γ-Secretase Activity Is Associated with a Conformational Change of Nicastrin

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γ-Secretase is a high molecular weight multicomponent protein complex with an unusual intramembrane-cleaving aspartyl protease activity. γ-Secretase is intimately associated with Alzheimer disease because it cleaves the proteolytic cleavage, which leads to the liberation of amyloid β-peptide. At least presenilin (PS), Nicastrin (Nct), APH-1, and PEN-2 are constituents of the γ-secretase complex, with PS apparently providing the active site of γ-secretase. Expression of γ-secretase complex components is tightly regulated, however little is known about the assembly of the complex. Here we demonstrate that Nct undergoes a major conformational change during the assembly of the γ-secretase complex. The conformational change is directly associated with γ-secretase function and involves the entire Nct ectodomain. Loss of function mutations generated by deletions failed to undergo the conformational change. Furthermore, the conformational alteration did not occur in the absence of PS. Our data thus suggest that γ-secretase function critically depends on the structural “activation” of Nct.

γ-Secretase plays a fundamental role in Alzheimer disease (AD) by catalyzing the final proteolytic cleavage, which leads to the formation of amyloid-β peptide (Aβ), the major component of the diseases defining senile plaques. By genetic and biochemical approaches several components of the γ-secretase complex have been identified. In addition to the presenilins (PS1 and PS2) (reviewed in Ref. 1), APH-1a/b, PEN-2, and nicastrin (Nct) (2–4) were recently identified. Apparently all four proteins assemble into a large 500–600-kDa complex (5–9), which displays the intramembranous proteolytic activity required for the cleavage of the β-amyloid precursor protein (APP) and other substrates such as Notch (for review see Ref. 1). Formation of the γ-secretase complex is coordinately regulated (2, 6–13) and depends on the presence of all known complex components. Although there is considerable evidence that PS constitutes the active site of γ-secretase (reviewed in Ref. 1), very little is known about the function of the individual PS binding partners. Previously we and others demonstrated that maturation of Nct is associated with γ-secretase complex assembly (6, 11–13). In addition, a conserved DYIGS motif is apparently involved in Nct function (9). Here we demonstrate that the major conformational change of Nct during the assembly of the γ-secretase complex., which displays the intramembranous proteolytic activity, is tightly regulated, however little is known about the assembly of the complex. Here we demonstrate that Nct undergoes a major conformational change during the assembly of the γ-secretase complex. The conformational change is directly associated with γ-secretase function and involves the entire Nct ectodomain. Loss of function mutations generated by deletions failed to undergo the conformational change. Furthermore, the conformational alteration did not occur in the absence of PS. Our data thus suggest that γ-secretase function critically depends on the structural “activation” of Nct.

EXPERIMENTAL PROCEDURES
cDNA Constructs—To down-regulate endogenous Nct by RNA interference (RNAi), oligonucleotides corresponding to Nct-1045 (6) were cloned into the pSUPER vector (14). Nct deletions (Del 1–5, Fig. 1a) were constructed by oligonucleotide-directed mutagenesis using PCR. Silencer mutations (aaagggaaattcccggtccaatt; the mutations are underlined) were introduced which affect amino acid sequences in the constructs to escape RNAi. All constructs were verified by DNA sequencing.

Cell Culture, Cell Lines, RNAi, and Transfections—Human embryonic kidney (HEK) 293 cells and mouse embryonic fibroblast cells were cultured as described (6). A stable Nct knock-down cell line was generated by stably co-transfecting HEK 293 cells overexpressing Swedish mutant APP (15) with pSUPER/Nct-1045 and pcDNA3.1/Hygro(−) (Invitrogen) and selection for hygromycin (100 μg/ml) resistance. This cell line was stably transfected with the indicated wt and mutant Nct constructs or the empty vector (pcDNA6) by Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer using selection for blasticidin (10 μg/ml) resistance. To inhibit mammadine 1, cells were cultured in the presence of the indicated amounts of kifunensine (Calbiochem) or vehicle for 48 h at 37 °C.

Antibodies—The polyclonal and monoclonal antibodies against the large cytoplasmic loop domain of PS1 (3027 and B1.3D7), the PS1 N terminus (PS1N), PEN-2 (1638), and the APP C terminus (6687), and Aβ (1–42) (3926) were described previously (see Refs. 6 and 7 and citations therein). The polyclonal antibody N1660 against the C terminus of Nct and monoclonal antibody 6E10 against Aβ (1–17) were obtained from Sigma and Senetek, respectively, the anti-APH-LaL (O2C2) antibody was described previously (9).

