Complex *N*-glycosylated form of nicastrin is stabilized and selectively bound to presenilin fragments

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Abstract The transmembrane glycoprotein nicastrin is a component of presenilin (PS) protein complex that is involved in γ-cleavage of βAPP and site-3 cleavage of Notch. PS undergoes endoproteolysis, and the proteolytic fragments are incorporated into the high molecular weight protein complexes that are highly stabilized. Here we show that Endo H-resistant, *N*-glycosylated form of nicastrin (p150-NCT) is highly stabilized and selectively bound to PS fragments. Moreover, loss-of-function mutations of nicastrin inhibited formation of fully glycosylated p150-NCT as well as stabilization of nicastrin, suggesting that glycosylation and stabilization of nicastrin polypeptides are tightly correlated with its function. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Nicastrin; Presenilin; γ-Secretase; Alzheimer's disease; *N*-glycosylation

1. Introduction

Presenilin (PS) 1 and PS2 are highly conserved polytopic transmembrane proteins that are implicated in the γ-cleavage of βAPP and site-3 cleavage of Notch, both of which take place within the transmembrane region [1]. PS polypeptides undergo endoproteolysis to generate N- and C-terminal fragments (NTF and CTF, respectively), and these fragments are incorporated into high molecular weight (HMW) protein complexes. HMW PS complexes are highly stabilized, whereas holoproteins are rapidly degraded. Systematic mutational analysis revealed that the stabilization and HMW complex formation of PS polypeptides, through the integrity of its C-terminal region, is obligatory for the γ-secretase and site-3 activities of PS [2–4]. Studies using the transition-state analogue γ-secretase inhibitors suggested that PS may represent the catalytic center within the HMW PS complex [5–8], although the precise organization of multiple protein components and their functional roles within this complex are yet to be elucidated.

Nicastrin (NCT) is a type I transmembrane glycoprotein identified as a component of HMW PS complex that was recovered from digitonin-solubilized membrane fractions [9]. Recently, genetic studies using *Caenorhabditis elegans* and *Drosophila* indicated that *aph-2/nicastrin* is required for *glp-1/notch* signaling and its intramembranous processing, suggesting that NCT is the indispensable component for PS complex [10,11–14]. It has been reported that NCT polypeptides undergo *N*-glycosylation, but the functional significance of glycosylation and its relationship to PS complex formation still remain unclear. It has also been reported that mutations in a conserved domain at residues 312–369 located at the luminal side of NCT alter the activities of γ-secretase for βAPP and site-3 protease for Notch [9–15]. Here we show that NCT undergoes Endo H-resistant *N*-glycosylation to form a 150 kDa polypeptide (p150-NCT) that is highly stabilized. Moreover, p150-NCT was selectively bound to PS fragments and the loss-of-function mutations of NCT inhibited the generation of p150-NCT, suggesting that p150-NCT is the mature, functional form within the HMW PS complex.

2. Material and methods

2.1. Plasmids

The cDNA encoding full-length NCT without first methionine (KIAA0253) was obtained from Kazusa DNA research institute. Mutant NCT constructs (NCT_{AAIGS}, $NCT_{\Delta 312}$, $NCT_{\Delta 694}$; Fig. 1) were generated by long-PCR method using *PfuTurbo* polymerase (Stratagene, La Jolla, CA, USA) as previously described [3,4,16]. The cDNAs were amplified by PCR using LA Taq polymerase (Takara, Kyoto, Japan) adding N-terminal kozak sequence and the first methionine, and then subcloned into pEF6/V5-His TOPO vector according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The expression vectors encoding $PS2_{wt}$, $PS2_{P414L}$ in pcDNA3, βAPP_{NL} in pCEP4 or NotchΔE in pCS2 are previously described [2–4,16–18].

