A Synthetic Antibody Fragment Targeting Nicastrin Affects Assembly and Trafficking of γ-Secretase*

Xulun Zhang§, Robert Hoey§, Akiko Koide§, Georgia Dolios§, Marcin Paduch§, Phuong Nguyen||, Xianzhong Wu**, Yueming Li***, Steven L. Wagner||, Rong Wang§, Shohei Koide§, and Sangram S. Sisodia††

From the Departments of§ Neurobiology and §Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois 60637, the ‡Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, New York 10029, the †Department of Neurosciences, University of California, San Diego School of Medicine, La Jolla, California 92039, and **Molecular Pharmacology and Chemistry, Memorial Sloan-Kettering Cancer Center, New York, New York 10065

Background: γ-Secretase is critical in Notch signaling and Alzheimer disease.

Results: Coexpression of a nicastrin antibody affects maturation and trafficking of γ-secretase and alters Notch and APP processing.

Conclusion: Nicastrin is a feasible target for modulating the targeting and substrate specificity of γ-secretase.

Significance: Modulation of γ-secretase through nicastrin may be of therapeutic value in cancer and Alzheimer disease.

The γ-secretase complex, composed of presenilin, nicastrin (NCT), anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2), is assembled in a highly regulated manner and catalyzes the intramembranous proteolysis of many type I membrane proteins, including Notch and amyloid precursor protein. The Notch family of receptors plays important roles in cell fate specification during development and in adult tissues, and aberrant hyperactive Notch signaling causes some forms of cancer. γ-Secretase-mediated processing of Notch at the cell surface results in the generation of the Notch intracellular domain, which associates with several transcriptional coactivators involved in nuclear signaling events. On the other hand, γ-secretase-mediated processing of amyloid precursor protein leads to the production of amyloid β (Aβ) peptides that play an important role in the pathogenesis of Alzheimer disease. We used a phage display approach to identify synthetic antibodies that specifically target NCT and expressed them in the single-chain variable fragment (scFv) format in mammalian cells. We show that expression of a NCT-specific scFv clone, G9, in HEK293 cells decreased the production of the Notch intracellular domain but not the production of amyloid β peptides that occurs in endosomal and recycling compartments. Biochemical studies revealed that scFvG9 impairs the maturation of NCT by associating with immature forms of NCT and, consequently, prevents its association with the other components of the γ-secretase complex, leading to degradation of these molecules. The reduced cell surface levels of mature γ-secretase complexes, in turn, compromise the intramembranous processing of Notch.

γ-Secretase catalyzes the intramembranous proteolysis of over 90 type I membrane substrates (1), including Notch receptors and the β-amidol precursor protein (APP). In all cases, ectodomain shedding is a prerequisite for intramembranous cleavage. In the case of Notch, a signaling receptor essential for cell lineage determination, cell proliferation, and survival, γ-secretase-mediated cleavage of a membrane-tethered stub of Notch, termed NEXT, on the cell surface leads to the generation of the Notch intracellular domain (NICD), which translocates to the nucleus to regulate gene expression (2). Hyperactivation of Notch signaling has been linked to many diseases, including several types of cancers, such as human T cell acute lymphoblastic leukemia, breast cancer, and lung cancer (3). Similarly, shedding of the APP ectodomain leads to the generation of membrane-tethered APP-CTFs, which are then subject to γ-secretase processing in late Golgi and endosomal compartments (4) to generate Aβ peptides. Aβ peptides are 38- to 43-amino acid peptides, the principal component of plaques in the brains of patients with AD (5). Therefore, it has been proposed that γ-secretase inhibitors may serve as promising treatments for AD, but recent clinical trials of such reagents have been terminated because of Notch-based liabilities. To overcome these confounding issues, investigations have focused on the development of small molecule γ-secretase modulators that can differentially affect the processing of APP and Notch (6).

*This work was supported, in whole or in part, by National Institutes of Health Grants P30 NS061777 and S10 RR022415 (to R.W.), U01 NS074501 (to S. L. W.), R01AG026660 (to Y. L.), and U54-GM087519 and R01-GM072688 (to S. K.). This work was also supported by the Adler Foundation, by the Edward H. Levy Fund, by the Alzheimer’s Association, by the American Health Assistance Foundation, and by the Cure Alzheimer’s Fund (to S. S. S.), and by the University of Chicago Comprehensive Cancer Center (to S. K.). S. S. S. is a paid consultant of Eisai Research Labs Inc., AZ Therapies Inc., and Janssen Pharmaceutica NV but is not a shareholder in any company that is a maker or owner of an FDA-regulated drug or device.

