appears to be present as an ~120 kDa full-length protein, as well as an ~10 kDa C-terminal membrane bound derivative (ctNct-His, Figure 1 and Figure S1A in the Supplemental Data available with this article online). Analysis of the Flag-peptide-eluted products by Western blotting or on Coomassie-stained SDS-polyacrylamide gels showed that only full-length Nct-His but not ctNct-His was coimmunoprecipitated with C99-Flag in high-salt Triton X-100 extracts (Figure 1B). Although the nature of the membrane bound ctNct is not clear, this result suggests that the cytoplasmic region and transmembrane region (TMR) of nicastrin do not bind to C99. Flag-peptide eluates from anti-Flag beads were also subjected to affinity pull-down using immobilized nickel-nitrilotriacetic acid (Ni-NTA). Retention of C99-Flag on Ni-NTA beads was dependent on the presence of Nct-His (Figure 1B). Addition of Flag peptide or EDTA prevented purification of the Nct-His:C99-Flag complex on anti-Flag or Ni-NTA beads (Figure S1A). Densitometry analyses of the Coomassie-blue-stained bands corresponding to Nct-His and C99-Flag showed an approximately equal molar ratio (Figure S1B). Under conditions that preserve the association of nicastrin and C99, neither nicastrin nor C99 binds to control membrane proteins (Figure S1C). Taken together, these observations indicate that the association of nicastrin with C99 is stoichiometric, specific, and direct.

In light of the absence of detectable association of C99 and the membrane bound C-terminal derivate of nicastrin, we decided to examine whether γ-secretase substrate directly binds to the large ectodomain of nicastrin using highly purified proteins (Figures 2A and 2B). A mixture of purified nicastrin ectodomain

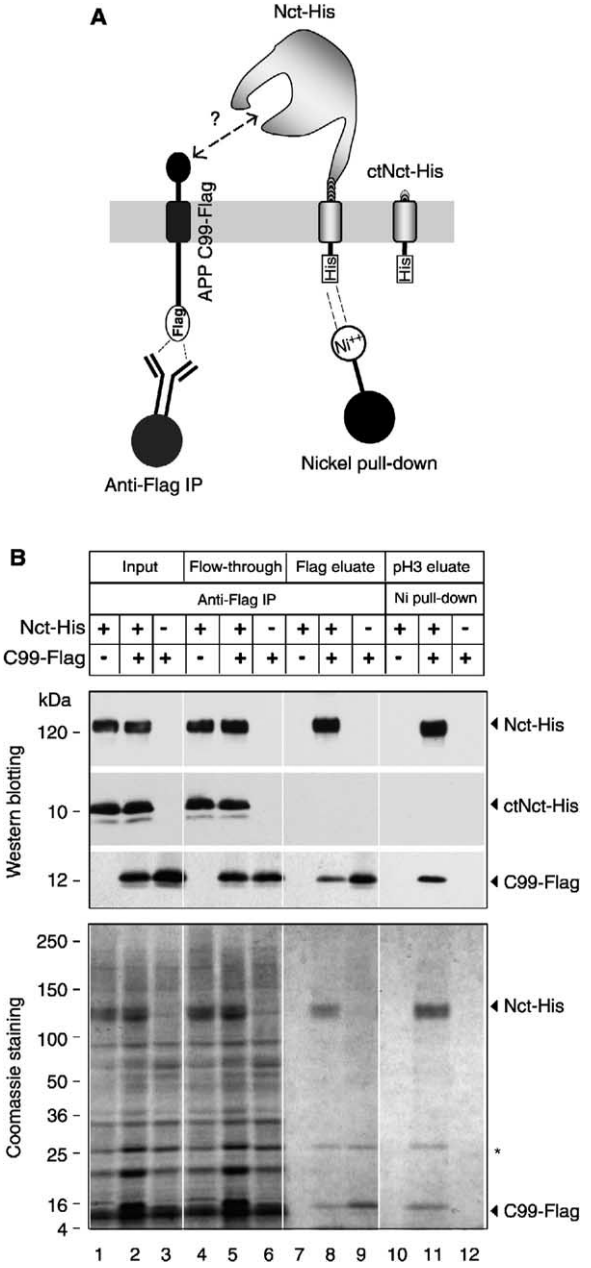


Figure 1. Nicastrin Stoichiometrically Copurifies with APP-Derived γ-Secretase Substrate

(A) Schematic representation of anti-Flag immunoprecipitation (IP) and Ni-NTA pull-down experiments. Dashed lines represent intermolecular interactions.

(B) Triton X-100 extracts of Sf9 cell membranes expressing Nct-His, Nct-His plus C99-Flag, or C99-Flag were incubated with anti-Flag beads. A portion of the input (lanes 1–3), flowthrough (lanes 4–6), and Flag-peptide-eluted products (lanes 7–9) were analyzed on SDS-polyacrylamide gels. The remaining Flag-peptide eluates were subjected to Ni-NTA pull-down (lanes 10–12). The SDS-polyacrylamide gels were either probed with anti-His or anti-Flag (top panels) or stained with Coomassie blue (bottom panel). Mass-spectrometry analysis identified the 25 kDa band (*) as the mitochondrion protein ADP/ATP translocase, which likely represents a nonspecific contaminant as it inconsistently coelutes with C99-Flag (also see Figure 2B).

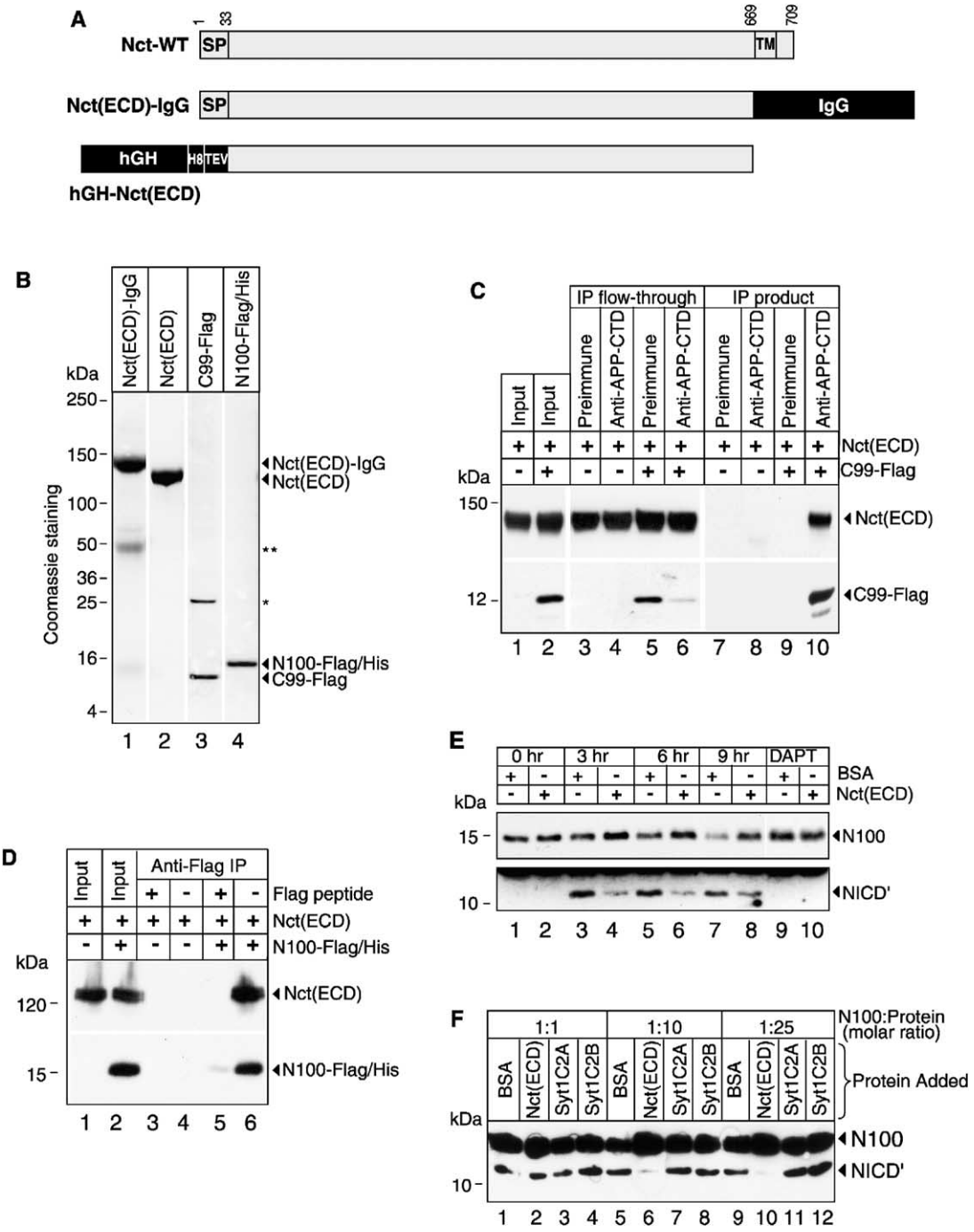


Figure 2. Nicastrin Ectodomain Physically and Functionally Interacts with APP- and Notch-Derived γ-Secretase Substrates

(A) Schematic representation of nicastrin-ectodomain fusion proteins. SP, signal peptide (residues 1–33); TM, transmembrane; black box represents sequence from proteins foreign to nicastrin. Numbers indicated refer to the primary structure of human nicastrin.