2.2. Cell culture, transfection and generation of stable cell lines

A stable Neuro2a (N2a) cell line doubly expressing βAPP_{NL} and NotchΔE (N2a NL/N) was generated by transfecting cDNAs (ratio of transfected DNAs; βAPP_{NL} : NotchΔE = 0.1 : 1 μg) using LipofectAMINE (Invitrogen, Carlsbad, CA, USA) and selection in DMEM containing hygromycin at 160 μg/ml. N2a NL/N cell line stably expressing PS2 and/or NCT was generated by transfecting cDNAs using LipofectAMINE and selection in DMEM containing G418 at 500 μg/ml and/or Blastcidin at 10 μg/ml. Individual resistant colonies were isolated and screened by examining the protein expression of βAPP_{NL}, NotchΔE, PS2 and NCT by immunoblotting.

2.3. Immunoblotting and co-immunoprecipitation

Immunoblotting and co-immunoprecipitation experiments were done as described [2–4,16–18]. Proteins were precipitated or detected by antibodies that recognize βAPP_{NL} (C4, gift of Dr. Y. Ihara), NotchΔE (anti-myc monoclonal antibody 9E10, Roche Applied Sciences), PS1 (G1L3), PS2 (G2N4 for NTF, G2L for CTF) and NCT (anti-V5 monoclonal antibody, Invitrogen) and visualized by Immunostar reagent (Wako, Japan) using LAS-1000plus (Fuji, Japan). To examine the glycosylation of NCT, cultured stable N2a cells at confluency in 10 cm plate were solubilized by RIPA buffer (150 mM Tris–

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HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and NCT polypeptides were immunoprecipitated by anti-V5 antibody. Eluted supernatants by boiling in sample buffer containing 0.1% SDS and 1% 2-mercaptoethanol were treated with 500 mU/ml of Endo H (Roche Applied Sciences) or 200 U/ml of PNGase F (Roche Applied Sciences), respectively, at 37°C for 12 h, and analyzed by immunoblotting. Half-lives of PS and NCT polypeptides were evaluated by blocking total cellular protein synthesis using cycloheximide (CHX; 30 μg/ml) treatment for indicated times and immunoblotting as described [3,4]. Scanned images were quantitated with Scion Image (Scion Corporation) software.

3. Results

3.1. Complex N-glycosylation of NCT is abolished by mutations in conserved luminal region as well as by deletion of the cytoplasmic region

To examine the post-translational maturation of wild-type NCT (NCT_{wt}) in N2a cells, we stably transfected NCT cDNA into N2a NL/N cell lines coexpressing wild-type PS2 (N2a NL/N/PS2) or an empty vector (N2a NL/N/mock). In lysates from stable N2a cells (N2a NL/N/mock/NCT_{wt}), polypeptides migrating at ~130 and 150 kDa (p130-NCT and p150-NCT, respectively) were detected by anti-V5 antibody (Fig. 2A), which were consistent with the molecular sizes of transfected NCT described previously [9], and the overexpression of PS2 did not affect the mobility pattern of these bands. Treatment of immunoprecipitated NCT polypeptides with Endo H yielded derivatives with molecular masses of 80 and 135 kDa (p80-NCT and p135-NCT, respectively) (Fig. 2B) [9]. The size of p80-NCT corresponded to that of a nascent NCT predicted from its amino-acid sequences, suggesting that this band represents the unmodified core-protein. To characterize p135-NCT, we next treated immunoprecipitated NCT by PNGase F. Immunoblot analysis revealed that PNGase F-treated NCT polypeptides were detected solely as a single band of p80-NCT, suggesting that p135-NCT represents an Endo H-resistant *N*-glycosylated form of NCT.