†To whom correspondence should be addressed: 947 E. 58th St., MC0928, Dept. of Neurobiology, The University of Chicago, Chicago, IL 60637. Tel.: 773-834-9186; E-mail: ssisodia@bsd.uchicago.edu.

‡The abbreviations used are: APP, amyloid precursor protein; CTFs, carboxy-terminal fragments; NEXT, extracellular Notch truncation; NICD, Notch intracellular domain; Aβ, amyloid β; AD, Alzheimer disease; PS, presenilin; NCT, nicastrin; ECD, ectodomain; scFv, single-chain variable fragment; APPSwe, amyloid precursor protein Swedish variant; Tricline, N-[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]glycine; AICD, APP intracellular domain; CHAPSO, 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonate; Fab, fragment antigen-binding; VH, heavy chain antibody domain; LH, light chain antibody domain; cpm, counts per minute.
Nicastrin scFv Affects γ-Secretase Assembly and Trafficking

γ-Secretase is composed of presenilin (PS1 and PS2), nicas- trin (NCT), anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) (7). Structural and functional analyses have demonstrated that PS is the catalytic subunit (8–13), whereas the other three cofactors execute functions as substrate recognition (NCT) (14), scaffolding (APH-1) (15) and enzyme maturation (PEN-2) (13, 16, 17). The assembly of γ-secretase is initiated in the endoplasmic reticulum. NCT and APH-1 form an initial NCT-APH-1 subcomplex (18, 19), which then associates with a catalytically inactive full-length PS to generate a NCT-APH-1-PS trimeric complex (16). During the last step of assembly, PEN-2 is recruited to this immature complex and promotes the endoproteolysis of full-length PS into fragments that remain associated in the mature complex (16). γ-Secretase then exits the endoplasmic reticulum, and the NCT subunit acquires complex glycosylation in the Golgi apparatus (20).

In view of evidence that NCT plays a critical role in substrate recognition (14, 21), we and others have examined the feasibility of modulating NCT function using antibody-based approaches (21–24) with the notion that NCT-specific antibodies could bind to and modulate the binding of NCT to individual substrates. In this regard, we reported that a synthetic body could bind to and modulate the binding of NCT to individual substrates. In this study, we generated additional NCT-specific synthetic antibodies using phage display technology and then reformatted the cDNAs encoding these antibodies to corresponding cDNAs encoding single-chain variable fragments (scFvs) (25) that were then stably expressed in HEK293 cells that constitutively express the APP “Swedish” (APPSwe) variant that causes early onset familial AD (26). We now describe the analysis of two anti-NCT-specific antibodies that, following conversion to scFvs, bind to the NCT ECD in vitro. In HEK293 cells that stably express one of these antibodies, termed scFvG9, NCT maturation is impaired and leads to reduced level on the cell surface, where the Notch derivative, NEXT, is subject to γ-secretase processing at the “e” site. On the other hand, γ-secretase that is present in the Golgi apparatus and endosomal/recycling compartments in cells expressing scFvG9 is fully capable of processing APPSwe to generate Aβ peptides. Therefore, we suggest that scFvG9 affects both the maturation of NCT and subcellular distribution of γ-secretase that leads to differential processing of APP versus Notch.

EXPERIMENTAL PROCEDURES

Cell Lines, cDNA Constructs, and Transfection—Full-length NCT was C-terminally tagged with a CT11 tag (27). The mouse NΔE construct (mNΔE) was C-terminally tagged with a myc tag (28). HEK293 cells and HEK293 cells stably expressing either wild-type human APP or the human APP Swedish variant were stably transfected with an empty vector or cDNAs encoding an scFv using Lipofectamine Plus reagent (Invitrogen). Stable cell pools were selected and maintained in the presence of 200 μg/ml zeocin (Invitrogen). HEK293S GnT1- cells (29) and HEK293 cells were maintained in DMEM containing 10% FBS and 1% PS (Invitrogen). To assess γ-secretase activity in HEK293 cells that stably express APPSwe and scFv, cDNA encoding mouse NΔE was transiently transfected into these cell pools for 48 h before detergent-solubilized cell lysates were prepared for analysis.

