Mutations in Nicastrin Protein Differentially Affect Amyloid β-Peptide Production and Notch Protein Processing

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Annelie Pamrén‡, Johanna Wanngren‡, Lars O. Tjernberg‡, Bengt Winblad§, Ratan Bhat§, Jan Näslund§, and Helena Karlström‡1
From the 1Department of Neurobiology, Caring Sciences and Society, Karolinska Institutet (KI)-Alzheimer Disease Research Center, Karolinska Institutet, Novum, SE-141 86 Stockholm and 2AstraZeneca CNS/Pain Research Area, SE-151 85 Södertälje, Sweden

The γ-secretase complex is responsible for intramembrane processing of over 60 substrates and is involved in Notch signaling as well as in the generation of the amyloid β-peptide (Aβ). Aggregated forms of Aβ have a pathogenic role in Alzheimer disease and, thus, reducing the Aβ levels by inhibiting γ-secretase is a possible treatment strategy for Alzheimer disease. Regrettably, clinical trials have shown that inhibition of γ-secretase results in Notch-related side effects. Therefore, it is of great importance to find ways to inhibit amyloid precursor protein (APP) processing without disturbing vital signaling pathways such as Notch. Nicastrin (Nct) is part of the γ-secretase complex and has been proposed to be involved in substrate recognition and selection. We have investigated how the four evenly spaced and conserved cysteine residues in the Nct ectodomain affect APP and Notch processing. We mutated these cysteines to serines and analyzed them in cells lacking endogenous Nct. We found that two mutants, C213S (C2) and C230S (C3), differentially affected APP and Notch processing. Both the formation of Aβ and the intracellular domain of amyloid precursor protein (AICD) were reduced, whereas the production of Notch intracellular domain (NICD) was maintained on a high level, although C230S (C3) showed impaired complex assembly. Our data demonstrate that single residues in a γ-secretase component besides presenilin are able to differentially affect APP and Notch processing.

The γ-secretase complex is an intramembrane protease that processes many type I transmembrane proteins, among them the amyloid precursor protein (APP)2 and Notch receptors (1). In the amyloidogenic pathway, processing of APP by the γ-secretase complex generates an intracellular domain (AICD) and the amyloid β-peptide (Aβ) (2). The latter constitute the main component in senile plaques in Alzheimer disease brains (3). Much attention has lately been focused on investigating how γ-secretase selects and processes the different substrates. This is important for therapeutic approaches aiming to selectively lower Aβ generation without affecting other vital functions of γ-secretase. The γ-secretase complex is composed of four members: presenilins (PS1 or PS2), nicastrin (Nct), Pen-2, and Aph-1 (Aph-1a or Aph-1b) (4–7). Although the presenilins are responsible for the catalytic cleavage of the substrates (5), less is known about the function of the other components.

Nct, a type I transmembrane protein itself, contains a region in its ectodomain that shows similarity to aminopeptidases and the transferrin receptor superfamily (8). This spawned the idea that Nct could act as a receptor. In 2005, Shah et al. (9, 10) reported that Nct physically interacts with APP- and Notch- derived γ-secretase substrates through a glutamate residue at position 333 in the so-called DAP (DYIGS and peptidase homologous region) domain of the Nct ectodomain (see Fig. 1A). This proposed substrate receptor-like role for Nct has lately been challenged by De Strooper and colleagues. They showed in vivo as well as in vitro that the mutation of glutamate 333 (mouse 332) instead was important for the maturation and assembly of the γ-secretase complex (11). Moreover, another member of the GXXGD-type aspartyl protease family, SPPL2b, possesses intrinsic enzyme activity without additional co-factors and shows similar substrate requirements as γ-secretase (i.e. length of the substrate ectodomain) (12), indicating that substrate selection may not depend on Nct. Thus, it remains unclear whether Nct is involved in substrate selectivity or has a more general role in the stabilization and maturation of the γ-secretase complex. Alignment of human, mouse, Drosophila, and Caenorhabditis elegans Nct sequences reveals four evenly spaced cysteines at positions 195, 213, 230, and 248. These residues are located in the extracellular region of Nct, close to the DAP domain, and the functional significance of these residues is not yet clear (4). Cysteine residues are in general involved in protein conformation and interactions, often via disulfide bonds and metal ions. Therefore, we wanted to further explore the role of these four conserved cysteines. To gain more insight, we mutated these residues and analyzed the Nct variants for their function in Nct-deficient mouse embryonic fibroblasts (MEF).
Nct Differentially Affects APP and Notch Processing