Protein Analysis—Cell lysates were prepared using STE-N-lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40). After a clarifying spin, cell lysates were subjected to immunoblot analysis. Where indicated Nonidet P-40 was substituted with DDM (0.7%), CHAPS (2%), or SDS (1%). For analysis of γ-secretase complexes DDM-solubilized membrane fractions were subjected to co-immunoprecipitation as described (7). Cell surface biotinylation was carried out as described (16). For deglycosylation, cell lysates were incubated with 50 milliunits/ml endoglycosidase H (endo H) for 16 h at 37 °C in 200 mM sodium citrate (pH 5.8), 0.5 mM phenylmethylsulfonyl fluoride, 100 mM 2-mercaptoethanol, 0.1% SDS) followed by immunoblot analysis. For detection of secreted Aβ following kifunensine treatment, media were replaced, conditioned for 3 h, and analyzed for Aβ by combined immunoprecipitation/immunoblotting using antibodies 9296/0E10.

Trypsin Resistance Assay—Cells were lysed as detailed above in the presence of 0.7% DDM or 1% SDS. Following a clarifying spin, cell lysates were incubated with the indicated amounts of trypsin in 150 mM...
FIG. 1. The entire ectodomain of Nct is required for its function in γ-secretase-mediated APP processing. a, schematic representation of Nct and the ectodomain deletion mutants generated. SP denotes the putative signal peptide and TM the transmembrane domain. Dotted boxes indicate conserved regions including the DYIGS motif-containing region (3). Potential glycosylation sites are indicated with black circles. b, Nct ectodomain deletion mutants are functionally inactive. HEK 293 cells stably co-expressing Swedish mutant APP (APPSw) and Nct-1045 small interfering RNA were stably transfected with the indicated cDNA constructs encoding wt Nct, Nct ectodomain deletion mutants (harboring silent mutations to escape RNAi; note that Del 3 escapes RNAi due to deletion of the RNAi-targeted region) or a vector control. Cell lysates were analyzed for levels of Nct (mature (m) and immature (im) forms), and PS1 CTF and APP-CTFs (generated by β-secretase (CTFβ) and α-secretase (CTFα)) by immunoblotting with antibodies N1660 (Nct), 3027 (PS1), and 6687 (APP). PEN-2 and APH-1αL levels were analyzed from membrane fractions of the same cells by immunoblotting with antibodies N1660 (Nct), 3027 (PS1), and 6687 (APP). PEN-2 and APH-1αL were detected in conditioned media by combined immunoprecipitation/immunoblotting with antibodies 16475 (PEN-2) and 02Δ8 (APH-1αL). Aβ was analyzed from conditioned media by combined immunoprecipitation/immunoblotting with antibodies 16475 (PEN-2) and 02Δ8 (APH-1αL). Aβ was analyzed from conditioned media by combined immunoprecipitation/immunoblotting with antibodies 16475 (PEN-2) and 02Δ8 (APH-1αL). Cell lysates were incubated with (+) or without (−) endo H and analyzed for Nct as in B.