Immunoblot Analysis and Antibodies—Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 5 mM EDTA and protease inhibitor mixture (Sigma). Protein concentrations were determined by BCA kit (Thermo Scientific, Rockford, IL). Equal amounts of protein lysates were resolved on SDS-PAGE and transferred to a nitrocellulose membrane. After blocking, the membrane was sequentially incubated with primary and secondary antibodies, and the secondary antibodies were detected with ECL (PerkinElmer Life Sciences). PS1NT antibody was used to detect full-length PS and the N-terminal fragment of PS1 (30). MAB5232 was used to detect the PS1 C-terminal fragment (EMD Millipore, Billerica, MA). PNT-2 antibody (Dr. Gopal Thinakaran) was used for the detection of PEN-2 protein (30). H2D antibody (Dr. Gang Yu) was used to detect endogenous APH-1αL (31). CT11 antibody was used to detect CT11-tagged NCT (30). Nicastrin (N-19) antibody (Santa Cruz Biotechnology) was used to detect endogenous NCT. 9E10 (Santa Cruz Biotechnology) was used to detect myc-, tagged mNΔE and NCT fragments as well as the scFv proteins. Anti-Hisγ antibody (Rockland Immunocochemicals) was used to detect Hisγ-tagged ECD, 716, as well as scFv protein. CTM1 polyclonal antibody was used for the detection of full-length APP and APP CTFs (21). 26D6 monoclonal antibody was used to detect APPs and Aβ (32). 4G8 monoclonal antibody (Covance) was used to immunoprecipitate Aβ from conditioned medium. Actin antibody was used to detect endogenous actin (Santa Cruz Biotechnology).

Synthetic Antibody Generation and Construction of scFv Vectors—Purification of secreted NCT fragments, screening, and expression of anti-nicastrin synthetic antibodies have been described previously (21), except that we used a new antibody phage display library (33) in this study. cDNAs encoding single chain variable fragments were generated by multiple rounds of PCR reactions. Heavy chain and light chain sequences of NCT-specific Fabs A9 and G9 as well as those of the negative control Fab2-2 were used as templates for the amplification of VH and VL regions by PCR. The VH region was amplified using the following primers: human transthyretin-VH, 5′-GAGACGGTGACCAGGGT-3′ (forward); LVH, 5′-GCCACGACCCACCACCGCCGCAGAGCAGGCTAGAGATCTCGAGGCTGCTGCTGCCTACCCGCCGCTTCTCCTCAGCTG-3′ (reverse). The VL region was amplified using the following primers: human transthyretin-VL, 5′-GAGTTTGTGTCTCGAGGCTGCTGCTGCCTACCCGCCGCTTCTCCTCAGCTG-3′ (forward); Xho-VL, 5′-AGC-ATCGAGAAGTGTAGAGATCTCGAGGCTGCTGCTGCCTACCCGCCGCTTCTCCTCAGCTG-3′ (reverse). The VH and VL regions were linked by a 15-amino acid peptide containing the three repeats of GGGGS. The human transthyretin signal peptide sequence was fused to the N terminus of the VH region, and a myc-Hisγ tag was attached.
Nicastrin scFv Affects γ-Secretase Assembly and Trafficking

to the C terminus of the VL region. The resulting cDNA was cloned into the pAG vector.

Immunoprecipitation of Secreted NCT Fragments with Anti-NCT Fab or scFvs—Conditioned medium from cells secreting ECD and 716 fragments was subjected to immunoprecipitation with Fab 2-2, 2, 12, A9, and G9 essentially as described earlier (21). In this study, a mixture of protein A and protein L beads (Thermo Scientific) was used for the capture of Fab-antigen complexes. To obtain secreted scFv proteins, cDNAs encoding scFv 2-2, scFvA9, and scFvG9 were transiently transfected into HEK293 cells for 48 h, and the conditioned media were collected. To assess the interaction between the ECD and these scFv proteins, conditioned medium from stable ECD-expressing cells was mixed with the conditioned medium containing secreted individual scFv protein. The mixture was adjusted with 5× PBS to a final concentration of 1× PBS and incubated at 4 °C overnight. The ECD-scFv complex was captured with protein A beads (Thermo Scientific) and detected by immunoblot analysis with anti-His<sub>s</sub> antibody.

