Single Chain Variable Fragment against Nicastrin Inhibits the γ-Secretase Activity

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γ-Secretase is a membrane protein complex that catalyzes intramembrane proteolysis of a variety of substrates including the amyloid β precursor protein of Alzheimer disease. Nicastrin (NCT), a single-pass membrane glycoprotein that harbors a large extracellular domain, is an essential component of the γ-secretase complex. Here we report that overexpression of a single chain variable fragment (scFv) against NCT as an intrabody suppressed the γ-secretase activity. Biochemical analyses revealed that the scFv disrupted the proper folding and the appropriate glycosylation maturation of the endogenous NCT, which are required for the stability of the γ-secretase complex and the intrinsic proteolytic activity, respectively, implicating the dual role of NCT in the γ-secretase complex. Our results also highlight the importance of the calnexin cycle in the functional maturation of the γ-secretase complex. The engineered intrabodies may serve as rationally designed, molecular targeting tools for the discovery of novel actions of the membrane proteins.

γ-Secretase catalyzes intramembrane proteolysis of a variety of substrates including amyloid β precursor protein (APP) to generate amyloid β peptide (Aβ), the latter being a major component of senile plaques in the brains of Alzheimer disease patients. Thus, agents that inhibit γ-secretase activity could serve as an effective therapeutics for Alzheimer disease, whereas the γ-secretase activity plays important roles in cell signaling pathways including Notch signaling (1, 2). γ-Secretase consists of at least four integral membrane proteins, i.e. presenilin (PS), nicastrin (NCT), APH-1, and PEN-2, all of which are essential to the proteolytic activity (3–5). Molecular and chemical biological analyses have revealed that PS forms a hydrophilic pore involving the transmembrane domain 6 and 7, where conserved catalytic aspartates reside to function as catalytic residues of γ-secretase complex (6, 7). APH-1 is a multipass membrane protein that plays a role in stabilization and trafficking of the γ-secretase complex (8), and PEN-2 is a cofactor for the activation and the regulation of the γ-secretase activity (3, 9).

NCT, which was identified as a PS-binding protein (10), is a single-pass membrane protein that harbors an extracellular domain (ECD) with a number of N-glycosylation sites. In mammalian cells NCT undergoes Endo H-resistant complex glycosylation and acquires trypsin resistance during the assembly process of the γ-secretase complex (11–17). Molecular and cellular analyses revealed that the trypsin resistance, presumably indicating the proper structural folding of NCT, might be directly linked to the enzymatic activity, whereas the complex glycosylation is dispensable. Moreover, multiple sequence alignment analyses revealed that NCT ECD have a similarity to an aminopeptidase (18), whereas certain catalytic residues are not conserved. Recently one study has suggested that NCT plays a critical role in substrate recognition (19). During the proteolytic process, NCT ECD captures the most N terminus of the substrate as a primary substrate receptor (i.e. exosite) for the γ-secretase via the aminopeptidase-like domain. However, this view has been recently challenged (20). Nevertheless, as structural information of NCT ECD is totally lacking, the functional role of the structural maturation of NCT in the formation and activity of the γ-secretase remains unclear.