(B) Tagged or untagged Nct(ECD), C99, and N100 were purified and stained with Coomassie blue. The ~50 kDa band (**) is a degradation product of Nct(ECD)-IgG as it reacts with an anti-Nct antibody.

(C) Nct(ECD) coprecipitated with C99-Flag in IP experiments using anti-APP-CTD but not preimmune serum. Nct(ECD) was not retained on anti-APP-CTD or preimmune serum in the absence (–) of C99-Flag.

(D) Nct(ECD) coprecipitated with N100-Flag/His on anti-Flag resin only in a mixture of the two proteins. Addition (+) of Flag peptide into the mixture prevented the precipitation of either component.

(E) N100-Flag/His preincubated with either BSA or Nct(ECD) at 1:10 (N100:BSA or N100:Nct(ECD)) molar ratio was added to cell-free γ-secretase from HeLa cells and incubated at 37°C for 0, 3, 6, and 9 hr. In one sample set, γ-secretase inhibitor DAPT was added before incubating for 9 hr at 37°C. Longer incubation (e.g., 9 hr) at 37°C resulted in partial degradation of N100-Flag/His and NICD′-Flag/His, presumably by contaminating proteases in the crude cell-free γ-secretase preparation. The exposure times in the two panels differ due to the higher abundance of N100-Flag/His relative to NICD′-Flag/His.

(F) N100-Flag/His preincubated with either BSA, Nct(ECD), Syt1 C2A, or Syt1 C2B at 1:1, 1:10, or 1:25 molar ratio was added to cell-free γ-secretase and incubated at 37°C for 3 hr. The samples in (C)–(F) were probed with anti-Nct N terminus for Nct(ECD) and anti-Flag for Flag-tagged C99, N100, and NICD′.

(Nct(ECD)) and C99-Flag was subjected to IP with an antibody against the APP cytoplasmic domain (anti-APP-CTD). We observed specific coprecipitation of Nct(ECD) with C99-Flag captured by anti-APP-CTD but not by preimmune serum (Figure 2C). We next tested whether nicastrin could physically interact with N100, a membrane-tethered Notch fragment resulting from TNFα-converting enzyme (TACE) cleavage. For ease of detection, a Flag/His-tag was added at the C terminus of N100. We found that untagged pure Nct(ECD) specifically coprecipitated with N100-Flag/His on immobilized anti-Flag only from a mixture of the two proteins (Figure 2D). Similarly, we observed specific association of C99 or N100 with IgG-tagged nicastrin ectodomain (Nct(ECD)-IgG, Figures 2A and 2B) but not with IgG fusion proteins for nicastrin residues 1–50, APP residues 1–205, and calcium channel subunit α2δ1 (Figure S2). These results indicate that the nicastrin ectodomain specifically and directly interacts with the APP- and Notch-derived γ-secretase substrates.

To determine the functional significance of nicastrin and substrate interaction, the effect of nicastrin ectodomain on intramembrane cleavage of N100-Flag/His was studied in a cell-free assay using a γ-secretase preparation from CHAPSO-extracted HeLa cell membranes. Mixing N100-Flag/His and purified Nct(ECD) prior to addition of cell-free γ-secretase resulted in reduced cleavage of N100-Flag/His to generate the Notch intracellular domain (NICD′-Flag/His) (Figure 2E). Conversely, addition of Nct(ECD) increased the lifetime of the N100-Flag/His substrate. The inhibitory effect on N100 cleavage correlates with the amount of Nct(ECD) added to the reaction mixture (Figure 2F). In contrast, bovine serum albumin (BSA) or the C2A and C2B domains of synaptotagmin-1 (Syt1) have no inhibitory effect on γ-secretase cleavage of N100. It is therefore likely that the purified nicastrin ectodomain acts as an inhibitor by sequestering γ-secretase substrate N100 and that binding of nicastrin ectodomain to the substrate is crucial for γ-secretase activity.

The DAP Domain of Nicastrin Is Critical for Substrate Recognition and Cleavage

To investigate the significance of the nicastrin ectodomain in γ-secretase-substrate recognition in mammalian cells, we analyzed the structural and functional effects of a chimeric nicastrin protein wherein the TMR is swapped with another type I membrane glycoprotein, E-selectin (Nct-TMS) (Figure 3A). His-tagged Nct-TMS mutant was expressed in a HEK293 cell line stably expressing SpC99 that consists of the signal peptide (Sp) of APP and the β-secretase-cleaved APP C-terminal fragment (C99). Ni-NTA pull-down revealed that SpC99 efficiently coprecipitated with both the wild-type (wt) and TMS mutant nicastrin (Figure 3B, top panels). In contrast, much less PS1-NTF, an active species of PS1, coprecipitated with Nct-TMS-His when compared to Nct-wt-His (Figure 3B, bottom panel). We next analyzed the effect of the Nct-TMS mutant on the production of the APP intracellular domain (a product of γ-secretase activity) in a sensitive cell-based Gal4/VP16-dependent luciferase transactivation assay. This assay, which indirectly measures γ-secretase activity, utilizes a chimeric protein wherein APP or C99 is fused with Gal4 and VP16 domains (Cao and Sudhof, 2001). When compared to Nct-wt-His, dose- and time-dependent inhibition of APP-GV cleavage was observed in HEK293 cells expressing Nct-TMS-His (Figure 3C and Figure S3). These results suggest that nicastrin TMR is important for γ-secretase assembly and that the ectodomain but not the TMR of nicastrin is involved in substrate binding.

To further study the role of the nicastrin ectodomain in substrate recognition and to delineate the critical substrate binding site in the ectodomain of nicastrin at physiological levels, we generated *nicastrin* knockout mice and obtained fibroblasts from these mice and their littermates. As expected, neither immature (hypoglycosylated) nor mature (hyperglycosylated) nicastrin was detectable in *Nct*$^{-/-}$ fibroblasts (Figure 4A). Presenilin endoproteolytic fragments were also not detectable in the *nicastrin*-deficient cells. In contrast, the β- and α-secretase-generated APP C-terminal fragments C99 and C83 were markedly increased. Membrane extracts from the *Nct*$^{-/-}$ cells were incapable of cleaving the Notch- and APP-derived γ-secretase substrates (N100 and C99) in cell-free assays (Figure S4). APP-GV transactivation assay also showed no detectable γ-secretase activity (Figure 4B). Transient expression of Nct-wt in the *Nct*$^{-/-}$ fibroblasts increased γ-secretase activity ~150-fold to a level comparable to that in the wild-type cells. Significantly, expression of Nct-TMS in *nicastrin*-deficient cells did not restore γ-secretase activity.

Having demonstrated that the *Nct*$^{-/-}$ fibroblasts were bona fide knockout cells that lack γ-secretase activity, we analyzed in these cells the structural and functional effects of several mutations in the conserved DAP domain of nicastrin (Figure 3A). The DAP domain was of interest because primary-structure analysis revealed potential substrate binding residues in this region based on known aminopeptidase structures. In addition, an earlier study indicated that the DYIGS motif was critical for nicastrin function in intramembrane proteolysis (Yu et al., 2000). APP-GV and C99-GV transactivation assays revealed that a deletion of 28 amino acids (residues 312–340) in the DAP domain of nicastrin abolished γ-secretase activity (Figures 4B–4D). Using known aminopeptidase structures as a model, we hypothesized that a conserved glutamate residue (Glu333) within this 28 amino acid region of nicastrin is positioned in an analogous substrate binding pocket. The equivalent glutamate residue in the active sites of many aminopeptidases is implicated in exopeptidase specificity via multiple interactions with the substrate, particularly the interaction of the carboxylate side chain of the active-site glutamate and the free N-terminal α-amino group of the substrate (Luciani et al., 1998; Vazeux et al., 1998). Accordingly, we examined whether mutation of Glu333 to Ala affects APP processing. Like Nct-TMS and Δ312–340, E333A mutation abolished intramembrane cleavage of APP in the *Nct*$^{-/-}$ cells (Figures 4B–4D) and inhibited γ-secretase activity in HEK293 cells (Figure 3C). Similarly, E333R, E333S, and E333T mutations inactivated γ-secretase activity in *nicastrin*-deficient cells, whereas the conservative E333Q mutation preserved residual γ-secretase activity (Figure 4C). In contrast, mutations of several other conserved residues in the DAP domain of nicastrin (e.g., T334A,