*Pulse Labeling*—To assess the conversion of full-length mouse NDE into the NCD fragment, HEK293 APPSwe/scFv cells were transfected with cDNA encoding mouse NAE and labeled for 40 min with 250 μCi [<sup>35</sup>S]methionine (PerkinElmer Life Sciences). Then the cells were chased for 2 h. In experiments to analyze the processing of APP, HEK293 APPSwe/scFv cells were either labeled for 10 min or 4 h with 250 μCi [<sup>35</sup>S]methionine. Cells were lysed in lysis buffer (50 mm Tris-Cl (pH 7.4), 150 mm NaCl, 5 mm EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate), and the total radioactivity (cpm) was determined after TCA precipitation by a liquid scintillation counter. Cell lysates of equal cpm were used for the immunoprecipitation of mouse NDE, NCD, and full-length APP and its derivatives. Immunoprecipitated mouse NDE and NCD fragments were resolved on 6% SDS-PAGE; soluble APP was resolved on 10% SDS-PAGE; and full-length APP, APP CTFs, and Aβ peptides were resolved on a Tris-Tricine gel. Resolved proteins were then transferred to a nitrocellulose membrane and exposed to a PhosphorImager screen or to an x-ray film. The PhosphorImager screen was scanned using a Storm Imager (GE Healthcare). Imagequant 5.0 software was used for the image analysis.

**Quantification of Aβ Peptides by ELISA**—Aβ38, Aβ40, and Aβ42 peptides in conditioned media of HEK293 APPSwe/scFv stable cells were quantitated by ELISA utilizing the Meso Scale Discovery Aβ triplex immunassay with 4G8 detection (catalog no. K15141E-1, MSD, Rockville, MD). The kit was run according to the instructions of the manufacturer. Briefly, 25 μl of MSD SULFO-TAG-conjugated 4G8 detection antibody, and 25 μl of sample was added to a blocked MSD multiplex plate pre-coated with capture antibodies specific for Aβ 38, Aβ 40, and Aβ 42 and incubated for 2 h with shaking at room temperature.

The plate was read on an MSD SECTOR® Imager 2400 after addition of read buffer. Concentrations of Aβ38, Aβ40, and Aβ42 in samples were back-fitted from the respective standard curves using MSD Workbench software.

**In Vitro γ-Secretase Activity Assay**—Membranes were prepared from HEK293swe/scFv-stable cells. Reactions were performed at 37 °C, and the production of NICD was determined using AlphaLISA signals (21).

**Mass Spectrometry Analysis of Immunoprecipitated Aβ Peptides**—Immunoprecipitation MS measurement of Aβ peptides was carried out as described previously using 4G8 antibody (34). Aβ peptides in 300 μl of conditioned medium from HEK293 cells stably expressing APPSwe were immunoprecipitated by incubating overnight with 1.5 μl of 4G8 antibody and 5.0 μl of protein A/protein G plus beads. Mass spectra were collected using a TOF/TOF 5800 mass spectrometer (AB Sciex). Each mass spectrum was accumulated from 2000 laser shots and calibrated using bovine insulin (an internal mass calibrant).

**Coimmunoprecipitation of γ-Secretase Complex Subunits under Native Conditions**—HEK293 APPSwe/scFv cells were lysed in a buffer containing 50 mm Tris-Cl (pH 8), 150 mm NaCl, 1% CHAPSO, 1 mm DTT, 5 mm EDTA, and 1× protease mixture (Sigma). For immunoprecipitation, 3 μl of 9E10 or N-19 antibody or 1 μl of PS1<sub>NT</sub> antibody was added to each reaction, respectively. A comparable amount of normal mouse IgG or rabbit IgG was added to the control reactions. The mixtures were then incubated overnight in a cold room. The immunoprecipitated complexes were captured either by protein G beads (for 9E10 and N-19) or by protein A beads (for PS1<sub>NT</sub>) that were washed three times with lysis buffer. γ-Secretase complexes were released from the beads by incubation in Laemmli buffer at 37 °C for 15 min.

