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

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Understanding of the structure of 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’s 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 assessed the 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 have any significant 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 have 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.
human γ-secretase at 3.4 Å resolution using cryo-EM approaches, and these studies also revealed a bilobar structure for the human NCT ECD (8). 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 137 to 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 occurs by rotation of the large and small lobes around a central pivot at F287 that and binding occurs by rotation of the large and binding occurs by rotation of the large and small lobes around a central pivot at F287 that leads to enhanced γ-secretase activity compared with WT NCT (4). Immunoblot analyses were done as described previously (10).

**Antibodies and Immunoblot Analysis—** PS1NT, MAB5232, Rat anti-human PS1 NTF, Anti-CT11, 9E10, Cleaved Notch1 (Val1744) (D3B8), CTM1, and 4G8 antibodies were describe 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-1αL-Myc-His, PS1, 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 of cycloheximide (Sigma) for 1 to 8 hours.

**EDTA Induction of Notch Cleavage—** NCT-deficient fibroblasts cotransfected with the cDNA encoding 6 x Myc-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 cDNA encoding APPSwe and cDNAs encoding either WT NCT, Δlid NCT or NCT F287P were labeled with 250 µCi of 35S-methionine (PerkinElmer, Waltham, MA) for 15 minutes and four hours, respectively, before full-length APP, APP CT11s, AB and P3 were immunoprecipitated and analyzed (10). NCT-deficient fibroblasts co-transfected with cDNA encoding mouse NΔE and 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 min, 60 min and 120 min, respectively, before mouse NΔE and NICD were immunoprecipitated and analyzed (10).

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs—** The cDNAs encoding human γ-secretase subunits, myc-tagged APPSwe, myc-tagged mNΔE and myc-tagged full length Notch 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.
RESULTS

The 1.95 Å resolution crystal structure of \textit{Dictyostelium 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 which 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 S137 to G168 (Figure 1A) and it has been suggested that displacement of the lid is essential for substrate access into a hydrophilic pocket. As it is well-established that NCT is required for assembly of a mature γ-secretase complex (6,13), we first asked whether expression of WT NCT or NCT lacking the lid (Δlid NCT) affects γ-secretase complex assembly. For these studies, we transiently cotransfected \textit{NCT}-deficient fibroblasts with cDNAs encoding human PS1, PEN-2, APH-1, and WT NCT or Δlid 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 (Figure 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 (Figure 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 (Figure 1B, lane 4), as previously described (6). Importantly, coexpression of Δlid 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 (Figure 1B, lane 5). We then examined the assembly of WT NCT and Δlid NCT into the γ-secretase complex by transiently expressing these polypeptides together with PEN-2, PS1 and APH-1 in \textit{NCT}-deficient cells, 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 Δlid NCT are immunoprecipitated, and to nearly identical levels in both cases (Figure 1C, lanes 3 and 6, respectively). Furthermore, when the stability of the γ-secretase complex containing Δlid NCT was assessed by treating cells with protein synthesis inhibitor cycloheximide, a similar turnover rate was observed between WT complex and Δlid NCT complex (Figure 1D, compare lanes 1-6 to lanes 7-12, respectively). These results suggest that Δlid NCT forms a complex with other γ-secretase components in a fashion similar to WT NCT.

Having established that Δlid NCT associates with all components of the γ-secretase complex, we then assessed the catalytic activity of the complex containing Δlid NCT. For these studies, we generated stable pools of \textit{NCT}-deficient fibroblasts that constitutively express either WT NCT or Δlid 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 L685, 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 Δlid NCT (Figure 1E, lanes 7 and 11, respectively), and that incubation with the unlabeled L685, 458 compound abolished the crosslinked JC8/PS1-NTF complex in both cases (Figure 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 Δlid NCT is as competent as WT NCT in generating an active γ-secretase complex.

