Complex N-linked Glycosylated Nicastrin Associates with Active γ-Secretase and Undergoes Tight Cellular Regulation*

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The intramembranous proteolysis of Notch and the amyloid precursor protein by γ-secretase exemplifies an unusual and newly recognized mechanism of signal transduction in multicellular organisms. Here, we show that only a form of nicastrin (NCT) containing N-linked complex oligosaccharides is present in active γ-secretase complexes. Overexpression of NCT does not generate more of this mature protein, a phenomenon analogous to the strictly regulated formation of mature presenilin heterodimers from immature holoprotein. The absence of presenilin severely limits the maturation of NCT, yet combined overexpression of both proteins does not increase respective mature types. Taken together, our findings describe unusual regulatory features of this key signaling protease: the association of NCT with γ-secretase is tightly regulated via glycosylation; at least one other cofactor exists; the least abundant member of the complex becomes limiting; and the cofactor that serves this role may vary by cell type.

γ-Secretase is a unique aspartyl protease whose active site appears to reside within the membrane (1). This elusive enzyme first garnered attention because it generates the amyloid-β protein Aβ, a ~42-residue peptide that accumulates to high abundance in the limbic and association cortices in Alzheimer’s disease (2). To generate Aβ, γ-secretase cleaves the amyloid β-protein precursor (APP),1 a type I glycoprotein that may serve as a cell surface receptor (3). A second substrate of γ-secretase is another cell surface receptor, Notch, which is critically required for various cell fate decisions during embryogenesis (4). Notch transduces its signal via the proteolytic release of the Notch intracellular domain by a γ-secretase-mediated intramembranous scission (5, 6). Additional single transmembrane substrates have been identified (7), suggesting that the normal function of γ-secretase is to serve as a signaling hub (8).

Despite significant progress in the characterization of its function in normal biology and disease, the identity of γ-secretase has not been definitively established. Nevertheless, there is substantial evidence that the presenilin 1 and 2 proteins (PS1 and PS2) constitute the active site of the protease (9). Genetic inactivation of PS1 or its orthologues in C. elegans, Drosophila melanogaster, and Mus musculus results in the disruption of γ-secretase-mediated processing of Notch and/or APP (6, 10–13). Mutation of either of two intramembrane aspartates that are conserved in all presenilins similarly interferes with γ-secretase activity (9, 14, 15), and aspartyl protease transition-state analogue inhibitors bind directly to PS1 and PS2 (16, 17). The latter data strongly implicate PS as a central component of γ-secretase but not necessarily the only one. The presenilins are themselves endoproteolytically cleaved into N- and C-terminal fragments (NTF and CTF, respectively), and these fragments appear to be the active form of the molecule (18). Yet overexpression of PS neither generates more fragments nor increases γ-secretase activity, suggesting that PS NTF and CTF levels are tightly regulated by unknown limiting cofactors (19).

Recently, Yu et al. (20) identified a single pass transmembrane protein via its association with PS1 and called it nicastrin (NCT). These authors suggested that NCT is a functional component of γ-secretase along with presenilin. Indeed, when the NCT gene was subsequently ablated in C. elegans or D. melanogaster, Notch function was blocked in a manner indistinguishable from ablation of PS (20–23). Furthermore, upon partial purification of γ-secretase on an inhibitor affinity matrix, PS NTF, PS CTF, and NCT all co-eluted with γ-secretase activity (24). Together, these data argue that both the PS NTF/CTF heterodimer and NCT are required components of γ-secretase.