**Cell Surface Biotinylation**—HEK293 APPSwe/scFv cells were grown to near confluence in a 10-cm plate and subjected to cell surface biotinylation with 0.8 mm sulfo-succinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate (Thermo Scientific) essentially as described previously (35). Cells were then lysed in immunoprecipitation buffer, and biotinylated proteins were captured with NeutrAvidin beads (Thermo Scientific). Biotinylated molecules were eluted from the beads by incubation in Laemmli buffer at 37 °C for 15 min.

**Quantification of Levels of PS1NTF, PS1 CTF, PEN-2, NCT, APH-1, and Full-length APP by ImageJ**—Immunoblot images were scanned, and the densities of γ-secretase components, APP, and actin were quantified by ImageJ. Levels of γ-secretase components and APP were normalized to the levels of actin. Data are represented as mean ± S.E.

**RESULTS**

**Generation of Synthetic Antibodies Recognizing the Nicastrin ECD**—We have previously reported the identification and characterization of two NCT-specific synthetic antibodies, Fab2 and Fab12, using phage display approaches (21, 25). In this work, we identified two additional anti-NCT antibodies using an NCT fragment encompassing the entire ectodomain, termed ECD, purified from the conditioned medium of N-acetylgalcosaminyltransferase 1-deficient (GnT1<sup>−/−</sup>) HEK293S cells that stably express the ECD fragment, as the antigen. To map the epitopes of these Fabs, termed FabA9 and FabG9, we performed immunoprecipitation studies under native conditions using His<sub>s</sub>-tagged ECD or an NCT fragment encompassing sequences encoded by exons 7–16, termed 716. As we showed earlier (21), Fab12 and Fab2 immuno-precipitated ECD (Fig. 1A, top panel, lanes 3 and 4), whereas Fab2, but not Fab12, also recognized 716. A negative control, Fab2-2,
Nicastrin scFv Affects γ-Secretase Assembly and Trafficking

failed to immunoprecipitate either ECD or 716, as expected (Fig. 1A, lane 2). In contrast, FabA9 and FabG9 recognized ECD but not 716, suggesting that these new Fabs recognize epitopes in the region encoded by exons 1–6. To examine whether these new Fabs bind to linear epitopes or conformational epitopes, purified ECD and 716 fragments were subjected to Western blot analysis using FabA9 or FabG9 as primary antibodies. FabA9 detected a denatured ECD fragment on the blot (Fig. 1B, top panel, lane 1), suggesting recognition of a linear epitope. In contrast, FabG9 failed to detect the NCT fragment in the Western blot, strongly suggesting that it recognizes a conformational epitope. Therefore, we generated two novel antibodies to the NCT ECD.

Stable Expression of scFvG9 in HEK293 Cells Decreases the Steady-state Levels of All γ-Secretase Components Except APH-1—To examine the function of NCT-specific synthetic antibodies in mammalian cells, we reformatted the Fabs into scFvs. The scFvs were fused to human transthyretin signal peptide to target the polypeptide to the lumen of the endoplasmic reticulum. To facilitate scFv detection and purification, we attached myc and His6 epitope tags at the very C terminus of the scFv. As a negative control, we prepared scFv 2-2, a non-binding antibody. The expression of scFvs and their secretion into conditioned medium were confirmed by immunoblot analysis (Fig. 1C, lanes 3–5). To confirm the function of scFvA9 and scFvG9, we tested their ability to capture ECD. Conditioned medium containing ECD and those from HEK293 cells expressing individual scFv were mixed and incubated at 4 °C overnight. A significant amount of ECD was immunoprecipitated by both scFvA9 and scFvG9 (Fig. 1C, lanes 4 and 5), confirming that both antibodies retain their binding function in the scFv format.