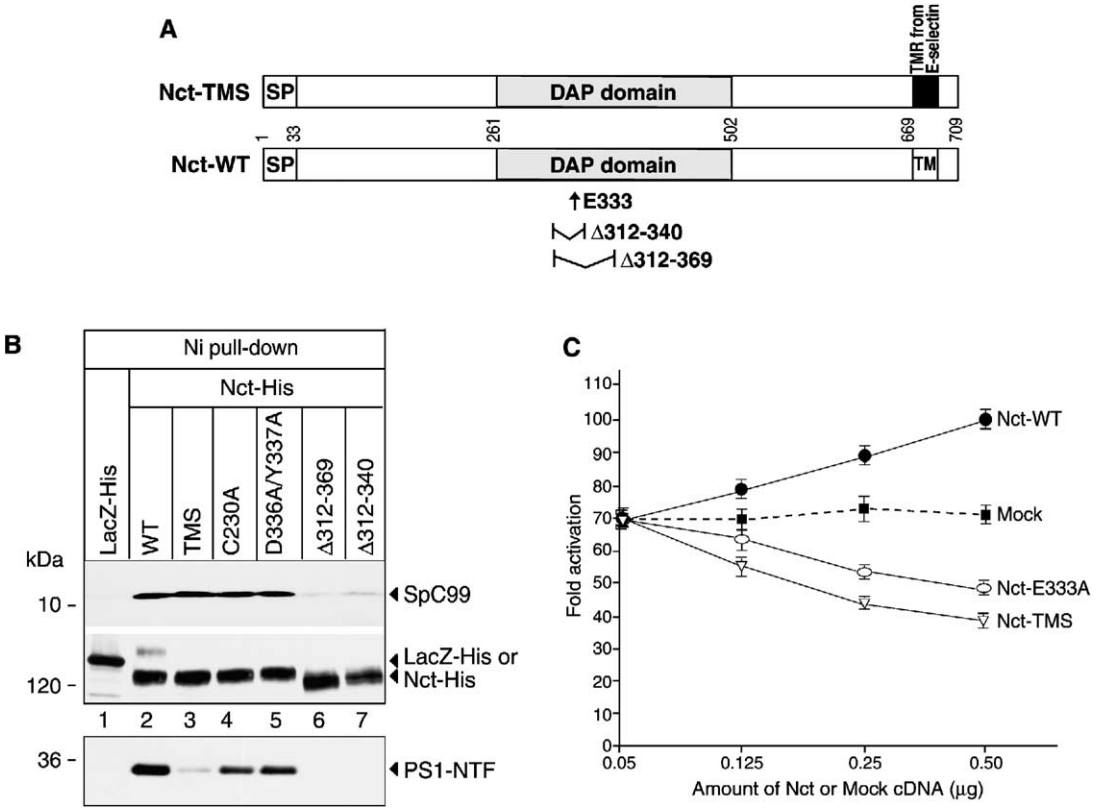


Figure 3. The Ectodomain but Not the TMR of Nicastrin Is Critical for γ-Secretase-Substrate Recognition

(A) Schematic representation of nicastrin proteins (Nct-wt, TMS, Δ312–340, and Δ312–369) and the position of Glu333. DAP domain, *D*YIGS and *p*eptidase homologous domain.

(B) HEK293 cells stably expressing SpC99 were transiently transfected with constructs encoding one of the following proteins: LacZ-His; His-tagged Nct-wt, TMS, C230A, D336A/Y337A, Δ312–369, or Δ312–340. After determining that similar amounts of SpC99, His-tagged proteins, and endogenous PS1 were expressed in each set of cells, we subjected equal amounts of Triton X-100 cell lysates to Ni-NTA pull-down in a high-salt buffer. The resultant products were probed with anti-APP-CTD or anti-His (top panels). In parallel experiments, CHAPSO lysates were subjected to Ni-NTA pull-down. The resultant products were investigated with anti-PS1-NTF (bottom panel).

(C) HEK293 cells were transiently cotransfected with the indicated amounts of *Nct* cDNAs or empty vector (Mock), APP-GV or a plasmid containing only the Gal4 DNA binding domain, and β-galactosidase. Samples were assayed for luciferase reporter activity on 96-well plates. Data collected were normalized to the β-galactosidase activity prior to presenting the results as fold activation over the Gal4-transfected samples. Data are represented as mean ± SEM.

T280A, S297A, and D360A) did not grossly affect γ-secretase cleavage of APP (Figures 4B and 4C and data not shown). To examine the role of the carboxylate side chain of Glu333 in mediating substrate interaction, we mutated nicastrin glutamate 333 to aspartate. If a similar molecular logic of substrate recognition applies to both aminopeptidase and nicastrin, the Nct-E333D mutation should not inactivate γ-secretase activity. Indeed, significant (albeit reduced) γ-secretase activity was observed in *Nct*$^{-/-}$ fibroblasts expressing Nct-E333D (Figures 4C and 4D). These observations support the model that Glu333 and the DAP domain of nicastrin are important in substrate recognition.

We next examined whether nicastrin physically interacts with γ-secretase substrates in vivo and whether this interaction is affected by mutations in the DAP domain. In our complementation experiments in *Nct*$^{-/-}$ cells, the steady-state level of exogenous nicastrin and the restored γ-secretase activity were comparable to those in the wild-type cells. This reflects the highly restricted nature of γ-secretase assembly and allows analysis of the substrate binding properties of nicastrin mutants at physiological protein levels. After confirming that endogenous nicastrin associates with C99/C83 in native wild-type cells but not in *nicastrin*-deficient cells (Figure 4A, bottom panel), we performed IP experiments under stringent conditions using high-salt Triton X-100 extracts from *nicastrin* knockout cell membranes expressing similar levels of Nct-wt, TMS, Δ312–340, E333A, and E333D. Because the level of C99/C83 is lower in the *nicastrin* knockout cells complemented with Nct-wt than in those with inactive nicastrin mutants (e.g., TMS, Δ312–340, and E333A), each set of cells was treated with γ-secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester) prior to detergent extraction so that the C99/C83 amount was comparable in each sample. While binding of nicastrin and C99/C83 was not grossly affected by the TMS or E333D mutation, significantly reduced amounts of Nct-E333A and Δ312–340 copre-