EXPERIMENTAL PROCEDURES

cDNA and Constructs—Wild type Nct, C-terminally tagged with a V5-epitope, was cloned into the pdcDNA5FRT/T0 vector (Invitrogen) on BamHI/NotI sites. cDNAs encoding Nct mutants C195S (C1), C213S (C2), C230S (C3), C248S (C4), and the combination C195S/C213S/C230S/C248S (C1–4) were generated by QuikChange multisite-directed mutagenesis (Stratagene). Del2 is a deletion mutant described previously (13) that spans over all four cysteines. The E333A construct has been described elsewhere (9). The wild type (WT) APP in pcDNA3 was cloned into the previously described pENTR2B vector (14) on NotI/EcoRV sites and then transferred to the pCAG-IRESS-Puro vector using the Gateway cloning technology (Invitrogen). The BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used to verify all DNA constructs. The reporter constructs, MH100, CMV-E-GVP, and C99-GVP, used in the luciferase-based reporter gene assay have been described previously (15).

Cell Culture and Generation of Stable Cell Lines—Nct#−/− MEF cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) (16). Flp-In™ cell lines were generated by transfection with the Flp-In target site vector pFRT/lacZeo and selected with 500 μg/ml Zeocin (Invitrogen). Transfected clones were screened by Southern blot and a β-galactosidase assay to identify the clone with the minimum of FRT integrated DNA. All clones were screened by Southern blot and a β-galactosidase activity was determined to adjust for transfection efficiency. Experiments were repeated 4–5 times.

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Co-immunoprecipitation—Co-immunoprecipitation experiments were carried out on Nct#−/− MEF cells transiently transfected with WT Nct, C1, C2, C3, or C4, together with CMV-β-gal and GFP. Cells were lysed in fresh medium containing either 1 μM L-685,458 or 1 μM vehicle (dimethyl sulfoxide (DMSO)) for 24 h before analysis of the conditioned medium as described previously (19). The corresponding concentration of the Aβ40-peptide in the samples was calculated using the Aβ-peptide standard curve. Next, β-galactosidase activity was determined to adjust for transfection efficiencies. Experiments were repeated 4–5 times.

Co-immunoprecipitation—Co-immunoprecipitation experiments were carried out on Nct#−/− MEF cells transiently transfected with WT Nct, C1, C2, C3, C4, C1–4, or Del2. Cells were lysed in co-immunoprecipitation buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% CHAPSO and Complete protease inhibitor mixture and thereafter briefly sonicated and subjected to ultracentrifugation (100,000 × g, 20 min). The supernatants were precleared with a mixture of protein G-Sepharose (GE Healthcare) and Complete protease inhibitor mixture and thereafter briefly sonicated and subjected to ultracentrifugation (100,000 × g, 20 min). The supernatants were precleared with a mixture of protein G-Sepharose (GE Healthcare) and Complete protease inhibitor mixture and thereafter briefly sonicated and subjected to ultracentrifugation (100,000 × g, 20 min). The supernatants were precleared with a mixture of protein G-Sepharose (GE Healthcare) and Complete protease inhibitor mixture and thereafter briefly sonicated and subjected to ultracentrifugation (100,000 × g, 20 min).

Affinity Capture of γ-Secretase Using GCB—To investigate the amount of active γ-secretase complexes in the cell lines, the complexes were pulled down with a γ-secretase inhibitor (L-685,458) coupled to biotin via a cleavable linker (GCB). This method has previously been characterized and described by Teranishi et al. (20). Membrane preparations of Nct#−/− MEF cells stably transfected with WT Nct, C1, C2, C3, or C4 were carried out as described earlier (21). The membrane preparations were resuspended in buffer H (20 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM EDTA) containing 0.5% CHAPSO and Complete protease inhibitor mixture. Endogenously biotinylated proteins were removed by magnetic streptavidin beads (Invitrogen), and samples were incubated with 200 nM GCB for 10

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min at 37 °C. As a negative control, the samples were incubated with 10 µM L-685,458 for 3 min at 37 °C prior to the addition of GCB. Magnetic streptavidin beads were added, and samples were incubated on rotation overnight at 4 °C. The beads were washed, and bound proteins were eluted with Laemmli sample buffer at room temperature for 20 min and subjected to SDS-PAGE and Western blotting.