We then expressed scFvA9 and scFvG9 in naïve HEK293 cells and examined their effects on the maturation of endogenous NCT and levels of γ-secretase components in stable pools. We chose to generate stable pools to avoid significant caveats associated with variable expression between individual cell lines and genomic insertion effects on endogenous genes. In the case of stable pools, these issues are essentially “normalized.” In the stable pools, the most striking observation is the accumulation of immature NCT and a reduction in levels of mature NCT in scFvG9 cells compared with naïve 293 cells or cells expressing scFvA9 (Fig. 2A, first panel, compare lane 4 with lanes 1 and 3). In parallel, we observed a reduction in steady-state levels of PS1 and PEN-2 but with no obvious decrease in the levels of APH-1 in lysates prepared from scFvG9 cells compared with naïve 293 cells (Fig. 2A, second through fifth panels, compare lanes 1, 3, and 4). To examine the effects of expressing the scFvs on APP processing, we stably transfected HEK293 cells that constitutively express WT APP or the familial AD-linked APPSwe variant with cDNAs encoding scFvA9, scFvG9, and scFv2-2 (control). As we observed in naïve 293 cells, APP wt cells and APPSwe cells stably expressing scFvG9 also exhibited partial impairment of NCT maturation (Fig. 2A, first panel, lanes 8 and 12), which was paralleled by a reduction in the steady-state levels of PS1 and PEN-2 (Fig. 2A, second, fourth, and fifth panels, lanes 8 and 12).

Expression of scFvG9 Does Not Enhance the Turnover of γ-Secretase Components—To assess the possibility that scFvG9 binding to NCT might lead to destabilization of the γ-secretase complex, we treated APPSwe/scFv stable cells with the protein synthesis inhibitor cycloheximide to examine the turnover rate of γ-secretase components. Immunoblot analysis showed that PS1 NTF, a subunit of the mature γ-secretase complex, was stable in scFvG9 cells as in other stable cell pools (Fig. 3A, first row, and B). Similarly, NCT also exhibited a similar level of stability in scFvG9 cell pools as in other stable cell pools (Fig. 3A, first row, and B). On the other hand, cycloheximide treatment lead to a rapid decrease in full-length APP in all cell pools, with a half-life of ~2 h, as described previously (36) (Fig. 3A, third row, and B). To provide additional confirmation of these results, we then asked whether the levels of γ-secretase components could be elevated by the proteasome inhibitor MG132. In this case, we observed a modest elevation of PS1 NTF in all stable cell pools after treatment over 8 h, which likely reflects new synthesis of PS1 and conversion (Fig. 3, C and D). Collectively, these results suggest that, in scFvG9 cells, the stability of mature γ-secretase components is no different from that observed in other stable cells analyzed, and we infer that the decrease in steady-state levels of γ-secretase complex components observed in scFvG9 cells is likely the result of rapid degradation of immature NCT molecules following synthesis, leading to reduced association and, hence, stabilization of subunits.
of the γ-secretase complex. This view further suggests that scFvG9 binds to NCT early in its biosynthetic processes.

Expression of scFvG9 Decreases Notch Processing in HEK293 Cells—To assess the impact of these scFvs on γ-secretase activity, we first analyzed Notch processing in the APPSwe cells that stably express vector, scFv2-2, scFvA9, or scFvG9. For these studies, we transiently transfected APPSwe/scFv stable cells with cDNA encoding myc6-tagged mN/T, a Notch derivative corresponding to NEXT (28). Western blot studies revealed that γ-secretase-mediated cleavage of NEXT at the e site that leads to production of NICD was reduced in scFvG9 stable cells compared with the other stable cells (Fig. 4A, top panel, compare NICD in lanes 7 and 8 with lanes 1–6). To confirm this result, we performed pulse-chase analyses. The results revealed that NICD production is reduced in scFvG9 cell pools compared with the other cell pools (Fig. 4A, center and bottom panels, compare NICD in lanes 7 and 8 to lanes 1–6). The production of NICD during the 40-min pulse in scFvG9 cell pools was about 26% of that in vector pools (Fig. 4B), and the production of NICD in scFvG9 cell pools after the 2-h chase was about 56% of that in vector pools (Fig. 4C). We considered the possibility that the reduction in NICD production in these cellular assays may reflect alterations in the trafficking of γ-secretase to subcellular compartments where the Notch derivative, NEXT, is processed to NICD. Therefore, we analyzed the production of NICD in in vitro γ-secretase activity assays in which whole cell membranes prepared from HEK293Swe/scFv double-stable cells were incubated with a truncated Notch substrate similar to NEXT. These assays confirmed a significant reduction in the production of NICD in membranes prepared from scFvG9 cells compared with membranes prepared from all other cell pools (Fig. 4D). These findings argue that the total γ-secretase activity is reduced in these cells.