RESULTS

In an attempt to identify the functionally important domains of Nct, we generated a set of deletions within the ectodomain (Fig. 1a). These cDNA constructs were investigated in a HEK 293 cell line stably expressing Swedish mutant APP (APPSw17) and a pSUPER-based Nct-1045 (6) small interfering RNA-encoding vector, which stably knocks down endogenous Nct expression by RNAi (Fig. 1b; lane 2). RNAi-mediated inhibition of Nct expression results in reduced PS1 CTF formation, reduced PEN-2 and APH-1αL (8) expression, the accumulation of the APP C-terminal fragments (APP-CTFs), and reduced Aβ generation (Fig. 1b). These observations are due to the inhibition of the γ-secretase activity upon down-regulation of Nct (6). Expression of a wt Nct cDNA with a cluster of silent mutations conferring RNAi resistance led to the formation of mature Nct (Fig. 1b), which has previously been shown to be associated with the functional γ-secretase complex (6, 11–13). In addition, an accumulation of large amounts of immature Nct due to its overexpression (6, 12, 17) was observed (Fig. 1b). In contrast, all deletion constructs apparently formed only one Nct polypeptide (Fig. 1b), indicating a failure of maturation. To investigate if the Nct deletion variants undergo complex glycosylation like wt Nct, cell lysates were treated with endo H. As shown in Fig. 1c, only mature Nct (endogenous and exogenous) was endo H-resistant, whereas immature Nct and all deleted variants failed to become endo H-resistant. Exogenous expression of wt Nct restored PS1/2 CTF formation and PEN-2 and APH-1αL expression and allowed full γ-secretase function as monitored by the significantly reduced levels of APP-CTFs accompanied by robust Aβ generation (Fig. 1b). In contrast to wt Nct, none of the deletion constructs restored PS1 CTF formation and PEN-2 or APH-1αL expression (Fig. 1b). Moreover, the deletion constructs did not allow the formation of a γ-secretase activity, because none of them reduced APP-CTF formation or increased Aβ production (Fig. 1b). Thus, all deletions within the ectodomain failed to restore γ-secretase function. This suggests an important role of not only the conserved DYIGS motif but the entire ectodomain in γ-secretase complex assembly and activity. The lack of a specific functional subdomain of Nct thus indicates that correct folding of the entire ectodomain is required for Nct function. The primary structure of Nct suggests a rather large luminal domain, which according to our findings plays a pivotal role in Nct function. To investigate if the luminal domain of functional Nct adopts a conformation, which is different from non-functional Nct, cell lysates were treated with increasing amounts of trypsin to monitor unmasking or masking of cleavage sites (18). Interestingly, the mature form of Nct, which is predominantly found in the mature γ-secretase complex (6, 12, 13, 17), was selectively trypsin-resistant whereas immature Nct remained trypsin-sensitive even at the lowest concentration of trypsin. The polypeptide migrating at 85 kDa is an intermediate degradation product of immature Nct. Other γ-secretase complex components such as the PS1 NTF and CTF and APH-1αL were fully sensitive to trypsin. b, all Nct deletion mutants are sensitive to trypsin. CHAPS-extracted HEK 293 cells stably transfected with wt Nct and the indicated Nct deletion mutants (as detailed in Fig. 1b) were incubated with (+) or without (−) 100 μg/ml trypsin and analyzed for Nct as in Fig. 1b. c, Nct not associated with the γ-secretase complex is trypsin-sensitive whereas mature Nct assembled into the γ-secretase complex is resistant. Cell lysates of PS1/2−/− or PS1/2−/+ mouse embryonic fibroblast cells were subjected to trypsin treatment as in b and analyzed for Nct as in Fig. 1b. Consistent with our previous results (7) immature Nct accumulates in the PS1/2−/− cells, whereas both mature and immature Nct is detected in PS1/2−/+ control cells. Mature Nct in PS1/2−/+ control cells is trypsin-resistant whereas immature Nct in PS1/2−/+ cells is trypsin-sensitive.
more, the γ-secretase complex components PS1 NTF, PS1 CTF, and APH-1aL were all fully sensitive to trypsin digestion (Fig. 2a), whereas PEN-2 was found to be less sensitive (data not shown). Because APH-1aL and the PS fragments are trypsin-sensitive, Nct is not simply protected by these γ-secretase complex components. In addition, the very small PEN-2 is unlikely to protect the large Nct ectodomain. Thus, Nct appears to undergo a conformational change independent of APH-1aL, PS, and also PEN-2. After demonstrating the selective trypsin resistance of mature Nct, the deletion variants (Fig. 1a), which all fail to restore γ-secretase activity (Fig. 1b), were investigated. Interestingly, none of them displayed trypsin resistance (Fig. 2b). This suggests that assembly of a biologically active γ-secretase complex is associated with the formation of a trypsin-resistant Nct variant. To further support this hypothesis, we analyzed Nct in mouse embryonic fibroblast cells derived from a PS1/2 gene knock-out. Because of the absence of PS in these cells no γ-secretase complex can be formed. As we and others have previously shown these cells are also deficient in Nct maturation (7, 11, 13).