Cycloheximide Treatment—Clone mixes of WT Nct, C1, C2, C3, and C4 in WT APP Nct−/− MEF were exposed to 50 µg/ml cycloheximide for 0, 0.5, 1, 2, 4, 6, and 8 h before being lysed in whole cell extraction buffer (20 mM HEPES, pH 7.8, 0.42 mM NaCl, 0.5% Nonidet P-40, 25% glycerol, 0.2 mM EDTA, 1.5 mM MgCl₂, 1 mM DTT) supplemented with Complete protease inhibitor mixture for 30 min at 4 °C (18). Forty-five µg of protein for each construct from the various time points were separated by SDS-PAGE and analyzed by immunoblotting using the α-V5 antibody. The expression was quantified by CCD camera, and GAPDH was used as loading control. The experiment was repeated 3–4 times.

RESULTS

Cysteine Residues in the Nct Ectodomain Differentially Affect γ-Secretase Processing of APP and Notch—To investigate the importance of the evenly spaced cysteines in Nct for γ-secretase activity, we replaced the cysteines with serines by mutagenesis (Fig. 1A). The cDNA constructs were transiently transfected into Nct−/− MEF cells, and the γ-secretase activity was monitored with the luciferase reporter gene assay (Fig. 1B). The single mutant C4, as well as the mutants C1–4 and Del2, did not mediate any γ-secretase activity. Del2 lacks a large region that covers all four cysteines including C4, which previously has been shown to be necessary for activity. The other single mutants C1, C2, and C3 showed no significant difference in Notch-cleaving activity as compared with wild type. Interestingly, there was a notable and significant difference between Notch and APP processing at the S3-cleavage site as compared with wild type. This difference was confirmed by Western blotting (C2; 0.9 versus 0.8 and C3; 0.8 versus 0.4 as compared with wild type, which was set to 1). When we normalized the AICD and NICD production to PS1-NTF formation, we observed that the intrinsic activity for C3 was reduced on APP as compared with Notch (0.6 versus 1.9), indicating that the C3-containing γ-secretase complex is not able to process APP as well as Notch (Fig. 1C). The mutant E333A generated ~10% of the wild type activity on both substrates (Fig. 1B) but had lower intrinsic activity on both APP and Notch (0.4 versus 0.4), which is in line with previous studies (9, 10) (Fig. 1C).

The Cysteines Affect γ-Site and e-Site Processing of APP Equally—To generate Aβ peptides, γ-secretase cleavage at the e-site is suggested to occur before γ-site cleavage (22). To explore whether the cysteine mutants had the same effect on APP processing at the γ-site as the e-site, an MSD sandwich immunoassay (Meso Scale Discovery) for quantification of secreted Aβ40 was performed. The cysteine mutants followed the same trend for Aβ40 generation (71% for C1, 61% for C2, and 57% for C3) as for AICD production (Fig. 1D), indicating that the cysteine residues affect both e-sites and γ-sites to the same extent.

C3 Gives Rise to Fewer Active γ-Secretase Complexes—Active γ-secretase requires four components: PS, Nct, Pen-2, and Aph-1 (23). A decrease in activity might therefore be due to reduced complex formation. Co-immunoprecipitation of Nct with the other γ-secretase components was performed to investigate whether interaction between the components was influenced. The mutants C1, C2, and C3 could all interact with the other components, whereas C4 failed to interact with any other component than Aph-1 (Fig. 2A), which has been shown previously (24) and explains its lack of activity on both substrates. To explore whether the complexes were active, a GCB pulldown was used. Interestingly, although C1 and C2 showed equal amount of active complexes as WT, there were clearly fewer active C3-containing complexes (Fig. 2B). The fact that Western blot analysis revealed that the C1 and the C2 mutants could be detected as mature and immature forms, whereas C3 showed only low levels of the mature form, supports this result (Fig. 2C).