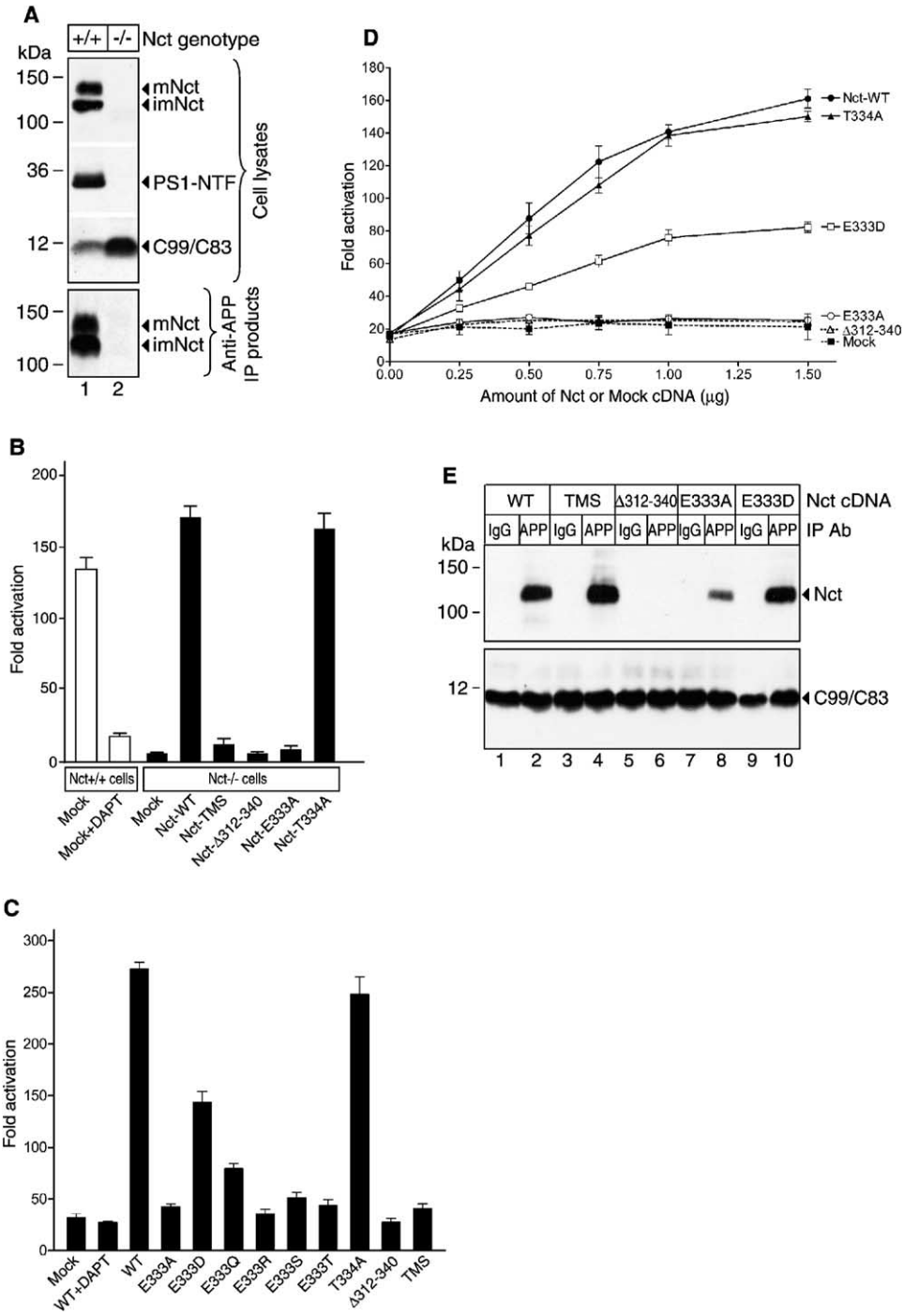


Figure 4. The DAP Domain of Nicastrin Is Required for γ-Secretase-Substrate Recognition In Vivo

(A) Top three panels: Western blotting for Nct, PS1-NTF, and APP C-terminal fragments C99/C83 in cell lysates from embryonic fibroblasts of *nicastrin* knockout mice (–/–) and their wild-type littermates (+/+). Bottom panel: Western blotting for nicastrin in the anti-APP-CTD IP products from Triton X-100 extracts of wild-type and *Nct*$^{-/-}$ fibroblasts. No detectable nicastrin was coprecipitated with preimmune serum in parallel experiments (not shown). mNct, mature nicastrin; imNct, immature nicastrin.

(B) Wild-type and *Nct*$^{-/-}$ cells transfected with 0.5 μg/well of *Nct* cDNAs or empty vector (Mock) were analyzed for APP cleavage on 96-well plates using the APP-GV transactivation assay as in Figure 3C. Data are represented as mean ± SEM.

(C) *Nct*$^{-/-}$ cells complemented with 0.5 μg/well *mock or Nct* cDNAs were analyzed with the C99-GV transactivation assay. Data are represented as mean ± SEM.

(D) *Nct*$^{-/-}$ cells transfected with the indicated amounts of cDNAs were analyzed with the C99-GV transactivation assay. Data are represented as mean ± SEM.

(E) *Nct*$^{-/-}$ fibroblasts complemented with the *Nct* cDNAs indicated on the top were subjected to overnight treatment with γ-secretase inhibitor DAPT. After confirming that each set of cells contained similar protein levels of C99/C83 (bottom panel) as well as nicastrin, Triton X-100 extracts were subjected to IP with anti-APP-CTD and control IgG. Samples in (A) and (E) were electrophoresed on SDS-polyacrylamide gels and probed with appropriate antibodies for Nct, PS1-NTF, and APP intracellular domain.

cipitated with C99/C83 (Figure 4E). The observation that C99/C83 binding was not significantly affected by E333D mutation is consistent with our earlier functional data and supports the model that carboxylate side chain of Glu333 is involved in substrate binding. We conclude that the nicastrin ectodomain, particularly the Glu333 and its nearby residues in the DAP domain, is required for substrate binding.

A defined experimental system amenable to rigorous biochemical analyses of γ-secretase in vitro would be invaluable for understanding γ-secretase biology. To this end, we chose to utilize purified recombinant enzyme from Sf9 cells expressing human PS1, Nct, APH-1aL, and PEN-2 (Figure 5A and Figure S5A). Using purified γ-secretase has the benefit of bypassing the highly restricted, glycosylation-dependent assembly and trafficking processes of the γ-secretase complex while maintaining all four recognized subunits at appropriate stoichiometric levels. γ-secretase was purified such that the known subunits were visible as prominent bands on Coomassie-blue-stained SDS-polyacrylamide gels (Figure 5B). The purified enzyme was enzymatically inactive unless reconstituted with lipids or liposomes, in which it cleaved N100-Flag/His intramembranously at the physiological S3 site of Notch (Figures S5B and S5C).

We obtained recombinant γ-secretase from Sf9 cells infected with baculoviruses expressing Nct (wt, E333A, or TMS), full-length PS1 (wt or D257A), APH-1aL, and PEN-2. PS1-NTF was efficiently generated in γ-secretase containing Nct-wt or Nct-E333A (Figure 5C), indicating that PS1 endoproteolysis was not impaired by the E333A mutation in the reconstituted γ-secretase. In contrast, PS1 endoproteolysis and resultant generation of PS1 endoproteolytic fragments were impaired by Nct-TMS or by the catalytic-site mutation PS1-D257A. We next tested whether cleavage of Notch or APP was affected by the Nct-E333A and TMS mutations in the recombinant γ-secretase. As expected, PS1-D257A mutation or γ-secretase inhibitor L685,458 prevented intramembrane cleavage of N100-Flag/His. Strikingly, γ-secretase cleavage of N100-Flag/His and generation of NICD′-Flag/His were also significantly inhibited by the Nct-E333A and TMS mutations (Figure 5D). Similarly, intramembrane cleavage of C99-Flag was inhibited when recombinant γ-secretase containing PS1-D257A, Nct-E333A, or Nct-TMS was used. Thus, the Nct-E333A mutation does not grossly affect the generation of steady-state presenilin endoproteolytic fragments but preferentially impairs intramembrane cleavage of type I membrane proteins, while the Nct-TMS mutation reduces both presenilin endoproteolysis and cleavage of type I membrane proteins. These data agree with a model in which assembly of nicastrin, APH-1, presenilin, and PEN-2 precedes presenilin endoproteolysis, which subsequently is required for activation of γ-secretase activity to cleave type I membrane proteins. Moreover, these results provide compelling evidence that nicastrin DAP domain plays a direct functional role in γ-secretase-substrate recognition.

We reasoned that a short reporter peptide mimicking the cleavage site (or sites) but lacking a recognizable N-terminal extracellular stub of γ-secretase substrate may be cleaved at the catalytic core without involvement of the substrate-recognition process associated with the nicastrin ectodomain. To test this hypothesis, we examined whether recombinant γ-secretase containing the Nct-E333A mutation was capable of cleaving a previously characterized fluorogenic peptide substrate that spans the cleavage sites at the C terminus of Aβ40 and Aβ42 (Farmery et al., 2003). We first confirmed and validated this experimental system by showing that cleavage of the APP-derived peptide substrate was sensitive to γ-secretase inhibitor L685,458 and was inhibited by the catalytic-site mutation PS1-D257A (Figure 5E). Cleavage of the reporter peptide was also inhibited by Nct-TMS, confirming the role of the nicastrin TMR in the assembly of the active γ-secretase. γ-secretase containing Nct-E333A could cleave the reporter peptide as efficiently as γ-secretase containing Nct-wt (Figure 5E). Considering the same γ-secretase preparation with Nct-E333A markedly inhibited intramembrane cleavage of the longer substrates C99 and N100 (Figure 5D), these results further implicate the nicastrin ectodomain in a substrate-recognition step that precedes the catalytic function of γ-secretase.

The Free Amino Terminus of Substrate Is Required for γ-Secretase-Mediated Intramembrane Proteolysis

The finding that a binding site for γ-secretase substrate resides in the ectodomain but not the transmembrane or cytoplasmic region of nicastrin implies that the N-terminal short extracellular stub of γ-secretase substrate participates in the interaction. We reasoned that blockage of the N terminus, but not the C terminus, of γ-secretase substrate would prevent the substrate from being recruited by nicastrin to the γ-secretase complex for intramembrane cleavage (Figure 6A). To test this hypothesis, we generated a baculovirus construct that expresses a chimeric C99 protein in which the seven residues (DAEFRHD) of C99 following the β-secretase-cleavage site were replaced by the Flag epitope (DYKDDDDK). Nct-His was coexpressed with either the N-terminally Flag-tagged C99 (nFlag-C99) or the C-terminally Flag-tagged C99 (C99-Flag, used earlier) in Sf9 cells. IP studies showed that Nct-His efficiently coprecipitated with either nFlag-C99 or C99-Flag on anti-APP-CTD beads (Figure 6B). Similarly, both nFlag-C99 and C99-Flag coprecipitated with Nct-His in Ni-NTA pull-down (Figure 6C). To test the effect of blocking the N terminus of γ-secretase substrate, we compared the amount of nicastrin coprecipitating with either the nFlag-C99 or C99-Flag on immobilized anti-Flag antibody. Although both nFlag-C99 and C99-Flag were efficiently retained on anti-Flag beads, nicastrin only readily and specifically coprecipitated with C99-Flag but not nFlag-C99 (Figure 6D). The most plausible explanation of this observation is that anti-Flag blocks the access of the N terminus of nFlag-C99 to the binding pocket in the ectodomain of nicastrin.