Expression of scFvG9 Has No Effect on Aβ Production but Impairs the Generation of AICD—We next examined APP processing in APPSwe cells that stably express vector, scFv2-2, scFvA9, or scFvG9 and observed a modest accumulation of the α- and β-secretase-generated APP CTFs in scFvG9 cells compared with CTFs in the other cell pools (Fig. 5, A, second panel, B, second panel, C, second panel, and D, second panel). Parallel analysis of full-length APP in cell lysates and soluble APP in conditioned medium showed no differences between each of the pools (Fig. 5, A, fourth panel, B, fourth panel, C, fourth panel, and D, fourth panel). Consistent with the immunoblot analyses, ELISA assays also revealed that the levels of secreted Aβ38, Aβ40, and Aβ42 in the conditioned medium of all APPSwe/scFv stable cell pools were indistinguishable (Fig. 5B). APP CTFs are also subject to processing by γ-secretase at the e site (37), which is distal to the carboxyl terminus of Aβ peptides, to generate 49- to 50-amino acid acid APP intracellular domain fragments, termed AICDs, that are virtually undetectable because of rapid intracellular turnover. To visualize this APP derivative, we performed pulse-chase analyses. APPSwe/scFv stable cells were pulse-labeled with [35S]me-
thionine for either 10 min or 4 h. In the 10-min pulse-labeled samples, the levels of immature full-length APP were similar in all cell pools (Fig. 5C, first panel). Similarly, after 4 h of labeling, the levels of both immature and mature APP in cell lysates (Fig. 5C, second panel) and soluble APP derivatives (APPs and Aβ) in the conditioned medium were essentially identical between all the cell pools (Fig. 5C, fifth and sixth panels). Notably, the maturation of APP was unaffected in scFvG9 cells compared with the other cell pools (Fig. 5A, first panel, and C, second panel), corroborating the selective effect of scFvG9 on NCT maturation. The increase of APP CTF levels in scFvG9 cells (Fig. 5A) was confirmed by radiolabeling and immunoprecipitation (Fig. 5C, third panel, lanes 7 and 8). The sensitivity of the prolonged 4-h labeling allowed us to detect AICD in all of the stable cells, and, importantly, the AICD levels in radiolabeled scFvG9 cell lysates were lower than those found in other stable cell pools (Fig. 5C, fourth panel, compare AICD in lanes 7 and 8 to lanes 1–6). Quantification of the labeled AICD fragment showed an ~54% reduction in scFvG9-expressing cells (Fig. 5D), similar to the reduction in the generation of NICD (Fig. 4). Finally, immunoprecipitation studies demonstrated that the levels of newly synthesized, radiolabeled, Aβ-related peptides that were

<table>
<thead>
<tr>
<th>APPSwe/vector</th>
<th>APPSwe/scFv2-2</th>
<th>APPSwe/scFvA9</th>
<th>APPSwe/scFvG9</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 1 2 4 6 8 8</td>
<td>0 1 2 4 6 8 8</td>
<td>0 1 2 4 6 8 8</td>
<td>0 1 2 4 6 8 8</td>
</tr>
<tr>
<td>mat, NCT</td>
<td>imm', NCT</td>
<td>mat, NCT</td>
<td>imm', NCT</td>
</tr>
<tr>
<td>PS1 NTF</td>
<td>PS1 NTF</td>
<td>PS1 NTF</td>
<td>PS1 NTF</td>
</tr>
<tr>
<td>APP FL</td>
<td>APP FL</td>
<td>APP FL</td>
<td>APP FL</td>
</tr>
<tr>
<td>Actin</td>
<td>Actin</td>
<td>Actin</td>
<td>Actin</td>
</tr>
</tbody>
</table>
Nicastrin scFv Affects γ-Secretase Assembly and Trafficking