In order to examine the putative role of the lid in regulating γ-secretase activity, we first aligned the NCT lid sequences from human, mouse, \textit{Drosophila}, \textit{C. elegans} and \textit{Dictyostelium}, and we identified P141, W164, N165 and G168 to be highly conserved between these evolutionally divergent organisms (Figure 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 Δlid. Importantly, the structural studies reported by Xie et al. (7) revealed that in the closed conformation, the indole ring of W164 makes several van der Waals contacts to the side chains of P424 and F448 and the aliphatic portion of Q420 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 cDNA encoding mouse N\(\Delta\)E (mN\(\Delta\)E), 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 \(\Delta\)lid exhibited any effect on NCT maturation (Figure 2A, NCT panel). In NCT-deficient cells, mN\(\Delta\)E failed to undergo intramembranous \(\gamma\)-secretase-mediated processing to generate the Notch Intracellular Domain (NICD) (Figure 2A, lane 1), as expected (14), while expression of WT NCT rescued production of NICD (Figure 2A, lane 2), and much to our surprise, so did all of the tested NCT variants (Figure 2A, lanes 3-12). Most noteworthy is the finding that the expression levels of these latter NCT variants are comparable (Figure 2B, NCT panel, lanes 2 and 3) as shown earlier (Figure 2A). Having established that expression of \(\Delta\)lid NCT in NCT-deficient cells leads to intramembranous proteolysis of N\(\Delta\)E, 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 cDNA encoding carboxy-terminally 6 x Myc-tagged full-length Notch and cDNAs encoding either WT NCT or \(\Delta\)lid NCT. Cells were briefly treated with 10 mM of 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 \(\gamma\)-secretase-mediated processing that generates the S3/NICD derivative that is subsequently translocated to the nucleus (15). Similar to the results obtained using mN\(\Delta\)E, expression of WT NCT or \(\Delta\)lid NCT led to the production of NICD upon EDTA treatment (Figure 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 (Figure 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 \(\gamma\)-secretase-mediated processing of Notch.

While the latter studies indicate that \(\gamma\)-secretase complexes containing either WT NCT or \(\Delta\)lid NCT did not show significant differences in processing Notch, these steady-state analyses may not accurately reflect the kinetics of the processing reactions. In order to assess this important issue, we performed \(^{35}\)S-methionine pulse-chase labeling of NCT-deficient fibroblasts that transiently coexpress myc-tagged mN\(\Delta\)E together with either WT NCT or \(\Delta\)lid NCT. Duplicate plates of cells were pulse-labeled with \(^{35}\)S-methionine for 20 min (Figure 2D, lanes 1, 2 and 9, 10) and then chased for 30 to 120 min (Figure 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 \(\Delta\)lid NCT (Figure 2D, compare lanes 1-8 with lanes 9-16).

Xie et al. claimed that rotation of the large lobe and the small lobe around F287 is “necessary and sufficient” to displace the lid during substrate recruitment (7), but this conclusion, while 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 hydrophobic interactions between F287 and the surrounding hydrophobic residues while providing conformational rigidity to the rotation. When compared with WT NCT, NCT F287P rescued \(\gamma\)-secretase activity, and to a similar extent
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(Figure 2E, lanes 2 and 3, respectively), findings which suggest that proposed model that opening of the “lid” by rotation of the large and small lobes around the F287 pivot that 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 (Figure 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 (Figure 2A), together with cDNA encoding the APP Swedish variant (APPSwe) containing a C-terminal myc epitope tag. As was shown in Figure 2A, all of the NCT variants were expressed at similar levels (Figure 2F, NCT panel), and the levels of full-length APP between samples was comparable (Figure 2F, APP FL panel). In NCT-deficient fibroblasts expressing APPSwe, we observed the accumulation of multiple APP carboxyl-terminal fragments (APP CTF) (Figure 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 (Figure 2F, lane 2), but not the CTFs with accelerated migration (Figure 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 was subject to processing by γ-secretase, while 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 Figure 2A, the expression of mutant human NCT variants (Figure 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 (Figure 2G, NCT panel, lanes 2 to 5), and the expression levels of full-length APP are similar (Figure 2G, APP FL panel). Consistent with earlier studies (3,16), expression of NCTΔ312-340 and NCT L571P led to the accumulation of APP CTFs (Figure 2G, APP CTF panel, lanes 4 and 5), indicative of diminished γ-secretase activity, while the levels of APP CTF in cells expressing Δlid NCT and WT NCT were largely reduced, thus indicating that γ-secretase activity was restored (Figure 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 APP Intracellular Domain (AICD) and Aβ peptides, and 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 minute 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 (Figure 2H, lanes 1, 2 and 3, 4, respectively and Figure 2K, lanes 1, 2 and 3, 4, respectively). Upon longer term labeling, we observe increased levels of mature APP and the presence of APP-CTFs (Figure 2H and Figure 2K). 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 (Figure 2H, lanes 5, 6 and 7, 8, respectively and Figure 2K, lanes 5, 6 and 7, 8, respectively). Importantly, the 4 hour labeling period allowed us to detect radiolabeled Aβ and related derivatives in the conditioned medium (Figure 2I and Figure 2L), 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 (Figure 2I, lanes 1, 2 and 3, 4, respectively; quantified in Figure 2J, and Figure 2L, lanes 1, 2 and 3, 4, respectively; quantified in Figure 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, and testable predictions pertaining to the function of domains within individual subunits. We found most intriguing, 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. Moreover, it was proposed that substrate access and binding would occur by rotation of the large and small lobes around a central pivot at F287 that results in displacement of the lid domain (7,8). Using cell based assays, we have extended the structural studies by examine the proposed role of the lid in mediating γ-secretase function, and 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 is 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 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 F287 serves as a pivot for the relative rotation between the large lobe and the small lobe by substituting the phenylalanine with proline in order to restrict potential conformational rotations, but again, failed to show that expression of F287P NCT had an impact on γ-secretase activity.