However, there has been some discrepancy regarding the molecular size of NCT. Transient and stable overexpression initially suggested that NCT is primarily a ~110-kDa glycoprotein (20). However, our partial purification of γ-secretase identified an NCT form that migrated at ~150 kDa (24). In an effort to resolve this apparent discrepancy, we have identified and analyzed several cell lines in which NCT is glycosylated to varying extents. In agreement with recently published work by Leem et al. (25) and Tomita et al. (26), only the most glycosylated, ~150 kDa form of NCT (which contains complex and high-mannose N-linked oligosaccharides) co-immunoprecipitated with PS heterodimers from cells. Furthermore, we found that this mature NCT form was located in the same subcellular vesicle fractions that contain PS1 NTF and CTF. When we used a potent γ-secretase inhibitor that we designed as an activity-
dependent affinity matrix, only the fully glycosylated (~150 kDa) form of NCT (along with PS heterodimers) was specifically bound to it. Surprisingly, we found that NCT overexpression did not generate increased amounts of mature protein, a result reminiscent of the strict limit on cellular PS NTF/CTF levels observed when PS holoprotein is overexpressed. Further, we show that the absence of PS1 limits NCT maturation, yet overexpression of PS1 does not lead to increased levels of mature NCT. Therefore, our findings indicate that the full glycosylation of NCT is tightly regulated in a manner analogous to the “replacement phenomenon” described for PS (19). Moreover, the full glycosylation of NCT correlates with active $\gamma$-secretase and depends on the presence of PS1. Our data also allow us to conclude that the assembly and maturation of $\gamma$-secretase is dependent on the association of PS and NCT with at least one other cofactor.

EXPERIMENTAL PROCEDURES

Preparation of Cell Lysates—Cell lysates were prepared essentially as described (29). Briefly, cells were grown in suspension (Jurkat and HeLa) or on plates (human embryonic kidney (HEK) and Chinese hamster ovary (CHO) and then collected and resuspended in MES buffer (50 mM MES, pH 6.0, 5 mM MgCl$_2$, 5 mM CaCl$_2$, and 150 mM NaCl) with complete protease inhibitors (Roche Molecular Biochemicals). Cells were broken by one passage through a French press above 1000 p.s.i. Nuclei and cell debris was pelleted for 10 min at 3000 $\times g$ and discarded. The postnuclear supernatant was pelleted at 100,000 $\times g$ for 1 h. This membrane pellet was washed in 0.1 M sodium carbonate (pH 11.3) to remove non-integral membrane proteins and then solubilized in 1% CHAPSO in MES buffer and diluted 4-fold in HEPS buffer (50 mM HEPES pH 7.0, 5 mM MgCl$_2$, 5 mM CaCl$_2$, and 150 mM NaCl). All lysates contained ~0.2 mg/ml protein, as measured by the BCA protein assay (Pierce).

Deglycosylation—For the initial analysis of glycosylated NCT species, the GlycoPro deglycosylation kit (Prozyme) was used. For deglycosylation with endoglycosidase H, Endo H (Prozyme) was used. All experiments were performed under SDS denaturing conditions according to the manufacturer’s instructions.

Subcellular Fractionation—Discontinuous iodixanol gradient fractionation was performed as described (34). Briefly, five 15-cm plates of CHO cells stably expressing APF895 with the Swedish mutation were broken by 10 strokes of a Dounce homogenizer. After four passages through a 25-gauge needle, the unbroken cells and nuclei were pelleted at 3000 $\times g$ for 10 min. The postnuclear supernatant was pelleted at 65,000 $\times g$ for 1 h, resuspended, and overlaid on a 2.5–30% discontinuous iodixanol gradient. Each 1-ml fraction was probed for the indicated proteins by direct Western blot. To verify individual runs, the fractions were probed with calnexin antibody (1:1000, StressGen) and synaptogamin 5 (1:1000, StressGen).

Pulse-chase Analysis—For each of the indicated conditions, a 10-cm dish of CHO cells was starved in media lacking methionine and cysteine for 30 min. Cells were pulsed for 30 min with 200 $\mu$Ci/ml of [35S]methionine (Express protein labeling mix, PerkinElmer Life Sciences). Cells were chased with cold medium for the indicated times and lysed in 1% Nonidet P-40. Immune precipitation was performed with 1:500 of antibody R302. IPs were washed three times in Nonidet P-40 lysis buffer and then run on an 8% Tris-glycine gel, fixed in 50% methanol with 20% acetic acid, dried, and exposed to a phosphorimager cassette.

