Evidence That the “Lid” Domain of Nicastrin Is Not Essential for Regulating γ-Secretase Activity*  

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Xulun Zhang‡, Eric Sullivan†, Maggie Scimeca‡, Xianzhong Wu‡, Yue-ming Li‡, and Sangram S. Sisodia‡1  
From the ‡Department of Neurobiology, The University of Chicago, Chicago, Illinois 60637 and the †Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10065  

Understanding of the structure of the γ-secretase complex consisting of presenilin (PS), anterior pharynx-defective 1 (APH-1), nicastrin (NCT), and presenilin enhancer 2 (PEN-2) is of significant therapeutic interest for the design of γ-secretase modulators for Alzheimer disease. The structure of γ-secretase revealed by cryo-EM approaches suggested a substrate binding mechanism for NCT, a bilobar structure that involved rotation of the two lobes around a central pivot and opening of a “lid” region that facilitates substrate recruitment. To validate this proposal, we expressed NCT that lacks the lid entirely, or a variety of NCT variants that harbor mutations at highly conserved residues in the lid region in NCT-deficient cells, and then assessed their impact on γ-secretase assembly, activity, and stability. In addition, we assessed the impact of mutating a critical residue proposed to be a pivot around which the two lobes of NCT rotate. Our results show that neither the mutations on the lid tested here nor the entire lid deletion has any significant impact on γ-secretase assembly, activity, and stability, and that NCT with the mutation of the proposed pivot rescues γ-secretase activity in NCT-deficient cells in a manner indistinguishable from WT NCT. These findings indicate that the NCT lid is not an essential element necessary for γ-secretase assembly, activity, and stability, and that rotation of the two lobes appears not to be a prerequisite for substrate binding and γ-secretase function.

γ-Secretase, an aspartyl protease that contains presenilin (PS),2 anterior pharynx-defective 1 (APH-1), nicastrin (NCT), and presenilin enhancer 2 (PEN-2), catalyzes intramembrane cleavage of a wide spectrum of membrane-bound substrates, including the amyloid precursor protein (APP) and the Notch receptors (1). γ-Secretase-mediated processing of APP generates Aβ peptides that play a central role in the pathogenesis of Alzheimer disease, whereas intramembranous cleavage of Notch is essential for modulating nuclear Notch signaling that is critical for cell lineage determination, proliferation, development, and the pathogenesis of cancers and other diseases (1). Investigations of the functional role of each subunit of the complex have established the following: PS is the catalytic center of the enzyme (2); NCT, a type I transmembrane protein, is responsible for recruiting substrates into the complex (3, 4); APH-1 is involved in assembly by providing a scaffold (5); and PEN-2 promotes PS endoproteolysis and enzyme maturation (6). Recent cryo-EM analyses of purified γ-secretase have revealed important aspects regarding the arrangement and interactions of the individual subunits within the complex. The 20 TMAs of the four subunits form a horseshoe-shaped structure inside the membrane, whereas the large ectodomain (ECD) of NCT sits on top of this structure on the extracellular side (7, 8). The crystal structure of the Dictyostelium purpureum NCT ECD at 1.95 Å resolution revealed the presence of two lobes (7). Extending these studies, Bai et al. (8) determined the atomic structure of human γ-secretase at 3.4 Å resolution using cryo-EM approaches, and these studies also revealed a bilobar structure for human NCT ECD. The larger of these lobes is encoded by exons 7–16 of the NCT gene and coincides with the “716” fragment that we showed earlier to play a role in substrate recognition, rather than enzyme assembly (4). The small lobe is composed of the first 252 residues of NCT and includes a short “loop” that extends from amino acids Ser-137 to Gly-168. This latter region is termed “lid” as it appears to cover the substrate entry pocket in the large lobe. Furthermore, it was proposed that substrate access and binding occur by rotation of the large and small lobes around a central pivot at Phe-287 that results in displacement of the lid domain (7, 8). From these findings, several implicit predictions arise. First, in NCT-deficient cells that lack γ-secretase activity, expression of NCT devoid of the lid should facilitate substrate engagement, thus leading to enhanced γ-secretase processing relative to cells expressing WT NCT. Second, in NCT-deficient cells, expression of a NCT variant harboring an F287P substitution that would restrict rotation of the two lobes would leave the lid in a “closed” conformation, thus suppressing γ-secretase-mediated processing of substrates when compared with cells expressing WT NCT. We now report that in contrast to the proposed models derived from the structural studies, expression of NCT variants that lack the “lid” domain or that harbor an F287P mutation had no discernable enhancement or suppression, respectively, of γ-secretase activity when compared with cells expressing wild type NCT.