To test the functional implications of blocking the N terminus of substrate, the N- or C-terminally Flag-tagged C99 was preincubated with anti-Flag before mixing with recombinant γ-secretase. In the absence of anti-Flag, C99-Flag and nFlag-C99 could be cleaved to generate AICD-Flag. Addition of increasing amounts of anti-Flag resulted in potent inhibition of AICD produc-

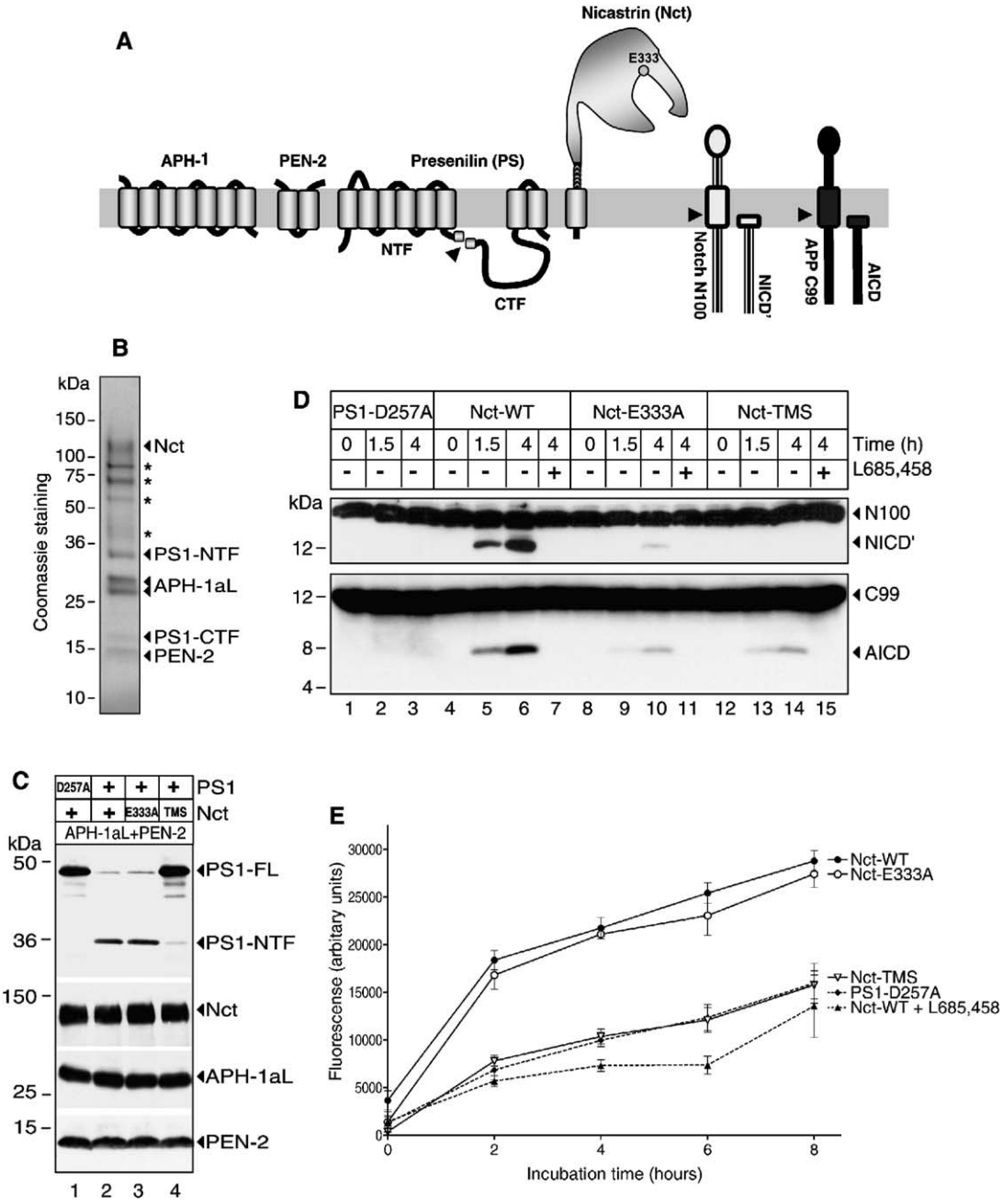


Figure 5. Nicastrin DAP Domain Is Required for Substrate Recognition In Vitro

(A) Schematic representation of γ-secretase subunits and substrates derived from APP and Notch. Arrow points to the site of presenilin endoproteolysis or γ-secretase cleavage. AICD, APP intracellular domain; NICD′, a truncated Notch intracellular domain.

(B) γ-secretase purified from Sf9 membranes coexpressing Nct, PS1, APH-1aL, and PEN-2 was separated on SDS-polyacrylamide gel and stained with Coomassie blue. The identities of several additional proteins (*) that copurified with γ-secretase activity are currently under investigation.

(C) Membrane-protein complexes isolated from Sf9 cells coinfected with baculoviruses harboring PS1 (wild-type [+] or D257A), Nct (wild-type [+], E333A, or TMS), APH-1aL, and PEN-2 were analyzed by Western blotting for full-length and endoproteolytic fragments of PS1 as well as the other γ-secretase components as indicated.

(D) Recombinant γ-secretase preparations from (C) were subjected to incubation with N100-Flag/His (top panel) or C99-Flag (bottom panel) at 37°C for 0, 1.5, and 4 hr, with (+) or without (–) γ-secretase inhibitor L685,458. Samples were electrophoresed on SDS-polyacrylamide gels and probed with anti-Flag or anti-APP-CTD.

(E) γ-secretases from (C) were incubated with 8 μM intramolecularly quenched fluorogenic peptide probe Nma-GGVVIATVK(Dnp)*rrr*-NH_2 at 37°C. Fluorescence was measured at the time points indicated. Background fluorescence of the peptide probe alone was subtracted from all readings. Data are represented as mean ± SEM.

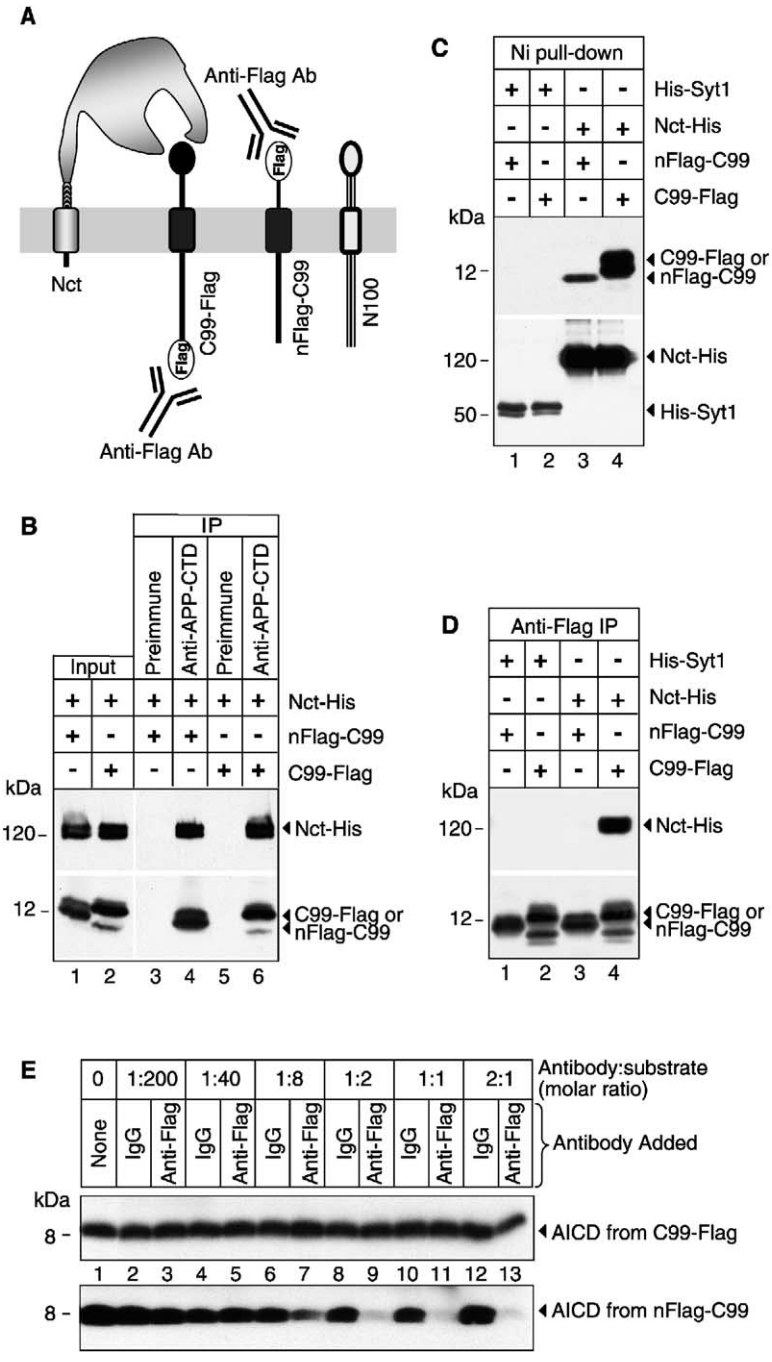


Figure 6. Antibody-Mediated Blocking of the N terminus of γ-Secretase Substrate Prevents Its Access to the Nicastrin Ectodomain

(A) Schematic representation of experimental design. Blocking the N terminus of nFlag-C99 by anti-Flag should prevent access to the binding site in nicastrin ectodomain and inhibit cleavage by γ-secretase. Blocking the C terminus of C99-Flag by anti-Flag should not grossly affect access to the binding site. Unblocked C99 or N100 should be able to access the substrate binding pocket in nicastrin.