Precipitation and Western Blotting—The III-31C resin used to precipitate active $\gamma$-secretase has been described (24). The cell lysates were adjusted to 1% CHAPSO and incubated with III-31C resin for 2 h at room temperature with gentle rocking. The resin was washed twice with several resin volumes of buffer (1% CHAPSO in HEPES buffer). The bound proteins were then released with 2× Laemmli sample buffer (4% SDS, 50 mM Tris, pH 7.2, 20% glycerol, and 10% $\beta$-mercaptoethanol). For PS1 NTF co-immunoprecipitations, anti-serum X81 (1:100, raised to the first 81 residues of the PS1 NTF) was added to lysates adjusted to 1% CHAPSO. Co-IPs were washed three times in 1% CHAPSO in HEPES buffer, and bound proteins were released with 2× Laemmli sample buffer. For III-31C or X81 precipitations, the samples were run on 8–16% Tris-glycine PAGE gels, transferred to polyvinylidene difluoride, and probed with R302 (for nicastrin, 1:4000), Ab14 (for PS1 NTF, 1:2000), and 13A11 (for PS1 CTF, 5 $\mu$g/ml) or guinea pig anti-nicastrin (1:2000, Chemicon), goat N-19 anti-PS1 NTF (1:200, Santa Cruz Biotechnology), and 13A11.

RESULTS

NCT is a glycoprotein with a predicted molecular mass of ~80 kDa that was initially reported to migrate at ~110 kDa (20). We had previously employed an affinity purification method to enrich for functional $\gamma$-secretase and had found NCT as a ~150-kDa polypeptide (24). To resolve this discrepancy, we prepared membranes from several cell lines including Jurkat, HeLa, HEK, and CHO cells. Following solubilization in 1% CHAPSO, we probed for endogenous NCT using antibody R302, which recognizes the C-terminal 20 amino acids. In all cases, we found a band that migrated at ~150 kDa (Fig. 1a). In addition, the HER lysates exhibited fainter bands at ~110 and ~80 kDa. In the CHO lysates, the higher (150 kDa) and intermediate (110 kDa) forms of NCT were also readily visible, suggesting that in certain cell lines there are at least two different forms of glycosylated NCT.

To further characterize the nature of the ~150-kDa species of NCT, we assessed the effects of different glycosidases. Using the endoglycosidase PNGase F, which removes all N-linked oligosaccharides, regardless of complexity, we were able to fully convert the 150-kDa NCT in HeLa lysates into an 80-kDa form, consistent with the size of the polypeptide backbone (Fig. 1b, lanes 1-4). The addition of glycosidases for O-linked sugars (endoglycosidase O and sialidase A) did not have any further effect on the migration of NCT (data not shown). This suggests that the

![Fig. 1. Mature and immature glycosylated forms of nicastrin exist in varying amounts in cells.](image-url)
shift in migration was exclusively caused by N-linked modifi-

In agreement with the presence of multiple N-linked glycosylation motifs in the ectodomain (20).

N-linked oligosaccharides can be further modified in the golgi/trans-golgi network compartments into either high-mannose or complex N-linked sugars. These two forms can be differentiated by Endo H, which can only cleave high-mannose residues (27). When we incubated Endo H with HeLa lysates, we found only a small downshift in the size of the protein to ~130 kDa (C-NCT) (Fig. 1b, lanes 5 and 6), indicating that most N-linked sugars in mature NCT (C+HM-NCT) are complex oligosaccharides, and only a minority are high-mannose residues. To further clarify the nature of the ~150- and ~110-
kDa NCT isoforms, we performed a similar experiment on CHO lysates. Analogous to its effect in the HeLa lysates, PNGase F reduced the migration of NCT to its native ~80-kDa position (Fig. 1c, lanes 1–4). The addition of Endo H generated both the intermediate Endo H-resistant band (C-NCT) as well as the 80-kDa band (Fig. 1c, lanes 5 and 6). This result suggests that the lower ~110-kDa NCT found endogenously in CHO cells is an immature form of the protein (N-NCT).