Experimental Procedures  

cDNA Constructs—cDNAs encoding human γ-secretase subunits, Myc-tagged APP Swedish variant (APP5Sw), Myc-tagged mouse NΔE (mNΔE), and Myc-tagged full-length Notch

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1 To whom correspondence should be addressed. Tel.: 773-834-9186; E-mail: ssisodia@bsd.uchicago.edu.

2 The abbreviations used are: PS, presenilin; APH-1, anterior pharynx-defective 1; NCT, nicastrin; PEN-2, presenilin enhancer 2; APP, amyloid precursor protein; APP5Sw, APP Swedish variant; Aβ, β-amyloid; ECD, ectodomain; CTF, C-terminal fragment; NTF, N-terminal fragment; NICD, Notch intracellular domain; mNΔE, mouse NΔE; CHAPSO, 3-[3-cholamidopropyl]dimethylammonio]-1-hydroxy-1-propanesulfonic acid.

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were described previously (4, 9). Constructs encoding various mutant nicastrin proteins were generated using PCR-based mutagenesis, C-terminally tagged with a sequence encoding the CT11 tag (4), and verified by sequencing. Human NCT Δ312–340 cDNA construct (3) was kindly provided by Dr. Gang Yu and was transferred to pAG3 vector so that it is under the same promoter for expression and it uses the same tag as other NCT constructs.

Antibodies and Immunoblot Analysis—PS1_VT, MAB5232, rat anti-human PS1 NT, anti-CT11, 9E10, cleaved Notch1 (Val-1744) (D3B8), CTM1, and 4G8 antibodies were described previously (10). Immunoblot analyses were done as described previously (10).

Photoaffinity Labeling of γ-Secretase Components—Membranes from NCT-deficient fibroblasts stably transfected with empty vector, WT NCT, or Δlid NCT were prepared as described earlier. Photoaffinity labeling with JC-8 was performed as described (11).

Coimmunoprecipitation of γ-Secretase Complexes under Native Conditions—NCT-deficient fibroblasts transiently cotransfected with cDNAs encoding human Aph-1L-Myc-His, PS1, and PEN-2-CT11, and cDNAs encoding either wild type nicastrin (WT ANPP) or mutant nicastrin with lid region deletion (Δlid ANPP) were lysed and immunoprecipitated with 1.5 μl of anti-human PS1 antibody. The immunoprecipitated complexes were visualized as described before (4).

Treatment with Cycloheximide—NCT-deficient cells were transiently cotransfected with cDNAs encoding either WT ANPP or Δlid ANPP, and were treated with 20 μg/ml cycloheximide (Sigma) for 1–8 h.

EDTA Induction of Notch Cleavage—NCT-deficient fibroblasts cotransfected with the cDNA encoding 6ΧMyc-tagged full-length Notch, and the cDNAs encoding either WT NCT or Δlid NCT were treated with 10 mM EDTA for 30 min before immunoblot analysis.

Pulse Labeling—NCT-deficient fibroblasts cotransfected with the cDNA encoding APPSwe and the cDNAs encoding either WT NCT, Δlid NCT, or NCT F287P were labeled with 250 μCi of [35S]methionine (PerkinElmer) for 15 min and 4 h, respectively, before full-length APP, APP C-terminal fragments (APP CTFs), Aβ, and P3 were immunoprecipitated and analyzed (10). NCT-deficient fibroblasts co-transfected with the cDNA encoding mouse NΔE and the cDNAs encoding either WT NCT or Δlid NCT were first pulse-labeled with 250 μCi of [35S]methionine for 20 mins, and then they were chased for 30, 60, and 120 min, respectively, before mouse NΔE and intracellular domain (NICD) were immunoprecipitated and analyzed (10).