(B) Membrane-protein extracts from Sf9 cells infected with baculoviruses expressing Nct-His plus either N- or C-terminally Flag-tagged C99 were subjected to IP with anti-APP-CTD or with preimmune serum.

(C) Membrane extracts from Sf9 cells expressing His-Syt1 or Nct-His plus either N- or C-terminally Flag-tagged C99 were subjected to Ni-NTA pull-down.

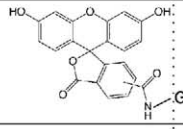
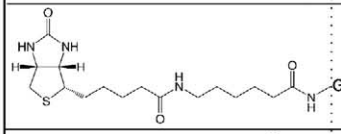
(D) Membrane extracts from (C) were subjected to anti-Flag IP. The resultant products in (B)–(D) were probed with anti-His for His-tagged nicastrin and Syt1 proteins and anti-Flag for Flag-tagged C99 proteins.

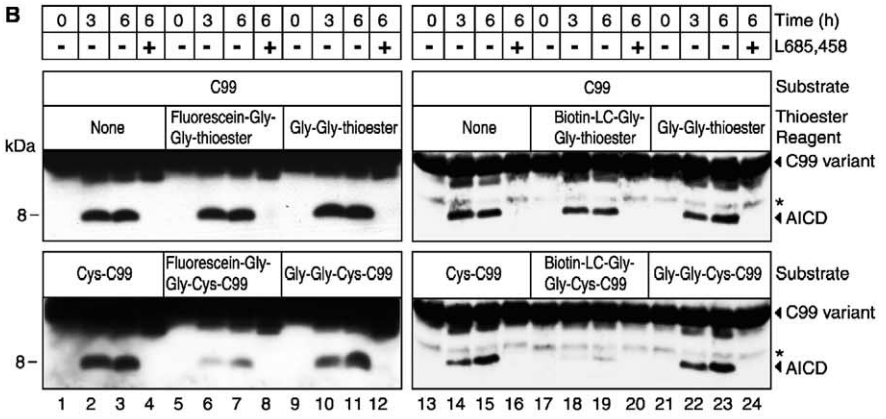
(E) C99-Flag or nFlag-C99 preincubated with either normal mouse IgG or anti-Flag at the indicated molar ratio was added to purified recombinant γ-secretase from Sf9 cells and incubated at 37°C for 4 hr. AICD generated from C99-Flag or nFlag-C99 was investigated with anti-APP-CTD.

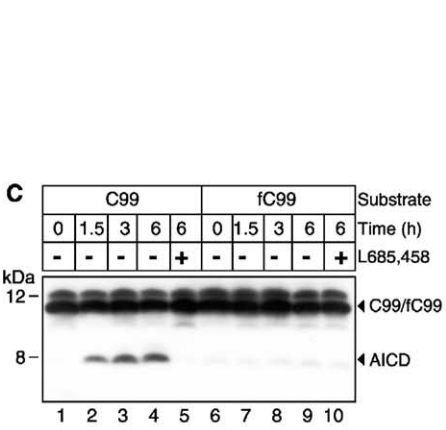
tion from nFlag-C99 (Figure 6E). Antibody-mediated blockage of the N terminus of nFlag-C99 did not prevent cleavage of N100 in the same reaction (Figure S6). It was suggested that dimerization inhibits substrate cleavage (Struhl and Adachi, 2000). However, addition of anti-Flag had no significant effect on C99-Flag cleavage (Figure 6E), suggesting that anchoring two molecules of C99 to the Fab arms of anti-Flag may not be comparable to the tight substrate dimerization by the GXXXG motif and the leucine zipper used in Struhl and Adachi (2000). Together, these observations suggest that the N-terminal stubs of type I membrane proteins are critical for intramembrane proteolysis.

We reasoned that the free N-terminal α-amino group of substrates may constitute the recognition site that is disrupted upon changes in the carboxylate group of Glu333 in the DAP domain of nicastrin. Thus, chemically blocking the free α-amino group of C99 may prevent it from being cleaved. Chemoselective modifications to the free α-amino group of C99 were initiated by employing a proteolytic cleavage approach to generate a C99-Flag-derived substrate, Cys-C99. The N-terminal cysteine of Cys-C99 was ligated to thioester derivative (dipeptide Gly-Gly thioester with or without N-terminal fluorescein or biotin-LC) using native chemical ligation (Dawson et al., 1994; reviewed in Dawson and Kent,

A Summary of C99 variants used in this study

Amino-terminal group	First five amino acids of C99 variants		γ-Secretase cleavage
H_2N—	Met-Asp-Ala-Glu-Phe···	*(C99)*	+
H_2N—	Met-Asp-Tyr-Lys-Asp···	*(nFlag-C99)*	+
H_2N—	Cys-Met-Asp-Ala-Glu···	*(Cys-C99)*	+
H_2N—	Gly-Gly-Cys-Met-Asp···	*(Gly-Gly-Cys-C99)*	+
	Gly-Gly-Cys-Met-Asp···	*(Fluorescein-Gly-Gly-Cys-C99)*	–
	Gly-Gly-Cys-Met-Asp···	*(Biotin-LC-Gly-Gly-Cys-C99)*	–
	Met-Asp-Ala-Glu-Phe···	*(fC99)*	–





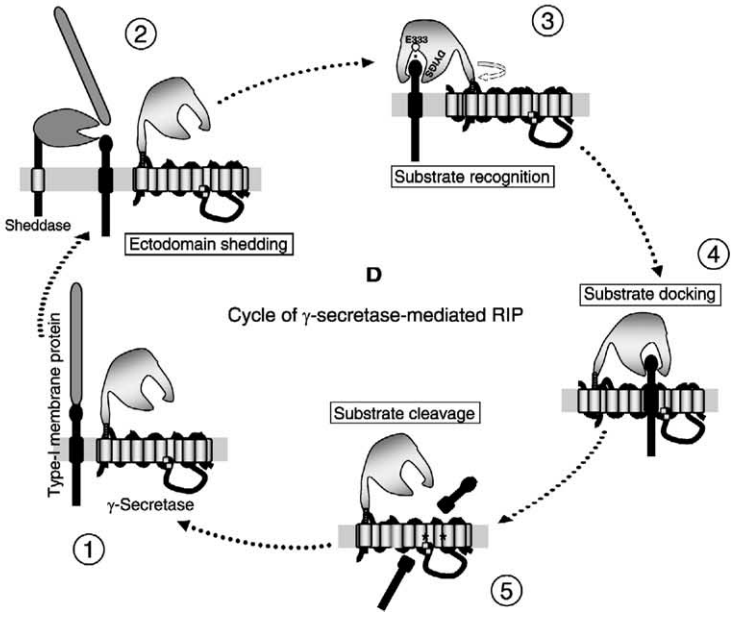


Figure 7. Chemical Blocking of the Free N Terminus Inhibits Substrate Cleavage by γ-Secretase

(A) Summary of C99 variants showing the N-terminal chemical groups and their effects on γ-secretase cleavage. All C99 proteins were Flag tagged at the C termini except nFlag-C99, which has an N-terminal Flag tag. fC99, N-formylated C99-Flag; +, efficient cleavage; –, impaired cleavage.

2000). (Figure S7). The resultant product (fluorescein-Gly-Gly-Cys-C99 or biotin-LC-Gly-Gly-Cys-C99) harbors fluorescein or biotin-LC at the N terminus (Figure 7A and Figure S7). γ-secretase cleavage of the fluorescein- or biotin-LC-labeled C99 was inhibited when compared to cleavage of C99 substrates with a free α-amino group: C99-Flag, Cys-C99, or Gly-Gly-Cys-C99, a ligation product of Gly-Gly-thioester and Cys-C99 (Figures 7A and 7B).