NCT is a conserved transmembrane protein that is present from nematodes to humans, and the middle portion of the luminal region is highly conserved [9,19]. It has been reported that mutations in this region of NCT affect the γ-secretase activities: an artificial missense mutation replacing the conserved DYIGS motif with AAIGS (residues 336–340) caused an increase, whereas a large deletion of the conserved region (residues 312–369) caused a reduction, in the generation of Aβ and NICD [9,15]. We constructed NCT expression plasmids carrying the same missense (NCT_{AAIGS}) or deletion ($NCT_{\Delta 312}$) mutations, and stably transfected them into N2a NL/N/mock or NL/N/$PS2_{wt}$ cell lines. To learn about the role(s) of the cytoplasmic region (residues 694–709) of NCT in its metabolism and function, we also generated and expressed a deletion mutant NCT lacking the entire cytoplasmic region ($NCT_{\Delta 694}$). Immunoblot analysis showed that the formation of p150-NCT was reduced by the NCT_{AAIGS} mutation, and was completely abolished in the deletion mutants ($NCT_{\Delta 312}$ and $NCT_{\Delta 694}$; Fig. 2A). Immunoblot analysis of immunoprecipitated mutant NCT after Endo H digestion revealed that these deletion mutations inhibited the formation of Endo H-resistant p135-NCT (Fig. 2B). These data suggest that NCT undergoes a two-step maturation process, i.e. (i) Endo H-sensitive *N*-glycosylation to form p130-NCT as an initial step, and (ii) Endo H-resistant *N*-glycosylation to generate the fully maturated form, p150-NCT, as the final step, in which the conserved luminal and cytoplasmic regions of NCT may play an important role(s).

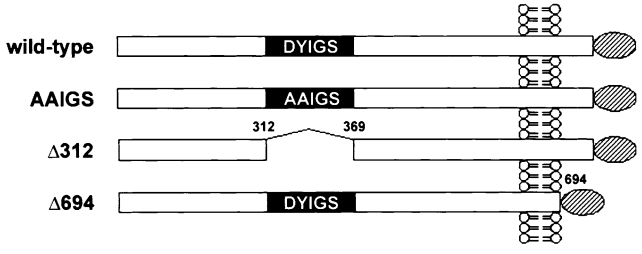

Fig. 1. Schematic representation of genetically engineered forms of NCTs used in this study. Highly conserved region is shown by a black box. C-terminally added V5-tag is indicated by shaded circle.

3.2. Complex N-glycosylated p150-NCT polypeptide is highly stabilized

Recent studies indicated that the stabilization of PS fragments is required for its γ-secretase activities [2–4]. If NCT is an integral component of the PS complex, it may undergo similar stabilization. To evaluate and compare the half-lives of NCT with those of PS polypeptides, we performed a chase experiment upon treatment with CHX that blocks total cellular protein synthesis, which has been employed to estimate the half-lives of PS proteins [3,4]. In CHX-treated N2a NL/N/PS2/NCT_{wt} cells, PS2 holoproteins were rapidly degraded, whereas fragments were stabilized during >24 h of chase as previously described [3,4]. Immunoblot and densitometric analysis using anti-V5 antibody revealed that p150-NCT polypeptides exhibit longer half-lives during >24 h of incubation, whereas the levels of p130-NCT declined faster than those of p150-NCT (Fig. 3A,B). These data suggest that p150-NCT is stabilized in a similar pattern with PS fragments and that NCT polypeptides acquire long half-lives during the maturation process, undergoing Endo H-sensitive and -resistant glycosylation.

To examine the effects of mutations on stabilities of NCT polypeptides, we performed immunoblot and densitometric analysis of the lysates from CHX-treated N2a cell lines expressing mutant NCT (Fig. 3C,D). The amounts of p150-NCT_{AAIGS} were low, although the levels were not altered during an incubation period of 28 h with CHX, whereas the levels of p130-NCT_{AAIGS} declined dramatically in a similar manner to that of wild-type p130-NCT ($t_{1/2}$: ~10 h). The levels of p130-$NCT_{\Delta 312}$ or $NCT_{\Delta 694}$ polypeptides also declined rapidly during the chase period. These data suggest that the complex *N*-glycosylation of NCT polypeptides is obligatory for its stabilization.

3.3. p150-NCT selectively binds to PS fragments

To examine whether stabilized forms of PS (i.e. fragments) and NCT (i.e. p150-NCT) specifically bind to each other, we performed co-immunoprecipitation of the lysates from N2a NL/N/mock/NCT_{wt} cell lines (Fig. 4A). The preponderant endogenous PS proteins accumulate as NTF and CTF in vivo [20]. Immunoblot analysis of immunoprecipitates from 1% CHAPSO-solubilized cell lysates revealed that p150-NCT polypeptides were the predominant species precipitated by the anti-PS1 antibody, whereas a tiny amount of p130-NCT was precipitated. Notably, p150-NCT polypeptides were the major co-immunoprecipitated species even in lysates