To further explore whether the higher form of endogenous NCT corresponds to mature protein, we subjected CHO membranes to iodoxanol gradient fractionation. This previously characterized method separates endoplasmic reticulum (ER)-rich vesicles from golgi- and post-golgi-rich vesicles (28). We verified the technique by probing for calnexin (an ER-resident protein) and syntaxin 6 (a protein enriched in the golgi/trans-golgi network; Fig. 2a, bottom two panels). As reported previously, the endogenous PS1 holoprotein was located in ER fractions, and the PS1 heterodimers were present principally in the golgi-enriched fractions (Fig. 2a). We found that the 150-kDa mature NCT isoform was located in the golgi-enriched vesicle fractions (Fig. 2a, lanes 4–8) where its quantitative distribution strikingly paralleled that of the PS1 NTF/CTF, whereas the 110-kDa immature isoform was present primarily in the ER fractions, with PS1 holoprotein (Fig. 2a, lanes 1–4). Because this technique effectively separated the 110-kDa from the 150-kDa NCT, we used fractions 1 and 12 to independently deglycosylate the two isoforms to confirm their Endo H sensitivities. In the ER-rich fraction 1, Endo H treatment converted the major 110-kDa band to 80 kDa (Fig. 2b, lanes 1–4). In fraction 12 the more abundant 150-kDa NCT was converted to an intermediate size of ~130 kDa (C-NCT; Fig. 2b, lanes 5–8). These results indeed suggest that the higher NCT band corresponds to a mature, fully N-glycosylated form of the protein and that it co-localizes to vesicular compartments containing PS1 heterodimers.

Because the subcellular distribution of mature NCT was indistinguishable from that of PS heterodimers (Fig. 2a), we reasoned that this isoform may be directly associated with PS1 NTFs/CTFs. We therefore performed a co-immunoprecipitation (co-IP) with antibody X81 to the N terminus of PS1. The lysates before (pre) and after (post) co-IPs were analyzed and compared with the X81-precipitated proteins (ppt). The same blots were probed for NCT (with guinea pig anti-nicastrin antibody), PS1 NTF (goat polyclonal antibody N-19), and PS1 CTF (mouse monoclonal antibody 13A11). b, CHO cells were pulsed with [35S]methionine (200 μCi/ml) for 30 min and chased in cold medium for the indicated times. Nonidet P-40 lysates were then IP’d with R302 (at 1:200) and separated by SDS-PAGE, and the NCT was detected using a phosphorimager. c, precipitation of γ-secretase with the III-3IC inhibitor affinity resin. CHAPSO lysate of CHO cells was precipitated with III-3IC beads in which excess amounts of PS would bind to the resin (excess lysate, left panels). Note that NCT, PS1 NTF, and PS1 CTF remain in the lysate after precipitation with the affinity matrix. For the right panels, one third of the CHO lysate was precipitated with the same volume of III-3IC beads (limiting lysate) to achieve quantitative binding (the amount required was determined empirically).

**Fig. 2.** Subcellular locations of endogenous nicastrin and presenilin 1 in a CHO cell line. a, CHO cells stably overexpressing APPs695 containing the KM → NL Swedish mutation were fractionated over a discontinuous iodixanol gradient (28). Individual fractions were probed for NCT, full-length PS1 (PS1 hol), PS1 NTF, and PS1 CTF. The gradient was also characterized for enrichment of ER or golgi/trans-golgi network compartments into either high-mannose or complex N-linked sugars (20).