In addition, we compared γ-secretase cleavage of N-formylated C99-Flag (fC99) and C99-Flag harboring a free α-amino group. fC99 was expressed in and purified from *E. coli* AG100A cells in the presence of actinonin as described (Spector et al., 2003). Bacterial protein synthesis initiates with formyl methionine followed by posttranslational removal of the formyl group by peptide deformylase (PDF). The PDF inhibitor actinonin blocks the deformylation process, and thus the resultant proteins retain an uncharged formyl group at the N terminus. Remarkably, cleavage of fC99 bearing this small modification (three atoms) was inhibited as compared to C99 containing a free α-amino group (Figures 7A and 7C). Together, our data indicate that the free N terminus of type I membrane proteins resulting from ectodomain shedding is a primary γ-secretase-recognition site.

Discussion

Our studies establish that nicastrin has two functions in the γ-secretase-mediated RIP: nicastrin via its ectodomain functions as a receptor that recognizes the short amino-terminal stubs generated by ectodomain cleavage of type I transmembrane proteins and via its TMR participates in assembly of the γ-secretase complex. The shedding of the extracellular domains of type I membrane proteins exposes the newly generated short N termini that are recognized by nicastrin. Considering that nicastrin and γ-secretase substrates are anchored in a two-dimensional lipid bilayer by their TMRs, the interaction of the short extracellular N termini of type I membrane proteins with the nicastrin ectodomain is constrained and depends on the spatial distance and steric compatibility of their respective binding sites. This interaction allows nicastrin to selectively recruit many different type I membrane proteins with short extracellular stubs into the γ-secretase complex. The captured substrates are aligned and docked in the lipid bilayer, likely at the substrate-docking site in the TMRs of presenilin (Kornilova et al., 2005), and presented to the catalytic core for intramembrane cleavage (Figure 7D).

The evidence for our model that nicastrin is a receptor for γ-secretase substrates is as follows. First, nicastrin ectodomain physically and functionally interacts with APP- and Notch-derived γ-secretase substrates in vitro and in vivo. This interaction requires the ectodomain but not the TMR of nicastrin. Second, deleting residues 312–340 within the DAP domain suppresses binding of nicastrin to substrates and inhibits γ-secretase activity. Mutation of the conserved Glu333 to Ala affects substrate binding and markedly inhibits intramembrane proteolysis in vivo. Nicastrin-dependent C99/C83 binding and cleavage are less affected by the E333D mutation than other Glu333 mutations examined in this study, suggesting that the carboxylate side chain of the conserved glutamate in nicastrin and in aminopeptidases similarly recognizes the N terminus of the respective substrates. Third, the Nct-E333A mutation in purified recombinant γ-secretase does not grossly affect presenilin endoproteolysis, which occurs when the γ-secretase complex is fully assembled. In contrast, purified γ-secretase containing Nct-E333A greatly inhibits intramembrane cleavage of APP and Notch. In addition, a short reporter peptide mimicking the γ-secretase-cleavage sites of APP but lacking a recognizable N-terminal stub could be efficiently cleaved by reconstituted γ-secretase that contains the E333A mutation. These results suggest that nicastrin is responsible for specific recognition of type I transmembrane substrates. Fourth, antibody-mediated blocking of the N but not the C terminus prevents C99 from binding to nicastrin and undergoing intramembrane processing. Furthermore, chemical blocking of the free N-terminal α-amino group drastically inhibits C99 cleavage. Together, these results indicate that the interaction between the extracellular DAP domain of nicastrin and the free N termini of type I membrane proteins is essential during the substrate-recognition process and is a primary determinant of substrate specificity of γ-secretase.

Our work shows that a recognition site for γ-secretase substrates resides in the DAP domain of nicastrin that is evolutionarily derived from a peptidase. The DAP domain retains substrate-recognition ability; however, not all residues required for peptidase activity are conserved. The catalytic activity of γ-secretase is believed to be provided by two transmembrane aspartates in presenilin (Wolfe et al., 1999) that are derived from an ancient aspartyl protease (Steiner et al., 2000). We propose that γ-secretase evolved in a unique and elegant convergence whereby the substrate-recognition func-

(B) Cys-C99 was subjected to native chemical ligation in the presence or absence of the thioester derivatives. C99-Flag was treated with the thioesters under the same conditions (top panel). The resultant C99 variants were assayed for γ-secretase cleavage similar to Figure 5D. Cleavage of the remaining unligated substrates may partly contribute to the residual AICD in lanes 6–7 and 18–19 of the bottom panel. Asterisk indicates a nonspecific protein insensitive to L685,458.

(C) Regular and N-formylated C99 were purified from bacterial cells in the absence and presence of actinonin. These substrates were subjected to γ-secretase-cleavage assays.

(D) Proposed role of nicastrin in γ-secretase-mediated RIP. γ-secretase is a high-molecular-weight complex composed of at least four membrane proteins: presenilin, nicastrin, APH-1, and PEN-2. γ-secretase substrates typically consist of type I membrane proteins with small extracellular stubs resulting from ectodomain shedding of the precursor proteins. The current study shows that nicastrin via its large ectodomain acts as a receptor for γ-secretase substrates by directly interacting with the free N termini of the substrates. The extracellular DAP domain of nicastrin, in which the conserved Glu333 residue and the DYIGS motif are critical, is involved in substrate recognition.

tion of an aminopeptidase was united with the catalytic function of an intramembrane aspartyl protease. This enables the γ-secretase complex to execute the unusual events associated with RIP of type I membrane proteins in the lipid bilayers.

The effects of mutations of the DAP domain on the binding of nicastrin to APP-derived substrates generally correlate with the inhibitory effects of these mutations on intramembrane proteolysis. The observation that functional effects are greater than the structural effects agrees with earlier studies that found that substrate binding was not grossly disrupted but might be subtly altered by specific point mutations of conserved glutamates in aminopeptidases (Thompson et al., 2003; Vazeux et al., 1998). The high sensitivity of the APP-GV and C99-GV transactivation assays also contributes to the ease of detecting smaller functional differences for γ-secretase catalysis. On the other hand, the observation that, under the stringent conditions used in this study, the E333A mutation partially reduces the nicastrin:substrate interaction while the Δ312–340 or Δ312–369 mutation abolished the interaction (Figures 3B and 4E) implies that other residues of the DAP domain may cooperate with Glu333 in substrate binding. Under gentle conditions (e.g., low-salt digitonin buffers), association of C99/C83 and Nct Δ312–340 has also been observed (Yu et al., 2000). This observation may reflect the role of the TMRs of the γ-secretase subunits in docking of substrates before their catalysis (see Figure 7D for model), which could be captured under the conditions that are compatible with γ-secretase assembly and activity. Although it is conceivable that sequences outside residues 312–340 of the DAP domain may contribute to substrate binding, our data clearly establish that a major substrate-recognition site consists of Glu333 and its nearby residues.

Our studies indicate that nicastrin is a receptor with broad specificity in recognizing short peptide-like extracellular domains of type I membrane proteins. However, the binding properties of nicastrin to different substrates could be differentially regulated by the number and composition of amino acids of the extracellular portion of the substrates. This view is supported by the finding that alteration of the N terminus of C99 subtly affects its binding to nicastrin (Figure 6C) and its cleavage by γ-secretase (data not shown) and that Notch and APP processing is differentially affected by mutations of the DYIGS motif of nicastrin (Chen et al., 2001). Designing or screening for compounds or antibody derivatives that specifically or preferentially block the binding of the N terminus of β-secretase-cleaved APP (i.e., C99) to the nicastrin ectodomain may prove to be a tractable strategy as a therapy for Alzheimer's disease.

Experimental Procedures

Reagents and General Methods

Wild-type and mutant cDNAs for γ-secretase subunits and substrates were cloned into baculoviral, bacterial, and mammalian expression vectors. *nicastrin*-deficient embryonic fibroblasts were obtained from *nicastrin* knockout mice, which will be reported elsewhere (K.T., G.Y., and T.S., unpublished data). Standard methods such as site-directed mutagenesis, transfection, Western blotting, and affinity precipitation were performed as described (Lee et al., 2002, 2004; Yu et al., 1998, 2000). All experiments in this paper were performed at least four times with multiple replications. The GraphPad Prism software was used for statistical analysis and graphing. More details of reagents and methods are described in the Supplemental Experimental Procedures.