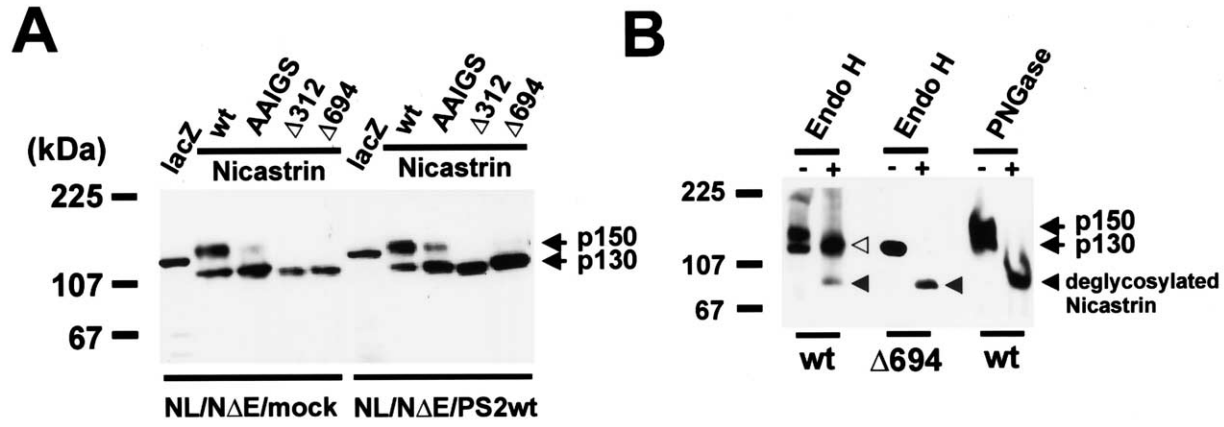

Fig. 2. Characterization of stably transfected NCT in N2a cell lines. A: Immunoblots of lysates from N2a NL/N/mock or NL/N/PS2 cells stably transfected with wild-type or mutant NCT probed with anti-V5 antibody. Arrows denote p130-NCT or p150-NCT polypeptides. B: Immunoblot analysis of NCT polypeptides digested by endoglycosidases. White and black arrowheads indicate Endo H-resistant (p135-NCT) and unmodified (deglycosylated; p80-NCT) polypeptides, respectively.

of cells expressing NCT_{AAIGS}, that significantly suppressed the maturation of NCT by *N*-glycosylation. In sharp contrast, $NCT_{\Delta 312}$ and $NCT_{\Delta 694}$ polypeptides that underwent only Endo H-sensitive *N*-glycosylation never interacted with PS proteins. These data suggest that the maturated p150-NCT selectively binds to the stabilized PS fragments.

To further determine whether immature p130-NCT bind to PS polypeptides, we performed co-immunoprecipitation studies in lysates of the N2a NL/N/PS2/NCT cell lines which accumulated fragments as well as holoproteins of PS2. We observed a predominant association of p150-NCT with PS2 proteins, although a small amount of p130-NCT was also precipitated by an anti-PS2 antibody in N2a cells coexpressing PS2 and NCT_{wt} (Fig. 4B). To examine whether PS2 holopro-