Results

The 1.95 Å resolution crystal structure of D. purpureum NCT (7), together with the 4.5 Å resolution EM map of human γ-secretase that has been recently refined to 3.4 Å (8, 12), revealed that NCT contains a short loop domain that extends from the small lobe and is positioned above the putative substrate-binding site of the large lobe. In human NCT, this region, termed “lid,” encompasses residues from Ser-137 to Gly-168.

FIGURE 1. Nicastrin carrying entire lid deletion forms a stable, active γ-secretase complex. A, lid amino acid sequence alignment between indicated species with highly conserved residues in bold type. The numbering above the sequence is based on human NCT sequence. B, Δlid NCT rescues presenilin endoproteolysis in NCT-deficient cells. FL, full-length. C, Δlid NCT coimmunoprecipitates with all the other γ-secretase subunits. D, Δlid NCT-containing γ-secretase shows a stability similar to WT γ-secretase in NCT-deficient fibroblasts when treated with 20 μg/ml cycloheximide (CHX) for the duration indicated above the NCT panel. E, photoaffinity labeling of PS1 NT using membrane preparations from NCT-deficient cells stably expressing either WT NCT or Δlid NCT.

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human PS1, PEN-2, APH-1, and WT NCT or ∆lid NCT singly or in combination. In the absence of NCT, expression of PS1 singly or in combination with PEN-2 resulted in very low levels of accumulated full-length PS1 or PEN-2 (Fig. 1B, lane 2), but additional expression of APH-1 led to elevated steady-state levels of both PS1 and PEN-2, and low levels of PS1 fragments generated by endoproteolytic processing of full-length PS1 (Fig. 1B, lane 3). Coexpression of WT NCT together with PEN-2, PS1, and APH-1 led to elevated steady-state levels of all four components, with a significant increase in the levels of PS1 derivatives (Fig. 1B, lane 4), as described previously (6). Importantly, coexpression of ∆lid NCT with the other three components also led to elevated levels of all four components and PS1 derivatives that are the active catalytic entities of the complex (Fig. 1B, lane 5). We then examined the assembly of WT NCT and ∆lid NCT into the γ-secretase complex by transiently expressing these polypeptides together with PEN-2, PS1, and APH-1 in NCT-deficient cells, and then prepared detergent-solubilized lysates under conditions that maintain γ-secretase activity. Lysates were subject to immunoprecipitation with an anti-human PS1 antibody, and we now show that all four γ-secretase components (ANPP) that contain either WT NCT or ∆lid NCT are immunoprecipitated, and to nearly identical levels in both cases (Fig. 1C, lanes 3 and 6, respectively). Furthermore, when the stability of the γ-secretase complex containing ∆lid NCT was assessed by treating cells with the protein synthesis inhibitor cycloheximide, a similar turnover rate was observed between WT complex and ∆lid NCT complex (Fig. 1D, compare lanes 1–6 with lanes 7–12, respectively). These results suggest that ∆lid NCT forms a complex with other γ-secretase components in a fashion similar to WT NCT.

Having established that ∆lid NCT associates with all components of the γ-secretase complex, we then assessed the catalytic activity of the complex containing ∆lid NCT. For these studies, we generated stable pools of NCT-deficient fibroblasts that constitutively express either WT NCT or ∆lid NCT. CHAPSO-solubilized membrane preparations from these stable cell pools were incubated with a photoactivatable γ-secretase inhibitor, termed JC-8 (11), in the presence or absence of the parental transition state inhibitor i-685,458 (11) prior to photoactivation. We observed that JC-8 can be efficiently crosslinked to PS1 NTF in γ-secretase complexes that contain either WT NCT or ∆lid NCT (Fig. 1E, lanes 7 and 11, respectively), and that incubation with the unlabeled i-685,458 compound abolished the crosslinked JC8-PS1-NTF complex in both cases (Fig. 1E, lanes 8 and 12, respectively). Thus, JC-8 crosslinking to PS1-NTF is specific, and more importantly, these results indicate, at least indirectly, that ∆lid NCT is as competent as WT NCT in generating an active γ-secretase complex.