Cysteine Mutations Do Not Affect the Stability of Nct—To address whether the low amount of active C3-containing complexes and its reduced γ-secretase processing of APP were due to impaired protein stability, we cycloheximide-treated cells. Stable transfected clone mixes of all four single cysteine mutants were treated with cycloheximide (50 µg/ml) between 0 and 8 h at six different time points. Interestingly, all four cysteine mutants were as stable as WT Nct when quantifying total Nct on Western blots between 0 and 8 h (Fig. 2D). Hence, the reduction in APP processing and the failure in complex assembly for C4 as well as less active C3-containing complexes are not due to protein instability.

DISCUSSION

Our results show that the Nct mutants C1, C2, and C3 can form γ-secretase complexes with sustained activity in terms of Notch processing, although the amounts of active complexes were lower in the case of C3. However, in terms of APP processing, the mutants C2 and C3 demonstrated a significant reduction as compared with the wild type molecule. We observed that the Aβ40 generation and the AICD production followed the same trend, suggesting that the Nct mutants affect the e- and the γ-site to the same extent. Chen et al. (25) showed that region 312–369 of Nct strongly modulated γ-secretase cleavage of APP, whereas the S3-cleavage of Notch was unaffected. This is consistent with our result, indicating that there are Nct-dependent differences in substrate processing by γ-secretase.

A recent study showed that Nct is S-palmitoylated at Cys-689 in lipid rafts (26). This post-translational modification is not important for γ-secretase assembly or intramembrane processing of APP, Notch, or N-cadherin. The protein stability was nevertheless affected when mutating Cys-689 to a serine residue. However, we did not observe any changes in protein stability with our cysteine mutants, suggesting that instability is not the reason for the reduced or impaired APP processing by C2, C3, and C4. We suggest that C4 is important for essential interactions required for complex assembly or protein folding and subsequently the maturation process. Another explanation
for differential processing of APP and Notch by C2 and C3 could be differences in subcellular localization between the cysteine mutants. Recently, Morais et al. (27) reported that APP processing indeed can be influenced by targeting Nct to different subcellular compartments. Furthermore, glycosylation of Nct has previously been demonstrated not to be essential for γ-secretase assembly, cell surface expression, or γ-secretase activity (13, 28).
The findings that C3, which shows a high activity on Notch processing, only presented a low level of active \( \gamma \)-secretase complexes as well as a lower amount of the mature Nct form, suggest that the remaining \( \gamma \)-secretase activity is sufficient to process Notch but not APP to the same extent. Our intrinsic \( \gamma \)-secretase activity data for C3 support this theory because the activity was lower for APP than for Notch. This is in line with the study from Chen et al. (25), where the deletion mutants 312–340 and 312–369 only had minor effects on NICD production despite their lack of mature Nct. C3 might also change the conformation of Nct and subsequently the whole \( \gamma \)-secretase complex so that the immature form could be part of the active complex and favor processing of Notch rather than of APP.

In conclusion, we present evidence that the evenly spaced cysteine residues in the Nct ectodomain affect APP and Notch processing differentially. This is to our knowledge the first study describing single residues in a \( \gamma \)-secretase component besides presenilin that differentially affect the cleavage of substrates (29–31). APP seems to be more dependent on the presence of a mature form of Nct for proper processing, but whether Nct is directly involved in substrate recognition or has a more indirect role such as gating the substrate into the catalytic site or stabilizing the complex is still not clear. A recent
study by Futai et al. (32) demonstrated that Nct is dispensable for γ-secretase activity with the PS1 mutant F411Y/S438P. These mutations are suggested to stabilize the complex and this, indirectly, suggests a stabilizing role for Nct. However, because modifications of Nct cause differences in APP and Notch processing, our results may favor the substrate recognition role rather than Nct being just a stabilizing factor.

Current γ-secretase inhibitors have failed in clinical trials because Notch signaling interference has led to, for instance, gastrointestinal side effects (33). Our findings could have an impact on how to generate molecules that specifically target γ-secretase without unwanted side effects.

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