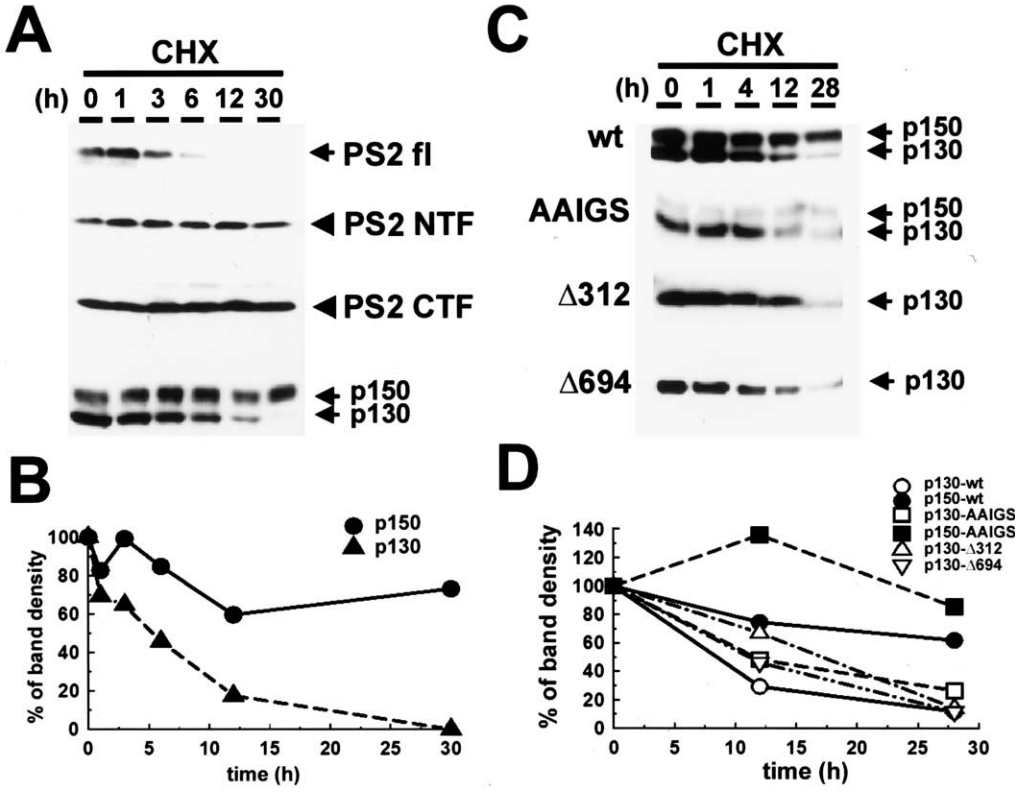

Fig. 3. Stabilization of NCT polypeptides. A: Analysis of the half-lives of PS2 (fragments and holoproteins, upper three lanes) and NCT (lower lane) in stably transfected N2a cells. Lysates prepared after treatment with CHX for various incubation time (shown above the lanes in hours) were analyzed by immunoblotting with anti-PS2 and V5 antibody. B: Densitometric analysis of the levels of p130-NCT (open circle) and p150-NCT (open triangle) after CHX treatment. The averages of intensities of each band in two independent experiments are shown. C: Half-lives of wild-type or mutant NCT in N2a stable cells were evaluated as in A. Names of transfected NCT are shown at the left of each lane. D: Densitometric analysis of the amount of wild-type or mutant NCT in CHX-treated cells after 12 or 28 h of incubation.