To examine the putative role of the lid in regulating γ-secretase activity, we first aligned the NCT lid sequences from human, mouse, Drosophila, Caenorhabditis elegans, and Dicyostelium, and we identified Pro-141, Trp-164, Asn-165, and Gly-168 to be highly conserved between these evolutionarily divergent organisms (Fig. 1A). We then generated constructs encoding mutant human NCT harboring single (P141A, P141T, W164E, W164A, N165K, N165G, G168D, G168S) or double (P141A/W164E) mutations, as well as ∆lid. Importantly, the structural studies reported by Xie et al. (7) revealed that in the closed conformation, the indole ring of Trp-164 makes several van der Waals contacts to the side chains of Pro-424 and Phe-448 and the aliphatic portion of Gln-420 in the large lobe, interactions that would have to be disrupted to allow substrate binding in a hydrophilic pocket of the large lobe. To assess the activity of the NCT variants with mutations in the “lid” domain, we transiently cotransfected NCT-deficient fibroblasts with cDNAs encoding wild type NCT or the mutant NCT variants together with the cDNA encoding mNΔE, a constitutively activated membrane-bound Notch 1 derivative. We observed that all of the transiently expressed NCT variants were expressed to a similar level and that neither the point mutations nor ∆lid exhibited any effect on NCT maturation (Fig. 2A, NCT panel). In NCT-deficient cells, mNΔE failed to undergo intramembranous γ-secretase-mediated processing to generate the NICD (Fig. 2A, lane 1), as expected (14), whereas expression of WT NCT rescued production of NICD (Fig. 2A, lane 2), and much to our surprise, did all of the tested NCT variants (Fig. 2A, lanes 3–12). Most noteworthy is the finding that expression of the ∆lid NCT polypeptide also rescued γ-secretase activity in a manner indistinguishable from the NCT variants harboring single or double point mutations (Fig. 2A, lane 3). To further validate these results, we transiently co-transfected NCT-deficient fibroblasts with the cDNAs encoding wild type NCT, ∆lid NCT, or two previously reported loss-of-function NCT variants, NCTΔ312–340 and NCT L571P (3, 4), together with the cDNA encoding mNΔE. We show that the expression levels of these latter NCT variants are comparable (Fig. 2B, NCT panel, lanes 2–5), but that oligosaccharide maturation of NCTΔ312–340 and L571P was severely reduced. As expected, neither empty vector nor NCTΔ312–340 and L571P rescued the production of NICD (Fig. 2B, NICD panel, lanes 1, 4, and 5, respectively), whereas WT NCT and ∆lid NCT rescues NICD generation (Fig. 2B, NICD panel, lanes 2 and 3) as shown earlier (Fig. 2A). Having established that expression of ∆lid NCT in NCT-deficient cells leads to intramembranous proteolysis of NΔE, we felt it was essential to extend the analysis to assess the impact on the processing of full-length Notch. For these studies, we transiently cotransfected NCT-deficient cells with the cDNA encoding C-terminally 6× Myc-tagged full-length Notch and the cDNAs encoding either WT NCT or ∆lid NCT. Cells were briefly treated with 10 mM EDTA, a manipulation that depletes calcium and results in shedding of the ectodomain segment and exposure of a site in the residual membrane-tethered fragment that is a substrate for ADAM10 (15). The resulting derivative, termed S2/NEXT, is a substrate for γ-secretase-mediated processing that generates the S3/NICD derivative that is subsequently translocated to the nucleus (15). Similar to the results obtained using mNΔE, expression of WT NCT or ∆lid NCT led to the production of NICD upon EDTA treatment (Fig. 2C, top panel, lanes 4 and 6, respectively). The identity of the NICD fragment was further confirmed with the Notch1744V-neo-epitope-specific antibody, D3B8 (Fig. 2C, middle panel, lanes 4 and 6, respectively). These results suggest that expression of NCT with a deletion of the entire lid region is still capable of activating γ-secretase-mediated processing of Notch.