Molecular engineering of monoclonal antibodies opens a venue for the functional analyses of targeted molecule and the therapeutic intervention for several diseases (21). A single-
chain antibody fragment (scFv) is comprised of heavy- and light-chain sequences of an antibody linked by a short linker and preserves binding abilities of its parental antibody. scFv can be expressed intracellularly as an intrabody (22, 23), which provides a powerful method for phenotypic knock-out of the genes. Intrabodies have been investigated as treatments for a variety of pathological conditions, including neurodegenerative diseases such as Parkinson disease and Huntington disease. Moreover, several recent publications have highlighted the therapeutic potential of intrabodies targeting intra- as well as extracellular epitopes (24–29). Here, we generated scFv against NCT from an anti-NCT monoclonal antibody. Unexpectedly, the overexpression of the anti-NCT scFv as an intrabody abolished the proteolytic activity by the destabilization of the γ-secretase complex and the inappropriate glycosylation of NCT. This is the first example showing that engineered antibody would be a useful tool for the direct modulation of the γ-secretase complex and its activity.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—C-terminal V5-His-tagged human NCT ECD inserted in pBlueBac4.5 (Invitrogen) was generated from NCT/ V5-His in pBlueBac4.5 (30) by long PCR. Cytoplasmic RNA was prepared from 1 × 10^7 hybridoma cells by using Isogen reagent (Nippongene, Tokyo, Japan). The cells were lysed by mixing with Isogen and incubated at room temperature for 5 min. After centrifugation of the lysate, the RNA was precipitated and dissolved in distilled water. This RNA was used as a template for first-strand cDNA synthesis with 3′ primers specific for the mouse IgG genes (Novagen, Darmstadt, Germany). The cDNA fragments were then amplified by PCR with LA Taq (Takara, Shiga, Japan) using 3′ and 5′ primers from the mouse Ig primer set as per the manufacturer’s instructions (Ig-Prime kit protocols; Novagen). The PCR products were subcloned into the pEF6/V5-TOPO vector (Invitrogen) by the TOPO cloning method. scFv cDNAs inserted into pSecTag2C (Invitrogen) were constructed as follows. The PCR-derived DNA fragments in pEF6/V5-TOPO were subjected to splice overlapping extension PCR to connect heavy- and light-chain genes to give a single fusion protein gene. In the first round PCR, heavy- and light-chain genes were amplified by using the following primers: the variable region heavy-chain gene, 5′-ggggatccGAAGTGAAAGCTGAGAGGAGAG-3′ (VHF#1) and 5′-caccacccggggtcagcggccaccacagcccctcGCTAGAGGAGAGT-3′ (VHR#1); the variable region light-chain genes, 5′-caccaccccggggtcagcggccaccacagcccctcGCTAGAGGAGAGT-3′ (VLF#1) and 5′-caccaccccggggtcagcggccaccacagcccctcGCTAGAGGAGAGT-3′ (VLR#2). In the second round PCR, the amplified heavy- and light-chain fragments were linked by using VHF#1 and VLR#1 or VLR#2. The amplified scFv cDNAs were digested with EcoRI and NotI to subclone into the EcoRI-NotI-digested pSecTag2C vector. Wild-type as well as mutant (i.e. A312, 648ATAA) human NCT inserted in the pEF6/V5-TOPO was generated as previously described (14). All cDNAs were sequenced by automated sequencer (LI-COR, Lincoln, NE). cDNAs encoding deletion mutants of human NCT fused with V5 tag were kindly gifted from Drs. Keiko Shirotani and Christian Haas (Ludwig-Maximilians-University, Munich, Germany) (17).

**Cell Culture and Transfection**—Maintenance of Sf9 cells, transfection, and recombinant baculovirus preparation were done as previously described (30, 31). Hybridoma cells were maintained in RPMI 1640 medium supplemented with l-glutamine (Nikken Bio Medical Laboratory, Kyoto, Japan) containing 15% (v/v) fetal bovine serum, 100 international units/ml of penicillin, 100 μg/ml of streptomycin, and 1 mM sodium pyruvate (Sigma) at 37 °C in 5% CO₂. All transfections were achieved by FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. HEK293 cell lines stably expressing scFv were selected by Zeocin (Invitrogen). Nestin knock-out fibroblasts (NKO cells) (32) stably expressing wild-type and mutant NCT were selected by Blasticidin (Calbiochem).

**Purification of NCT ECD and Secreted scFv**—For NCT ECD production, Sf9 cells were infected with recombinant virus encoding NCT ECD at multiplicity of infection 2 and incubated for 72 h. For scFv, Sf91F-expressing cells (clone 2) were incubated in regular media for 72 h. NCT ECD or scFv was recovered from the culture media by using a nickel-chelating column (GE Healthcare). Bound proteins were eluted by a stepwise gradient of imidazole (5–300 mM) in phosphate-buffered saline.
Eluted fractions were analyzed by Coomassie or silver staining as well as immunoblotting.