**Fig. 3.** Mature nicastrin is stably associated with the γ-secretase complex. a, CHAPSO-solubilized CHO membranes were co-immunoprecipitated (co-IP) with antibody X81 to the N terminus of PS1. The lysates before (pre) and after (post) co-IPs were analyzed and compared with the X81-precipitated proteins (ppt). The same blots were probed for NCT (with guinea pig anti-nicastrin antibody), PS1 NTF (goat polyclonal antibody N-19), and PS1 CTF (mouse monoclonal antibody 13A11). b, CHO cells were pulsed with [35S]methionine (200 μCi/ml) for 30 min and chased in cold medium for the indicated times. Nonidet P-40 lysates were then IP’d with R302 (at 1:200) and separated by SDS-PAGE, and the NCT was detected using a phosphorimager. c, precipitation of γ-secretase with the III-3IC inhibitor affinity resin. CHAPSO lysate of CHO cells was precipitated with III-3IC beads in which excess amounts of PS would bind to the resin (excess lysate, left panels). Note that NCT, PS1 NTF, and PS1 CTF remain in the lysate after precipitation with the affinity matrix. For the right panels, one third of the CHO lysate was precipitated with the same volume of III-3IC beads (limiting lysate) to achieve quantitative binding (the amount required was determined empirically).
Mature Nicastrin Is Tightly Regulated

FIG. 4. Mature nicastrin undergoes the replacement phenomenon and is limited by presenilin and an unknown cofactor(s). For each experiment, the same membrane was probed with the indicated antibodies. a, solubilized membranes from an HEK cell line expressing APP695 with the Swedish mutation (HEKsw) or HEKsw cells stably co-expressing wild type NCT were probed with R302. The right hand panel shows only the exogenous NCT in the latter cells by stripping and reprobing for the V5 epitope. b, a comparison of NCT and PS in three different CHO cell lines. Nonidet P-40-solubilized membranes from CHO cells expressing just human APP (CHO), human APP plus wild type PS1 (PS70), and human APP plus PS1 D257A and PS2 D366A (2A-2) were probed with R302 for NCT, AB14 for PS1 holoprotein and NTF, and 13A11 for PS1 CTF. c, Nonidet P-40-solubilized membranes from fibroblasts isolated from wild type (PS1 +/+) or PS1 knockout (PS1 −/−) mouse embryos were compared for NCT and PS1 as described in b. Lane 3 is a 10-min exposure of NCT from lane 2.

The levels of PS NTF/CTF heterodimers are regulated by unknown limiting cofactors in the cell (19). Because the results above indicate that mature NCT is tightly associated with PS heterodimers, the mature isoform may be a limiting cofactor and/or may itself be limited by another cofactor. We addressed these possibilities by first evaluating the effect of overexpressing NCT on its glycosylation pattern. In HEK cells stably overexpressing wild type NCT, very little additional 150-kDa mature protein was formed compared with the endogenous control level (Fig. 4a, lanes 1 and 2). Indeed, comparison of the HEK cells with or without stable expression of NCT demonstrated a selective accumulation of the 110-kDa isoform. When just the exogenously expressed NCT was specifically detected via its V5 epitope, a large majority was found to be in the immature form, and only a small fraction was able to replace endogenous mature NCT (Fig. 4c, lane 3). This result suggests that mature NCT undergoes a replacement phenomenon similar to mature (heterodimeric) PS. When either protein is overexpressed, mostly immature forms accumulate; for NCT it is the immature N-linked glycosylated form, and for PS it is the immature holoprotein.

Because the maturation of both NCT and PS1 appears to be strictly regulated, we wondered whether each protein was the limiting factor for the other. We therefore examined CHO cells stably overexpressing wild type PS1 (PS70) because these cells have excess endogenous NCT (see Fig. 1a, lane 4) and excess PS1 (30). Compared with a CHO cell line that does not stably express exogenous PS1 (designated CHO), we observed no increase in mature NCT nor in PS1 heterodimers (Fig. 4b, lanes 1 and 2). Indeed, the CHO cells already have detectable endogenous PS1 holoprotein (see Figs. 2a and 4a, lane 1), suggesting that these cells express limiting amounts of an unidentified additional cofactor(s) that prevents further maturation of both PS1 and NCT.