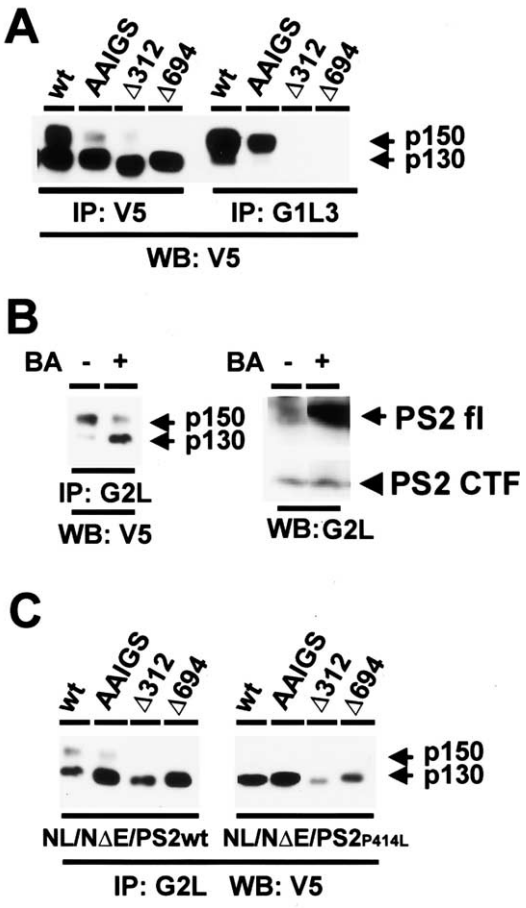

Fig. 4. Co-immunoprecipitation analysis of NCT with PS. A: Immunoblot analysis of wild-type and mutant (AAIGS, Δ312 and Δ694) NCT polypeptides associated with endogenous PS1 fragments. 1% CHAPSO-solubilized membrane fractions from N2a NL/N/mock cells were immunoprecipitated by anti-V5 or anti-PS1 (G1L3) antibodies. B: Co-immunoprecipitation of p130-NCT with PS2 holoprotein. Immunoprecipitates or total cell lysates from N2a NL/N/PS2/NCT_{wt} with (+) or without (−) butyric acid (BA) treatment were analyzed by immunoblotting with anti-V5 or anti-PS2 (G2L) antibody. Note that overexpression of PS2 holoprotein by the addition of BA shifts the predominantly precipitated species from p150-NCT to p130-NCT. C: Co-immunoprecipitation of mutant NCT with wild-type ($PS2_{wt}$) or loss-of-function mutant PS2 ($PS2_{P414L}$). Immunoprecipitated lysates from N2a NL/N/$PS2_{wt}$ or $PS2_{P414L}$ stably coexpressing wild-type and mutant NCT by anti-PS2 antibody were analyzed by immunoblotting with anti-V5 antibody.

tein interacts with some specific form of NCT, we upregulated the level of full-length PS2 by inducing the transcription of CMV promoter-driven cDNAs by the addition of butyric acid [21]. An induction by butyric acid for 24 h dramatically increased the levels of PS2 holoprotein, whereas those of PS2 fragments were almost unchanged (Fig. 4B, right panel). Co-immunoprecipitation by anti-PS2 antibody clearly showed that p130-NCT was the preponderant species that interacted with PS2 holoproteins in the lysates treated with butyric acid (Fig. 4B, left panel). Moreover, in contrast to the results obtained in N2a NL/N/mock cells (see Fig. 4A), p130-NCT was the predominantly co-immunoprecipitated species with PS2 polypeptides in N2a NL/N/$PS2_{wt}$ cells coexpressing $NCT_{\Delta 312}$ or $NCT_{\Delta 694}$ (where PS2 holoprotein is predominant; see Fig. 4B, right panel), whereas p130- and p150-NCT were both precipitated from N2a NL/N/PS2/NCT_{AAIGS} cells with PS2 (Fig. 4C). Taken together, it is suggested that PS2 holoproteins preferentially interact with p130-NCT polypeptides.

Finally, we examined the effect of coexpression of P414L mutant PS2, that completely abrogates the stabilization and HMW complex formation of PS2 (Fig. 4C) [4]. Co-immunoprecipitation analysis showed that only p130-NCT was recovered from immunoprecipitates of the cell lysates of N2a NL/N/$PS2_{P414L}$ stably coexpressing NCT_{wt} or NCT_{AAIGS}. These data suggest that p130-NCT, a partially maturated form of NCT, preferentially interacts with the PS2 species that is rapidly degraded.

4. Discussion

In this study, we showed that (i) NCT undergoes a maturation process from partially glycosylated immature form (p130-NCT) to a complex *N*-glycosylated form (p150-NCT); (ii) mature p150-NCT is highly stabilized, whereas immature p130-NCT is rapidly degraded; (iii) p150-NCT interacts preferentially with the stabilized PS fragments. These results suggest that the assembly of PS and NCT occurs within ER, and that maturation of PS complex (i.e. processing to generate PS fragments, complex *N*-glycosylation of NCT and stabilization of PS fragments as well as of p150-NCT) may take place in the medial Golgi or later compartments for the reasons below.