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Although the latter studies indicate that γ-secretase complexes containing either WT NCT or Δlid NCT did not show significant differences in processing Notch, these steady-state analyses may not accurately reflect the kinetics of the processing reactions. To assess this important issue, we performed [35S]methionine pulse-chase labeling of NCT-deficient fibroblasts that transiently coexpressed Myc-tagged mNΔE together with either WT NCT or Δlid NCT. Duplicate plates of cells were pulse-labeled with [35S]methionine for 20 min (Fig. 2D, lanes 1, 2, 9, and 10) and then chased for 30–120 min (Fig. 2D, lanes 3–8 and 11–16), and detergent-solubilized lysates were subject to immunoprecipitation with anti-Myc-specific 9E10 antibody. These studies reveal that the rate of production of NICD in cells that express WT NCT is not different from that in cells that express Δlid NCT (Fig. 2D, compare lanes 1–8 with lanes 9–16).

Xie et al. (7) claimed that rotation of the large lobe and the small lobe around Phe-287 is “necessary and sufficient” to displace the lid during substrate recruitment, but this conclusion, although plausible, was never formally tested. To address this issue, we expressed a NCT variant harboring an F287P mutation, which would be expected to disrupt the proposed hydro-
phobic interactions between Phe-287 and the surrounding hydrophobic residues while providing conformational rigidity to the rotation. When compared with WT NCT, NCT F287P rescued γ-secretase activity, and to a similar extent (Fig. 2E, lanes 2 and 3, respectively), findings suggesting that the proposed model, in which opening of the “lid” by rotation of the large and small lobes around the Phe-287 pivot allows substrate access to the large lobe, needs revision.

γ-Secretase plays an essential role in catalyzing intramembranous processing of a variety of substrates. Having validated that expression of a panel of NCT lid domain variants can promote γ-secretase processing of Notch (Fig. 2A), we extended the analysis to assess the processing of APP. For these studies, we transiently cotransfected NCT-deficient fibroblasts with cDNAs encoding the mutant human NCT proteins, described above (Fig. 2A), together with a cDNA encoding the APPSwe containing a C-terminal Myc epitope tag. As was shown in Fig. 2A, all of the NCT variants were expressed at similar levels (Fig. 2F, NCT panel), and the levels of full-length APP between samples were comparable (Fig. 2F, APP FL panel). In NCT-deficient fibroblasts expressing APPSwe, we observed the accumulation of multiple APP CTFs (Fig. 2F, lane 1), indicative of the absence of γ-secretase activity. Expression of WT NCT dramatically reduced the levels of the CTFs with retarded migration (Fig. 2F, lane 2), but not the CTFs with accelerated migration (Fig. 2F, lane 2). As the transfection efficiency of fibroblasts is less than 10%, we would conclude that in the small fraction of cells that coexpress WT NCT and Myc-tagged APPSwe, the larger Myc-tagged CTFs derived from APPSwe were subject to processing by γ-secretase, whereas the more rapidly migrating CTFs that are present in all cells must be derived from endogenous APP, wherein γ-secretase activity is absent. Similar to the results in Fig. 2A, the expression of mutant human NCT variants (Fig. 2F, lanes 3–12) led to proteolysis of the APP CTF derived from APPSwe, and to levels similar to that observed by expressing WT NCT, suggesting their active involvement in restoring γ-secretase activity. We further validated these results by expressing the NCTΔ312–340 and NCT L571P variants together with APPSwe. We show that the NCT variants accumulate to comparable levels (Fig. 2G, NCT panel, lanes 2–5), and the expression levels of full-length APP are similar (Fig. 2G, APP FL panel). Consistent with earlier studies (3, 16), expression of NCTΔ312–340 and NCT L571P led to the accumulation of APP CTFs (Fig. 2G, APP CTF panel, lanes 4 and 5), indicative of diminished γ-secretase activity, whereas the levels of APP CTF in cells expressing Δlid NCT and WT NCT were largely reduced, thus indicating that γ-secretase activity was restored (Fig. 2G, APP CTF panel, lanes 2 and 3). Notably, we were unable to detect the products of γ-secretase-mediated processing of APP CTF, including the AICD and Aβ peptides, and this likely reflects inherent limitations in transfection efficiency and rapid turnover of the products. To overcome these issues, we chose a more sensitive assay, one that employed [35S]methionine labeling of NCT-deficient fibroblasts that transiently express Myc-tagged APPSwe together with either WT NCT, Δlid NCT, or NCT F287P. A 15-min pulse labeling showed that nascent full-length APP synthesis is comparable in cells expressing either WT NCT, Δlid NCT, or the NCT pivot variant, F287P (Fig. 2H, lanes 1–4, respectively, and Fig. 2K, lanes 1–4, respectively). Upon longer term labeling, increased levels of mature APP and the presence of APP CTFs are observed (Fig. 2, H and K). However, there is no significant difference in the extent of APP maturation or levels of APP CTFs between cells expressing WT NCT, Δlid NCT, or NCT F287P (Fig. 2H, lanes 5–8, respectively, and Fig. 2K, lanes 5–8, respectively). Importantly, the 4-h labeling period allowed us to detect radiolabeled Aβ and related derivatives in the conditioned medium (Fig. 2, I and L), and again, we failed to detect a difference in the production of these secreted derivatives in cells expressing either WT NCT, Δlid NCT, or NCT F287P (Fig. 2I, lanes 1–4, respectively; quantified in Fig. 2J, and Fig. 2L, lanes 1–4, respectively; quantified in Fig. 2M).