Analysis of A5201A Binding Ability by Enzyme-linked Immunosorbent Assay—Purified NCT ECD or bovine serum albumin was coated on 96-well plates at the concentration of 2 g/ml in a coating buffer (0.1 M sodium bicarbonate, pH 8.6), and the plates were incubated overnight at 4 °C. After the coating, the plates were blocked by a blocking buffer (phosphate-buffered saline containing 1× BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) and 0.02% (w/v) sodium azide) and stored at 4 °C until used. A5201A, anti-V5 antibody (Invitrogen), as a positive control or mouse IgG fraction (SIGMA) as a negative control was added at various concentrations to the wells, and the plates were incubated overnight at 4 °C. Binding of antibody was detected by anti-mouse IgG antibody conjugated with horseradish peroxidase (GE Healthcare) and tobacco mosaic virus substrate. A450 was measured and quantitated by SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

Antibodies, Immunological Analyses, and in Vitro γ-Secretase Assay—Anti-G1Nr3, G1L3, and PNT3 polyclonal antibodies against glutathione S-transferase-fused human PS1 N terminus, cytoplasmic loop region, or synthetic peptide corresponding to the N-terminal 26 amino acids of human/mouse PEN-2, respectively, were previously described (30, 33–35). Anti-PS1NT polyclonal antibody was kindly gifted from Drs. Gopal Thianakaran and Sangram Sisodia (The University of Chicago, Chicago, IL). Other antibodies were purchased from Cell Signaling Technology (Danvers, MA) (anti-c-myc (9B11)), Covance (Princeton, NJ) (anti-APH-1aL (O2C2)), Santa Cruz Biotechnology (Santa Cruz, CA) (anti-NCT N terminus (N19)), Sigma (anti-NCT C terminus (N1660), anti-α-tubulin (DM1A)), or Stressgen (Ann Arbor, MI) (anti-Calnexin). Cells were solubilized with HEPES buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) containing 1% (w/v) CHAPSO. Immunoprecipitation, immunoblot analysis, metabolic labeling, and enzymatic digestion experiments were previously described (14, 33–36). For detection of the γ-secretase activity in vitro, solubilized HeLa cell membranes were coincubated with the substrates in the presence (+) or absence (−) of 100 nM DBZ. De novo generation levels of Aβ40 (open columns) or Aβ42 (filled columns) peptides were normalized by the γ-secretase levels, which were assessed by densitometric analysis of PS1 CTF on the immunoblotting (n = 3, *, p < 0.01, Student’s t test).
Anti-NCT Intrabody Decreases the Expression Levels of NCT and Suppresses the γ-Secretase Activity—Budded baculovirus from Sf9 cells infected with recombinant virus displays the recombinant proteins on its virion membrane (30, 40). Thus, budded baculovirus can be used as an optimal immunogen to generate monoclonal antibodies against the membrane proteins. Using this technology, we have generated a monoclonal antibody A5201A that specifically binds to NCT ECD. A5201A showed specific binding ability to V5-tagged NCT ECD in a similar manner to anti-V5 antibody, whereas an irrelevant IgG exhibited no reactivity (Fig. 1). Next, we generated two intrabodies based on A5201A, named 5201C and 5201F. Both intrabodies consist of light and heavy chain variable regions that were cloned from hybridoma cells producing A5201A, conjugated with three GGGGS pentapeptide repeats as a linker by PCR. Each cDNA was cloned into a pSecTag2C vector, which enables the targeting of the intrabodies into the lumen by the IgG leader sequence and detection with c-myC tag attached to the C terminus (Fig. 2A). 5201C and 5201F harbored a difference only in the light chain variable region sequences, whereas the heavy chain variable region sequences were totally identical. As NS-1 cells, the mouse myeloma cells used for the generation of the hybridoma (40) endogenously express κ light chain gene, one of the two light chain sequences might be derived from NS-1 cells.