Endo H digestion removes immature, *N*-linked oligosaccharide side chains that are conjugated to proteins within ER, but not those added in medial-Golgi, whereas PNGase F removes all *N*-linked carbohydrates. Thus, *N*-linked sugars resistant to Endo H digestion, but labile to PNGase F, are interpreted to have been incorporated within Golgi. A number of potential *N*-glycosylation sites have been predicted in the N-terminal hydrophilic domain of NCT [9]. In our stably transfected cells, NCT polypeptides were detected as p130-NCT and p150-NCT, showing molecular weights higher than that of a nascent polypeptide. Both Endo-H and PNGase F treatments yielded p80-NCT that corresponded to a nascent NCT. In addition, a p135-NCT resistant to Endo H treatment also was detected, whereas PNGase F treatment converted all NCT polypeptides into p80-NCT. These results suggest that the core-sugar moieties at a subset of glycosylation sites in NCT undergo maturation process and form complex glycoconjugates after export from ER. Consistent with our findings, Leem et al. recently showed that NCT undergoes Endo-H resistant glycosylation and sialylation, but not *O*-glycosylation and addition of chondroitin sulfate glycosaminoglycan [22]. Notably, deletion mutation of NCT (i.e. $NCT_{\Delta 312}$ or $NCT_{\Delta 694}$) abolished the generation of Endo H-resistant p150-NCT. Thus, it is most likely that p150-NCT represents the complex *N*-glycosylated form of NCT, and that the deletion of N-terminal (luminal) or C-terminal (cytoplasmic) domains inhibits the maturation of NCT polypeptides.

CHX treatment and co-immunoprecipitation experiments revealed that p150-NCT is highly stabilized and preferentially bound to PS fragments, whereas p130-NCT was rapidly degraded and interacted with PS species that are unstable. Recently, Leem et al. demonstrated the selective stabilization of mature form of NCT by pulse-chase method [22], which was in agreement with our observations using CHX treatment. Although the molecular mechanism of the stabilization of PS fragments still remains unclear, the observation that formation of the HMW form (>250 kDa) of PS complex is

closely related to its stabilization strongly supports the idea that the proper assembly, folding and maturation of each component are required for its formation [3,4]. Fractionation experiments revealed that PS holoproteins form low molecular weight (<250 kDa) complex and are predominantly located in ER, where Endo H-sensitive *N*-glycosylation occurs [4,16,18]. Moreover, $PS2_{P414L}$ polypeptides, that locate within ER as unstable holoprotein and form only low molecular weight complexes, associated exclusively with p130-NCT. This possible interaction of p130-NCT with PS holoprotein suggests that the assembly of PS complex is initiated in ER. In contrast, p150-NCT was associated with the stabilized PS fragments, that are known to form HMW PS complex in the Golgi compartments [4,18]. Furthermore, deletion mutant NCT polypeptides did not associate with PS fragments and were rapidly degraded similarly to PS holoproteins or loss-of-function mutant PS proteins (e.g. $PS2_{P414L}$) [3,4]. These data suggest that a full *N*-glycosylated form of NCT is incorporated into the stabilized PS complex and that maturation of PS complexes containing NCT occurs during or after transport through medial Golgi. Consistent with our hypothesis, Leem et al. showed that maturated NCT as well as PS fragments located in Golgi fractions, whereas immature form of NCT and PS holoproteins were fractionated in ER by sucrose density gradient separation [22]. Although the 'paradox' between the subcellular localization of functional γ-secretase activity and that of PS protein has not been clarified yet, further investigation into the metabolism and function of fully *N*-glycosylated NCT in PS complex would shed lights on this problem [23].

The deletion of cytoplasmic region inhibited the complex *N*-glycosylation and stable interaction of NCT with PS fragments. It is likely that this region plays an important role in the maturation of PS complex. However, no specific functional domains or motifs are found within the cytoplasmic domain. It is noteworthy that the amino acid sequence of the cytoplasmic region of human NCT is highly divergent among mammals, *C. elegans aph-2* and *Drosophila nicastrin*, only a single valine residue proximal to transmembrane region being conserved. It is possible that this valine residue has some critical role in the maturation of NCT, or alternatively, the mechanism of maturation of NCT/PS complex is different between mammals and invertebrates.

Our mutational analysis of NCT provided evidence that the maturated NCT polypeptides are stabilized and selectively interact with PS fragments, that are the seminal components of HMW PS complex, and that functional mutations of NCT affect its maturation. Further attempts to define the molecular mechanism of stabilization of PS complex containing p150-NCT and to identify other components of PS complexes relevant to intramembranous proteolytic activity will facilitate our understanding of the pathogenic mechanisms of AD.

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