Discussion

The recent description of the atomic structure of γ-secretase at 3.4 Å resolution (8) has provided important new insights into the arrangement and interactions of the individual subunits within the complex, as well as testable predictions pertaining to the function of domains within individual subunits. We found aspects of the bilobar structure of NCT and the role of a short “loop” in the small lobe termed “lid” that appears to cover a substrate entry pocket in the large lobe to be most intriguing. Moreover, it was proposed that substrate access and binding would occur by rotation of the large and small lobes around a central pivot at Phe-287 that results in displacement of the lid domain (7, 8). Using cell-based assays, we have extended the structural studies by examining the proposed role of the lid in mediating γ-secretase function, and we now offer several insights.

First, we demonstrate that NCT that lacks the entire lid domain or a variety of NCT variants containing amino acid substitutions of specific residues proposed to play a role in the association of the “lid” region with the large lobe are as competent as WT NCT in promoting γ-secretase activity when expressed in otherwise catalytically inactive NCT-deficient cells.

Second, we show that the absence of the NCT lid region has no impact on the assembly and stability of the γ-secretase complex in NCT-deficient cells when compared with cells expressing WT NCT. Collectively, these findings suggest that the interaction between the lid and the large lobe is dispensable for stabilizing the γ-secretase complex and that the domain has little, if any, impact on enzymatic activity.

Finally, we tested the proposed model that Phe-287 serves as a pivot for the relative rotation between the large lobe and the small lobe by substituting the phenylalanine with proline to restrict potential conformational rotations, but again, we failed to show that expression of F287P NCT had an impact on γ-secretase activity.

Although we have failed to demonstrate that the “lid” domain and putative pivot that allows rotation of the large and small lobes of NCT have any significant impact on γ-secretase processing, it is conceivable that these moieties play alternative roles that have yet to be defined. In any event, our findings lead to the inescapable conclusion that the proposed model (7, 8), in
which the “lid” region in the small lobe of NCT plays an essential role in substrate engagement, is in need of revision.

**Author Contributions**—X. Z. designed and conducted experiments, analyzed the data, and wrote the manuscript, E. S. and M. S. conducted experiments, X. W. and Y. L. provided assays, and S. S. S. analyzed the data and wrote the manuscript.

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