We then generated HEK293 cell lines stably expressing 5201C or 5201F (Fig. 2B, supplemental Fig. S1). Immunoblot analysis revealed that both intrabodies were expressed intracellularly as a ~36-kDa protein and secreted into culture media. Intriguingly, the expression levels of NCT, especially that of mature NCT, were markedly reduced in 5201F-expressing cell lines, and the remaining “mature-like” NCT showed slightly longer migration on SDS-PAGE than that of mock- or 5201C-expressing cells. Hereafter, we refer to this mature-like NCT of ~115 kDa observed in 5201F-expressing cells as NCT*. Moreover, the protein levels of other components of the γ-secretase complex, i.e. PS1, APH-1aL, and PEN-2, were also decreased in 5201F-expressing cells. In contrast, none of the γ-secretase components was affected in 5201C-expressing cells. Next, we examined whether the intrinsic γ-secretase activity was affected in the intrabody-expressing cells by in vitro assay using an APP-based recombinant substrate (3, 38). We then normalized the activity against the levels of PS1 CTF to measure the specific activity per active complex (20). 5201F-expressing cells showed significant reduction in the AB*-generating activities (for Aβ40, 34.5% (#2) and 23.9% (#4) compared with that of mock cells; for Aβ42, 21.3% (#2) and 39.4% (#4)) (Fig. 2C). These results suggest that the overexpression of intrabody 5201F, but not 5201C, reduces the steady-state expression levels as well as the intrinsic activity of the γ-secretase complex.

RESULTS

Anti-NCT Intrabody Decreases the Expression Levels of NCT and Suppresses the γ-Secretase Activity—Budded baculovirus from Sf9 cells infected with recombinant virus displays the recombinant proteins on its virion membrane (30, 40). Thus, budded baculovirus can be used as an optimal immunogen to generate monoclonal antibodies against the membrane proteins. Using this technology, we have generated a monoclonal antibody A5201A that specifically binds to NCT ECD. A5201A showed specific binding ability to V5-tagged NCT ECD in a similar manner to anti-V5 antibody, whereas an irrelevant IgG exhibited no reactivity (Fig. 1). Next, we generated two intrabodies based on A5201A, named 5201C and 5201F. Both intrabodies consist of light and heavy chain variable regions that were cloned from hybridoma cells producing A5201A, conjugated with three GGGGS pentapeptide repeats as a linker by PCR. Each cDNA was cloned into a pSecTag2C vector, which enables the targeting of the intrabodies into the lumen by the IgG leader sequence and detection with c-myC tag attached to the C terminus (Fig. 2A). 5201C and 5201F harbored a difference only in the light chain variable region sequences, whereas the heavy chain variable region sequences were totally identical. As NS-1 cells, the mouse myeloma cells used for the generation of the hybridoma (40) endogenously express κ light chain gene, one of the two light chain sequences might be derived from NS-1 cells.

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Incorporation of 5201F into the γ-secretase complex. Co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from intrabody-expressing stable HEK293 cells with control IgG (c) or anti-c-myc antibody 9B11 (m). Immunoprecipitates were analyzed by immunoblotting with each antibody indicated below the panel. The white circle indicates the immunoglobulin chain. imNCT immature NCT.

FIGURE 3. Incorporation of 5201F into the γ-secretase complex. Co-immunoprecipitation analysis of 1% CHAPSO-solubilized fractions from intrabody-expressing stable HEK293 cells with control IgG (c) or anti-c-myc antibody 9B11 (m). Immunoprecipitates were analyzed by immunoblotting with each antibody indicated below the panel. The white circle indicates the immunoglobulin chain. imNCT immature NCT.