We also looked at the effect of aspartate mutant PS1 and PS2 (in the stable CHO cell line 2A-2) on NCT maturation. Similar to overexpression of wild-type protein (in the PS70 cells), we found no significant alteration in the pattern of the NCT isoforms in these cells (Fig. 4b, lane 3). Thus, even though aspartate-mutant presenilins are nonfunctional (9, 14, 15) and cannot form PS heterodimers (Fig. 4b, lane 3), they are nonetheless capable of stabilizing the 150-kDa NCT. Therefore, the assembly of γ-secretase does not require normal proteolytic function; i.e. the presence of inactive PS is sufficient to stabilize NCT. On the other hand, when we examined PS1-deficient fibroblasts derived from PS1 knockout mice, the amount of mature 150-kDa NCT was dramatically reduced (Fig. 4c). This result strongly suggests that the ability of NCT to enter into the γ-secretase complex requires the presence of PS. In other words, in PS1 −/− fibroblasts, the limiting cofactor for γ-secretase is PS itself. In the absence of PS1 the complex cannot form, and NCT is apparently prevented from acquiring complex N-linked oligosaccharides. On long exposures, a small amount of mature NCT was still present in these cells (Fig. 4c, lane 3), which may be because of the presence of the less abundant PS2 molecule.

DISCUSSION

γ-Secretase is an unusual aspartyl protease that has resisted full elucidation. The data reported here yield new insights into the nature of its assembly and the maturation of two of its key components, PS and NCT. Both proteins exhibit conversions from immature to mature forms, and these conversions correlate with the formation of the active γ-secretase complex. For PS, endoproteolysis is generally required for entry into γ-secretase, although certain unusual mutations in or near the cleavage site of holo-PS can bypass that requirement (31). For NCT, complex N-linked glycosylation is associated with its incorporation into the active enzyme. Other groups have recently reported partly related findings on the maturation of NCT (25, 26). Both these studies convincingly demonstrated that the 150-kDa NCT is highly stable and is physically associated with
PS1 (25, 26) (compare Fig. 3, a and b). We further show that the 150-kDa NCT is associated with functional γ-secretase molecules because our III-31C resin specifically enriches for active enzyme. Importantly, under limiting lyase conditions, we demonstrate that all mature NCT binds to the affinity resin (Fig. 3c). Therefore, the NCT that possesses the full N-linked modification (150 kDa) is associated with active γ-secretase. With this affinity method, we also observed a very small amount of immature NCT that was precipitated by the matrix (see Fig. 3c, lanes 3 and 6). This result raises the possibility that immature NCT may associate with PS1 heterodimers just prior to the maturation (complex glycosylation) of NCT in the Golgi compartment.

Our analysis of NCT function has additional implications for its function. We find that for any exogenously expressed NCT to enter into the γ-secretase complex, significant overexpression is required (see Fig. 4c). These findings imply that cellular transfection methods used to study the functional effects of engineered mutations in either protein (particularly dominant negative ones) require substantial overexpression. This phenomenon has been observed previously for the dominant negative effect of the aspartate-mutant PS proteins (9, 14, 15). It may not be appreciated without achieving expression sufficiently high to replace the endogenous mature (150 kDa) protein.

Our data also demonstrate that at least one other cofactor for γ-secretase exists. Because CHO cells endogenously express excess amounts of both PS and NCT (i.e. immature forms of both proteins are present; see Fig. 4b), we can assume the existence of one or more additional cofactor(s) that is likely to be limiting in this cell line. In this regard, the recently identified APH-1 gene in C. elegans is an attractive candidate (32). A loss-of-function mutation of this protein causes an identical phenotype to loss-of-function mutations in APH-1 (22) when they recently showed that RNA interference (19). Because our III-31C resin can fully deplete mature C. elegans NCT orthologue) (33) or in

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