Protein Purification and Modification

Purification of recombinant γ-secretase and its substrates (N100-Flag/His, C99-Flag, and nFlag-C99) from insect cell membranes and the nicastrin ectodomain from conditioned media of mammalian cells is described in the Supplemental Experimental Procedures. N-formylated C99-Flag (fC99) was purified on anti-Flag beads from detergent extracts of Δ*acrAB E. coli* strain AG100A(DE3) (a gift from Dr. R.T. Sauer) transformed with pET21b/C99-Flag and induced by 1 mM IPTG plus 2 μg/ml actinonin for 5 hr at room temperature. Cys-C99 was expressed in *E. coli* as a fusion protein with an N-terminal His_6 tag and a recognition site for tobacco etch virus (TEV) protease as well as a C-terminal Flag tag (Figure S7B). The fusion protein purified on Ni-NTA beads was cleaved with His-tagged TEV protease. Purified Cys-C99 was obtained after Ni-NTA depletion of the undigested fusion protein and TEV protease. Chemical synthesis of fluorescein- and biotin-LC-Gly-Gly-thioester and their ligation to the α-amino group of Cys-C99 are described in detail in the Supplemental Experimental Procedures.

γ-Secretase Assays

N100-Flag/His, nFlag-C99, C99-Flag, Cys-C99, and their N-terminally modified variants were incubated at 37°C with recombinant γ-secretase purified from Sf9 cells or with cell-free γ-secretase from HeLa cells in a reaction buffer containing 0.25% CHAPSO, 50 mM PIPES (pH 7.0), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.0125% phosphatidylethanolamine, and 0.1% phosphatidylcholine. A typical 50 μl reaction uses 25 μg γ-secretase preparation and 0.1 μg substrate. AICD-Flag or NICD′-Flag/His was analyzed on Tris-Tricine SDS-polyacrylamide gels using anti-APP-CTD, anti-Flag, or anti-cleaved Notch-1 (Val1744) antibody. γ-secretase cleavage of APP or C99 was also analyzed in HEK293 or fibroblast cells using the luciferase reporter gene assay system as described (Cao and Sudhof, 2001; Lee et al., 2004). The fluorogenic reporter peptide mimicking the γ-secretase-cleavage sites of APP was assayed as reported (Farmery et al., 2003).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and seven figures and can be found with this article online at http://www.cell.com/cgi/content/full/122/3/435/DC1/.

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References

Brown, M.S., Ye, J., Rawson, R.B., and Goldstein, J.L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell *100*, 391–398.

Cao, X., and Sudhof, T.C. (2001). A transcriptionally [correction of

transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science *293*, 115–120.

Chen, F., Yu, G., Arawaka, S., Nishimura, M., Kawarai, T., Yu, H., Tandon, A., Supala, A., Song, Y.Q., Rogaeva, E., et al. (2001). Nicastrin binds to membrane-tethered Notch. Nat. Cell Biol. *3*, 751–754.

Dawson, P.E., and Kent, S.B. (2000). Synthesis of native proteins by chemical ligation. Annu. Rev. Biochem. *69*, 923–960.

Dawson, P.E., Muir, T.W., Clark-Lewis, I., and Kent, S.B. (1994). Synthesis of proteins by native chemical ligation. Science *266*, 776–779.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., et al. (1999). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature *398*, 518–522.

De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998). Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature *391*, 387–390.

Fagan, R., Swindells, M., Overington, J., and Weir, M. (2001). Nicastrin, a presenilin-interacting protein, contains an aminopeptidase/transferrin receptor superfamily domain. Trends Biochem. Sci. *26*, 213–214.

Farmery, M.R., Tjernberg, L.O., Pursglove, S.E., Bergman, A., Winblad, B., and Naslund, J. (2003). Partial purification and characterization of gamma-secretase from post-mortem human brain. J. Biol. Chem. *278*, 24277–24284.

Herreman, A., Van Gassen, G., Bentahir, M., Nyabi, O., Craessaerts, K., Mueller, U., Annaert, W., and De Strooper, B. (2003). gamma-Secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. J. Cell Sci. *116*, 1127–1136.

Kopan, R., and Ilagan, M.X. (2004). Gamma-secretase: proteasome of the membrane? Nat. Rev. Mol. Cell Biol. *5*, 499–504.

Kornilova, A.Y., Bihel, F., Das, C., and Wolfe, M.S. (2005). The initial substrate-binding site of gamma-secretase is located on presenilin near the active site. Proc. Natl. Acad. Sci. USA *102*, 3230–3235.

Lee, S.F., Shah, S., Li, H., Yu, C., Han, W., and Yu, G. (2002). Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-beta precursor protein and Notch. J. Biol. Chem. *277*, 45013–45019.

Lee, S.F., Shah, S., Yu, C., Wigley, W.C., Li, H., Lim, M., Pedersen, K., Han, W., Thomas, P., Lundkvist, J., et al. (2004). A conserved GXXXG motif in APH-1 is critical for assembly and activity of the gamma-secretase complex. J. Biol. Chem. *279*, 4144–4152.

Lemberg, M.K., and Martoglio, B. (2002). Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. Mol. Cell *10*, 735–744.

Lichtenthaler, S.F., Wang, R., Grimm, H., Uljon, S.N., Masters, C.L., and Beyreuther, K. (1999). Mechanism of the cleavage specificity of Alzheimer's disease gamma-secretase identified by phenylalanine-scanning mutagenesis of the transmembrane domain of the amyloid precursor protein. Proc. Natl. Acad. Sci. USA *96*, 3053–3058.

Luciani, N., Marie-Claire, C., Ruffet, E., Beaumont, A., Roques, B.P., and Fournie-Zaluski, M.C. (1998). Characterization of Glu350 as a critical residue involved in the N-terminal amine binding site of aminopeptidase N (EC 3.4.11.2): insights into its mechanism of action. Biochemistry *37*, 686–692.

Marchler-Bauer, A., Anderson, J.B., Cherukuri, P.F., DeWeese-Scott, C., Geer, L.Y., Gwadz, M., He, S., Hurwitz, D.I., Jackson, J.D., Ke, Z., et al. (2005). CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res. *33* (Database issue), D192–D196.

Shirotani, K., Edbauer, D., Kostka, M., Steiner, H., and Haass, C. (2004). Immature nicastrin stabilizes APH-1 independent of PEN-2 and presenilin: identification of nicastrin mutants that selectively interact with APH-1. J. Neurochem. *89*, 1520–1527.

Spector, S., Flynn, J.M., Tidor, B., Baker, T.A., and Sauer, R.T. (2003). Expression of N-formylated proteins in Escherichia coli. Protein Expr. Purif. *32*, 317–322.

Steiner, H., Kostka, M., Romig, H., Basset, G., Pesold, B., Hardy, J., Capell, A., Meyn, L., Grim, M.L., Baumeister, R., et al. (2000). Glycine 384 is required for presenilin-1 function and is conserved in bacterial polytopic aspartyl proteases. Nat. Cell Biol. *2*, 848–851.

Struhl, G., and Adachi, A. (2000). Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. Mol. Cell *6*, 625–636.

Thompson, M.W., Govindaswami, M., and Hersh, L.B. (2003). Mutation of active site residues of the puromycin-sensitive aminopeptidase: conversion of the enzyme into a catalytically inactive binding protein. Arch. Biochem. Biophys. *413*, 236–242.

Urban, S., and Freeman, M. (2003). Substrate specificity of rhomboid intramembrane proteases is governed by helix-breaking residues in the substrate transmembrane domain. Mol. Cell *11*, 1425–1434.

Urban, S., Lee, J.R., and Freeman, M. (2001). Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. Cell *107*, 173–182.

Vazeux, G., Iturrioz, X., Corvol, P., and Llorens-Cortes, C. (1998). A glutamate residue contributes to the exopeptidase specificity in aminopeptidase A. Biochem. J. *334*, 407–413.

Weihofen, A., Binns, K., Lemberg, M.K., Ashman, K., and Martoglio, B. (2002). Identification of signal peptide peptidase, a presenilin-type aspartic protease. Science *296*, 2215–2218.

Wolfe, M.S., Xia, W., Ostaszewski, B.L., Diehl, T.S., Kimberly, W.T., and Selkoe, D.J. (1999). Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature *398*, 513–517.

Xia, W., Ray, W.J., Ostaszewski, B.L., Rahmati, T., Kimberly, W.T., Wolfe, M.S., Zhang, J., Goate, A.M., and Selkoe, D.J. (2000). Presenilin complexes with the C-terminal fragments of amyloid precursor protein at the sites of amyloid beta-protein generation. Proc. Natl. Acad. Sci. USA *97*, 9299–9304.

Ye, J., Dave, U.P., Grishin, N.V., Goldstein, J.L., and Brown, M.S. (2000). Asparagine-proline sequence within membrane-spanning segment of SREBP triggers intramembrane cleavage by site-2 protease. Proc. Natl. Acad. Sci. USA *97*, 5123–5128.

Yu, G., Chen, F., Levesque, G., Nishimura, M., Zhang, D.M., Levesque, L., Rogaeva, E., Xu, D., Liang, Y., Duthie, M., et al. (1998). The presenilin 1 protein is a component of a high molecular weight intracellular complex that contains beta-catenin. J. Biol. Chem. *273*, 16470–16475.

Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y.Q., Rogaeva, E., Chen, F., Kawarai, T., et al. (2000). Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. Nature *407*, 48–54.