Secreted 5201F failed to bind NCT. A, culture media of 5201F-expressing cells were applied to a nickel chelating column, and the bound proteins were eluted with stepwise increased concentrations of imidazole and EDTA. Eluates were dialyzed against phosphate-buffered saline and then analyzed with silver staining (upper panel) and immunoblotting with anti-c-myc antibody 9B11 antibody (lower panel). B, partially purified secreted scFv from the 100 mM imidazole fraction in A was mixed with 1% CHAPSO-solubilized HEK293 cell lysates and immunoprecipitated with control IgG or anti-c-myc 9B11 antibody. Immunoprecipitates were analyzed by immunoblotting using each antibody indicated below the panels. Note that secreted scFv failed to bind with endogenous NCT. mNCT and imNCT represent mature and immature NCT, respectively.

FIGURE 4. Secreted 5201F failed to bind NCT. A, culture media of 5201F-expressing cells were applied to a nickel chelating column, and the bound proteins were eluted with stepwise increased concentrations of imidazole and EDTA. Eluates were dialyzed against phosphate-buffered saline and then analyzed with silver staining (upper panel) and immunoblotting with anti-c-myc antibody 9B11 antibody (lower panel). B, partially purified secreted scFv from the 100 mM imidazole fraction in A was mixed with 1% CHAPSO-solubilized HEK293 cell lysates and immunoprecipitated with control IgG or anti-c-myc 9B11 antibody. Immunoprecipitates were analyzed by immunoblotting using each antibody indicated below the panels. Note that secreted scFv failed to bind with endogenous NCT. mNCT and imNCT represent mature and immature NCT, respectively.
Intrabody against Nicastrin

All γ-secretase components were also detected in the immunoprecipitates with 5201F. These data suggest that 5201F directly interacts with NCT and that 5201F-bound NCT is incorporated into the γ-secretase complex. Unexpectedly, however, 5201F purified from conditioned media failed to pull down NCT from HEK cell lysates (Fig. 4). Then, we transiently expressed intrabody in the presence or absence of human NCT in NKO cells (32) and performed the immunoprecipitation analysis using mixed lysates. The coexpressed 5201F precipitated NCT polypeptides, whereas the intrabody in NKO cells failed to interact with the independently expressed NCT (Fig. 5, A and B), suggesting that the intrabody 5201F is incorporated into the γ-secretase complex during its assembly process. Moreover, significant reduction of mature NCT was observed in stable NKO cells coexpressing 5201F and human NCT in a similar manner to that in HEK293 cells expressing 5201F, suggesting that 5201F was able to form NCT* in NKO cells (supplementary Fig. S2). Next, we analyzed the location of the epitope of the intrabody 5201F using systematically deleted constructs (NCT/Δ1–Δ5) of NCT ECD (17) in NKO cells. Previous results have suggested that these deletion constructs encode loss-of-function mutant forms of NCT. Although 5201F bound to all deletion NCT mutants, the immunoreactivities against NCT/Δ2, Δ3, Δ4, and Δ5 were significantly reduced, suggesting that 5201F directly recognizes a broad region in NCT ECD irrespective of the formation of functional γ-secretase complex (Fig. 5, C and D). Collectively, these results suggest that the intracellularly expressed scFv 5201F directly targets the nascent or newly synthesized NCT polypeptides during the biosynthetic pathway and is incorporated into the γ-secretase complex. NCT were converted to the 100 kDa immature form by trimming of glucose and mannose in the ER. Then these NCT matured into the complex glycosylated forms that exhibited retarded migration at 120 kDa. This mature NCT was long-lived, and significant levels of labeled mature NCT were still present 48 h after labeling as previously reported (11). In 5201F-expressing cells, however, the levels of immature NCT were relatively low at 3 h of chase and the conversion to NCT* was completed within 6 h. Moreover, 48 h after synthesis, NCT* was still present but clearly lesser in amount compared with that of mature NCT in mock- or 5201C-expressing cells, suggesting that the binding of 5201F caused rapid and inappropriate maturation of NCT polypeptides and rendered the NCT* unstable.