Thus, fibroblasts derived from a PS1/2 gene knock-out are ideally suited to investigate the association of trypsin-resistant Nct with γ-secretase complex formation. Interestingly, immature Nct in PS1/2−/− cells was degraded by trypsin, whereas mature Nct in the corresponding PS1/2−/− control cells was fully trypsin-resistant (Fig. 2c). Thus, the conversion of trypsin-sensitive to a trypsin-resistant Nct is indeed tightly associated with γ-secretase complex formation. Furthermore, the selectivity of trypsin resistance of mature Nct versus immature/non-functional Nct suggests a major conformational change of Nct during γ-secretase complex assembly and maturation. However, the selective resistance of mature Nct does not exclude the possibility that proteases could not interact with mature Nct due to the rather large and abundant sugar side chains added during maturation. Indeed, 16 putative glycosylation sites are present in the ectodomain (3). To denature and unfold mature Nct, cells were lysed in the presence of 1% SDS, and lysates were then digested with increasing amounts of trypsin. Under these conditions mature Nct became sensitive to trypsin digestion, whereas non-denatured mature Nct extracted under conditions which preserve the γ-secretase complex remained protease-resistant (Fig. 3a). However, glycosylation could protect even partially denatured mature Nct and thus indirectly prevent trypsin-mediated degradation. To exclude this possibility we blocked complex glycosylation by incubating untransfected HEK 293 cells (expressing endogenous Nct) in the presence of kifunensine, which potently inhibits mannosidase I (19). As shown in Fig. 3b, treatment with kifunensine strongly blocked maturation of Nct as manifested by the appearance of a novel Nct species (termed immature-like Nct, see below) migrating at lower molecular weight. However, in contrast to the immature form of Nct, the immature-like species observed upon kifunensine treatment was still trypsin-resistant like the mature fully glycosylated Nct variant (Fig. 3c). These data suggest that a conformational change of Nct associated with trypsin resistance must take place upon assembly and/or maturation of the γ-secretase complex. To investigate if the γ-secretase complex is still active upon inhibition of mannosidase I, Aβ was isolated before and after kifunensine treatment. Consistent with Herreman et al. (13), Aβ production was not inhibited by kifunensine (Fig. 3d). Moreover, expression levels of PS1 CTFs and PEN-2 were not significantly reduced by kifunensine treatment (Fig. 3e, left panel) demonstrating that kifunensine does not interfere with the assembly of the γ-secretase complex. Furthermore, immature-like Nct and PEN-2 co-immunoprecipitated with PS1 upon kifunensine treatment for 2 days (Fig. 3e, right panel). Finally, cell surface biotinylation revealed that immature-like Nct reaches the plasma membrane in cells treated with kifunensine (Fig. 3f) like endogenous Nct in untreated cells (13, 16).

**DISCUSSION**

Our findings demonstrate that trypsin resistance of the Nct ectodomain is associated with γ-secretase complex assembly, maturation, and activity. Thus, we conclude that γ-secretase activity requires a conformational alteration of Nct. Immature and all functionally inactive deletion mutations fail to undergo the conformational switch required for γ-secretase activity and remain trypsin-sensitive. Complex glycosylation does not protect itself against proteolytic degradation, because its inhibition by kifunensine does not affect the protease resistance and function of Nct. In addition, binding of Nct to other γ-secretase complex components does not protect from trypsin degradation, because APH-1aL and the PS1 NTF and CTF are all sensitive to trypsin as well, whereas mature Nct is selectively resistant. Thus, non-functional Nct is structurally “activated” by a conformational alteration. The conformational alteration may be similar to that of the sterol regulatory element-binding protein-activating protein (SCAP) (18). In the latter case cholesterol addition leads to a conformational change of SCAP,
which unMASKS additional cleavage sites of trypsin. Moreover, similar to the loss of function mutations of Nct (Fig. 1), mutations in SCAP also affect its conformational alteration as monitored by trypsin sensitivity (18). A successful conformational change of Nct requires the presence of the complete luminal domain. All ectodomain deletions analyzed not only lead to a loss of function but also fail to undergo the conformational alteration of Nct upon γ-secretase complex assembly and maturation. Previously, a deletion of the DYIGS motif was shown to affect Aβ production (3). This is fully confirmed by our findings, which demonstrate that the same deletion (deletion construct 3 in Fig. 1a) does not restore γ-secretase activity in a Nct knock-down background. However, not only the deletion of the DYIGS motif but all other deletions investigated within the ectodomain inhibit the formation of biologically active Nct and consequently a functional γ-secretase complex. Certainly, this does not exclude the possibility that smaller deletions and point mutations may be tolerated.

Taken together, our findings provide the first insights into the assembly and maturation of the γ-secretase complex. Not only PS may exist as a “premature” variant (the PS holoprotein) but also Nct. In the case of Nct, “activation” is associated with a rather substantial conformational alteration that is required for γ-secretase assembly and activity.

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