While 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) that the “lid” region in the small lobe of NCT plays an essential role in substrate engagement is in need of revision.

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The Authors declare no conflicts of interests.

Author contributions—XZ designed and conducted experiments, analyzed the data and wrote the manuscript, ES and MS conducted experiments, XW and YL provided assays, SSS analyzed the data and wrote the manuscript.

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FIGURE LEGENDS

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, and the numbering above the sequence is based on human NCT sequence. *B*, Δlid NCT rescues presenilin endoproteolysis in NCT-deficient cells. *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 for the duration indicated above the NCT panel. *E*, Photoaffinity labeling of PS1 NTF using membrane preparations from NCT-deficient cells stably expressing either WT NCT or Δlid NCT.

FIGURE 2. Nicastrin carrying lid mutation and deletion or carrying pivot mutation rescues mNΔE and APP processing in NCT-deficient fibroblasts. *A*, NCT carrying lid mutation or entire lid deletion rescues mNΔE processing in NCT-deficient fibroblasts, both mNΔE and the NICD are indicated by
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arrows. B, Δlid NCT rescues mNΔE processing in NCT-deficient fibroblasts compared with negative controls NCT Δ312-340 and NCT L571P. C, Δlid NCT rescues full-length Notch processing upon EDTA treatment in NCT-deficient fibroblasts, the top panel was probed with 9E10 antibody, while the middle panel was probed with D3B8 antibody. D, Pulse-chase labeling of NCT-deficient fibroblasts transiently coexpressing mNΔE and either WT NCT or Δlid NCT shows a similar level of NICD production in these cells, mNΔE and NICD are indicated by arrows. E, NCT pivot mutant, F287P, rescues mNΔE processing in NCT-deficient fibroblasts. F, NCT carrying lid mutation or entire lid deletion rescues APP processing in NCT-deficient fibroblasts as indicated by reduced APP CTF levels. G, Δlid NCT rescues APP processing in NCT-deficient fibroblasts compared with negative controls NCT Δ312-340 and NCT L571P. H-J, Pulse labeling of NCT-deficient fibroblasts transiently coexpressing APPSwe and either WT NCT or Δlid NCT shows similar levels of APP CTF and Aβ and P3 production (in J, Aβ data are normalized to full-length APP and are represented by mean±sem, n=2). K-M, Pulse labeling of NCT-deficient fibroblasts transiently coexpressing APPSwe and either WT NCT or NCT F287P shows similar levels of APP CTF and Aβ and P3 production (in M, Aβ data are normalized to full-length APP and are represented by mean±sem, n=2).
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