Next we examined the glycosylation state of NCT* by Endo H digestion. Mature NCT in mock- or 5201C-expressing cells was Endo H-resistant and migrated at ~120 kDa in SDS-PAGE, whereas immature NCT was completely deglycosylated as previously described (Fig. 6B) (11–17). Unexpectedly, NCT* showed Endo H resistance, too. Moreover, cell surface biotinylation experiments revealed that mature NCT* was displayed on the plasma membrane in a similar manner to the mature NCT of the control cell lines (Fig. 6C). The levels of PS1 N-terminal fragment in mock- and 5201F-expressing cells were almost comparable (Fig. 6B), suggesting that the steady-state level of the γ-secretase complex containing 5201F on the cell surface is not significantly altered. Finally, we examined the trypsin resistance of NCT*. As previously described (17), substantial levels of mature NCT in mock- or 5201C-expressing cells remained intact after 30-min of incubation with trypsin, whereas immature NCT was completely digested (Fig. 6D). In contrast, in 5201F-expressing cells, NCT* was completely digested by trypsin in a similar manner to that

**FIGURE 5. Direct binding of intracellular 5201F to NCT.** A, immunoblotting of the NKO cells overexpressing intrabodies with or without human NCT. mNCT and imNCT represent mature and immature NCT, respectively. B, immunoprecipitation (IPed) of 1% CHAPSO-solubilized NKO cells with control IgG (c) or anti-c-myc antibody 9B11 (m). Coexpressed samples were the lysates from NKO cells coexpressing intrabodies and human NCT. Mixed samples were the mixture of the lysate from NKO cells either expressing intrabodies or NCT. C, immunoblotting of the NKO cells expressing the deletion mutant of human NCT (Fig. 5, C) or anti-c-myc antibody 9B11. White circles indicate nonspecific proteins appeared in NKO cells. D, immunoprecipitation of 1% CHAPSO-solubilized NKO cells in C with anti-c-myc antibody 9B11.
cells partially rescued the generation of mature NCT and PS1 fragments (Fig. 7B). Cycloheximide treatment caused rapid degradation of PS1 CTF in NCT/648ATAA-overexpressing cells, whereas PS1 CTF in cells expressing wild-type NCT was stable as previously reported (42), indicating that the reconstituted γ-secretase complex by NCT/648ATAA mutant is unstable (Fig. 7C). The trypsin digestion experiment revealed that mature NCT/648ATAA was readily degraded, suggesting that mutant NCT failed to acquire the conformational maturation, thereby causing the instability of the γ-secretase complex (Fig. 7D). The de novo Aβ generating activity in NCT/648ATAA-expressing NKO cells was also decreased (66.3% of that in wild-type human NCT-expressing cells). Notably, however, the de novo activity normalized by PS1 CTF levels in cells expressing NCT/648ATAA was not reduced compared with that in wild-type human NCT-expressing cells (Fig. 7E). These data suggest that the defect in the conformational maturation of NCT ECD caused the decrease in the total Aβ generating activity by loss of stability of the functional γ-secretase complexes, whereas the intrinsic activity of the enzyme was unaltered.

Glucose Trimming in ER Is Required for the Intrinsic Activity of the γ-Secretase Complex—N-Glycosylated proteins are fold by ER-resident chaperones (e.g., calnexin (CNX) or calreticulin) that recognize a monoglucose on unfolded polypeptides (43, 44). During the folding process, the transfer and the trimming of glucose are executed by the ER-resident glucosyltransferase and glucosidase, respectively. Thus, unfolded, but still glucose-attached proteins are captured by CNX to be folded; this process is called “the CNX cycle.” To test the possibility that the CNX cycle is involved in the maturation of NCT, the immunoprecipitation analysis was performed. We confirmed the association of CNX and NCT as previously described (Fig. 8A) (45). Moreover, this interaction was significantly reduced by the overexpression of 5201F (Fig. 8B), suggesting that the glucose trimming and/or the CNX cycle would be inhibited by the scFv. Castanospermine (CST) is an α1,2-glucosidase inhibitor that causes the inhibition of interaction between N-linked glycoproteins and CNX (46, 47). The CST treatment caused the accumulation of aberrant NCT polypeptides, which presumably represent the glucosylated form of NCT (glucoNCT) (Fig. 9A). Intriguingly, glucoNCT showed a similar molecular weight to that of NCT* and acquired the Endo H resistance (Fig. 9B). In contrast, the levels of the γ-secretase components and the trypsin resistance of NCT were unaffected, suggesting that the glucose trimming is dispensable for the proper folding of NCT and the trafficking of the stable γ-secretase complex (Fig. 9, A and C). However, specific de novo Aβ generating activity normalized by PS1 CTF levels in CST-treated cell membrane was significantly decreased to a similar extent to that in 5201F-expressing cells (for Aβ40, 32.0% compared with that of mock-treated cells; for Aβ42, 33.4%) (Fig. 9D). These data indicate that the proper glucose trimming of NCT ECD accompanied by the CNX cycle is required for the intrinsic γ-secretase activity but not for the formation of the stable enzyme complex. Taken together, the binding of scFv 5201F has detrimental effects both on the
conformational maturation and the glucose trimming of NCT ECD, thereby causing the destabilization and the loss of the enzymatic activity of the γ-secretase complex, respectively.

**DISCUSSION**

Recently, much attention is being focused on the use of scFv fragments as intrabodies. Intrabodies have been used for phenotypic knock-out of endogenous target proteins by several different strategies. In this study, we generated two intrabodies by using an anti-NCT ECD monoclonal antibody A5201A as a template. Biochemical analyses revealed that the specific binding of 5201F on NCT ECD inhibited the conformational change and the proper glycosylation of NCT, thereby causing the destabilization of the γ-secretase complex and the loss of proteolytic activity. Our results suggest that the functional maturation of NCT ECD regulates the proper trafficking, stability, and the specific activity of the γ-secretase complex.

γ-Secretase is an unusually stable protease that has >24 h of half-life in mammalian cells (42). Biochemical studies have shown that NCT forms a subcomplex with APH-1 within the biosynthetic pathway (48) and functions as a stabilizing cofactor as well as a substrate receptor for the γ-secretase complex (19, 20). The assembly of the γ-secretase complex occurs in the ER (49, 50), and only “functionally” assembled γ-secretase is subsequently sorted out to the Golgi apparatus in Rer1- and COPII-regulated manners (5, 51–53). However, the molecular information on the quality control of a prefunctional γ-secretase complex in the ER remains unknown. Here, we showed that the overexpression of 5201F on NCT ECD inhibited the conformational change and the proper glycosylation of NCT, thereby causing the phenotypic “knock-out” of the γ-secretase components. Notably, we found that 5201F accelerated the inappropriate

**FIGURE 7.** The role of conformational maturation of NCT in the γ-secretase activity. A, schematic depiction of NCT mutants analyzed in this experiment. The black box indicates highly conserved region containing DYIGS motif (312–369). The C-terminal V5 tag is indicated by shaded circle. B, immunoblot analysis of wild-type and mutant NCT-expressing NKO cell lysates. mNCT and imNCT represent mature and immature NCT, respectively. C, immunoblot analysis of wild-type and mutant NCT-expressing NKO cell lysates treated with cycloheximide (CHX). D, trypsin digestion of wild-type or mutant NCT polypeptides. E, de novo γ-secretase activity of the mutant NCT-expressing cells measured by in vitro assay. White bars indicate the proteolytic activity in the solubilized membrane containing equal protein amounts. Black bars denote the relative activity normalized by the γ-secretase levels, which were assessed by densitometric analysis of PS1 CTF on immunoblotting.

**FIGURE 8.** Interaction of CNX and NCT. A, immunoprecipitation (IP) analysis of NKO cell lysate transiently coexpressing human NCT and intrabodies. Immunoreactivities of NCT-bound CNX were quantified and normalized by the amount of total NCT in B (n = 7; *, p < 0.05, Student’s t test).
Endo H-resistant glycosylation of NCT, suggesting that the quality control and the trafficking system for the prefunctional γ-secretase in the ER are altered upon 5201F expression. In general, the folding and maturation states of glycoproteins are monitored by the CNX cycle. Properly folded glycoproteins escape this cycle and are sorted out from the ER. Trypsin resistance of NCT is tightly correlated with the proteolytic activity of the γ-secretase complex and may reflect its structural change in the ER (17), whereas the molecular basis of this conformational change has not been clarified to date. Of note, NCT fused with ER retention dilysine signal at the C terminus retained the ability to form the functional ER retention dilysine signal at the C terminus.

The role of glucose trimming in NCT maturation and the γ-secretase activity. A, immunoblot analysis of CST-treated HEK293 cell lysates. Note that CST treatment caused the accumulation of aberrant molecular weight NCT (glucoNCT). mNCT and imNCT represent mature and immature NCT, respectively. NTF, N-terminal fragment. B, Endo H and C, trypsin digestion of glucoNCT accumulated by CST treatment. D, specific γ-secretase activity of the CST-treated HEK293 cells analyzed by in vitro assay as in Fig. 1C (n = 3; *, p < 0.01, Student’s t test).

An ineffective CNX cycle is also caused by the inhibition of glucose trimming. The treatment with CST induced the accumulation of the glucosylated NCT polypeptides and reduced the γ-secretase activity. In general, aberrant glucosylated proteins generated by CST treatment are rapidly transported out from the ER or degraded by ER-associated degradation (46, 54, 55). However, our results showed that biochemical characteristics of the γ-secretase were unaffected by CST, whereas the intrinsic proteolytic activity was reduced. In contrast, it has been shown that treatment by kifunensine, which inhibits the mannos trimming occurring at the later stage of glycosyl maturation, has no effect on the γ-secretase activity (16, 17), suggesting that the glucose/mannose trimming was dispensable for the proper folding of NCT and the formation of the γ-secretase complex. Rather, our data indicate that the glucosyl state of NCT per se is involved in the specific enzymatic activity, suggesting the functional significance of the CNX cycle in the biosynthesis of the γ-secretase complex. The analysis of 648/ATAA mutant NCT also supported our notion that the conformational maturation of NCT determines the amount, but not the intrinsic activity, of the functional γ-secretase complex. Although the molecular mechanism(s) by which the glycosylation of NCT modulates the intramembrane cleavage remain unknown, aberrant glucosylated NCT might have lesser activity in the substrate-capturing function.

We propose a model for the mode of action of 5201F. As depicted in Fig. 10, NCT would be an essential component in the γ-secretase complex with dual functions, which were disrupted by the binding with 5201F. The properly glycosylated and folded NCT might function not only as a substrate receptor (19) but as a gatekeeper for the trafficking and the stability of the γ-secretase complex (20). Our data presented here also expand the repertoire of antibody-based functional reagents valuable for the cell biology research on the γ-secretase. To modify/engineer their binding profile, scFvs can be applicable to phage display, to which “rational design and directed molecular evolution” process with high throughput platform is applicable (56). In addition, structural studies using scFv have provided crucial information for the rational development of the small compound targeting protein-protein interactions (57).
Intrabody against Nicastrin

Notably, amino acid substitutions at certain sites in NCT ECD differently affected the Aβ generation and Notch processing (20). Thus, search for small compounds or peptides targeting NCT ECD might provide a new class of biological tools as well as therapeutics for γ-secretase modulation. Further investigations including structural analyses of NCT ECD complexed with scFvs would shed light on the molecular mechanism of the functional assembly of γ-secretase and the role of NCT ECD in the γ-secretase-mediated intramembrane cleavage.

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