scFvG9 Interacts with Immature but not Mature γ-Secretase Complexes—The studies shown in Fig. 3, A and B, clearly demonstrate that the reduced levels of γ-secretase components in scFvG9 cell pools (Fig. 2) is not the result of enhanced degradation or decreased stability of the mature complex. Therefore, we asked whether the effect of scFvG9 on the steady-state levels of γ-secretase components may occur at an earlier stage of complex assembly. To address this issue, we solubilized membrane fractions from APPSwe/scFv stable cells with 1% CHAPSO, conditions under which γ-secretase remains assembled (38). As expected, anti-myc antibody immunoprecipitated scFv2-2, scFvA9, and scFvG9 from corresponding scFv-expressing cell lysates (Fig. 6A, sixth row, lanes 7, 11, and 15), whereas scFv2-2, a negative control antibody, did not coimmunoprecipitate any endogenous γ-secretase component (Fig. 6A, lane 7). scFvA9 immunoprecipitated only a small amount of endogenous, immature NCT (Fig. 6A, first row, lane 11) but no other γ-secretase component (Fig. 6A, lane 11). In contrast, scFvG9 immunoprecipitated elevated levels of immature NCT species (Fig. 6A, first row, lane 15). Moreover, scFvG9 coimmunoprecipitated full-length PS1 and APH-1 that would be expected if early assembled NCT-associated subcomplexes are being recognized. In support of this view, scFvG9 failed to coimmunoprecipitate PEN-2 or PS1 NTF, components of mature, active γ-secretase complexes (Fig. 6A, lane 15). Conversely, an anti-NCT N-terminal antibody immunoprecipitated endogenous NCT from all APPSwe/scFv stable cell lysates (Fig. 6A, first row, lanes 4, 8, 12, and 16), but, more importantly, the only scFv that was coisolated was scFvG9 (Fig. 6A, sixth row, lane 16). To validate the findings above, we performed coimmunoprecipitation studies with an antibody raised against an aminoterminal epitope of PS1. As expected, this antibody coimmunoprecipitated PS1 CTF and other γ-secretase components from all of the APPSwe/scFv stable cells (Fig. 6B, lanes 3, 6, 9, and 12), but in no case did we detect scFv coisolated with this antibody (Fig. 6B, fourth row, lanes 3, 6, 9, and 12). These results strongly suggest that scFvG9 binds to immature NCT in isolation and/or in immature γ-secretase complexes.

On the basis of the cycloheximide/MG132 experiments (Fig. 3) and coimmunoprecipitation studies (Fig. 6, A and B) and the earlier demonstration that NCT maturation was impaired in scFvG9 cells (Fig. 2), we asked whether the binding of scFvG9 to either NCT alone or to NCT-containing immature complexes leads to retention of the complexes in intracellular compartments of the secretory pathway, therefore excluding residence on the cell surface where intramembranous processing of NEXT occurs (39). To test this possibility, we used the membrane-impermeable cross-linker sulfosuccinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate to assess the levels of γ-secretase complexes on the surface of APPSwe/scFv stable cells. Although the total levels of NCT in all scFv cells were similar, the levels of surface NCT in scFvG9 cells were reduced significantly (Fig. 6C, compare lanes 4 with lanes 1–3 and lanes 8 with lanes 5–7). Collectively, these findings suggest that, although APP-CTFs can be efficiently processed by γ-secretase complexes that are present in endosomal and/or recycling compartments in cells expressing scFvG9, these complexes fail

secreted from the stable cell pools were equal between the cell pools, findings that fully corroborate the results obtained by Western blot analysis (Fig. 5A) and ELISA (Fig. 5B) studies of accumulated Aβ in the medium. Although we did not observe any significant differences in levels of Aβ between the stable cell pools, two polypeptides that migrated between the Aβ and p3 appeared to be more pronounced in the conditioned medium of scFvG9 cells compared with the conditioned medium of the other cell pools (Fig. 5C, sixth panel). To determine the spectrum of secreted Aβ-related peptides, we immunoprecipitated Aβ-related species from the conditioned medium and subjected the recovered material to MALDI-TOF mass spectrometry. This approach failed to identify any differences in the spectrum of Aβ-related peptides between cells expressing scFvG9 and cells expressing scFv2-2 or scFvA9 (Fig. 5E). scFvG9 has no perceptible effect on γ-secretase-mediated APP processing to generate Aβ peptides, but expression of this scFv reduces AICD production.

FIGURE 4. Stable expression of the single-chain fragment variable G9 decreases NICD production. A, top panel, HEK293 APPSwe/scFv double-stable cells were transiently transfected with cDNA encoding mouse NCT for 48 h, and immunoblot analysis was performed on cell lysates to examine full-length mNCT substrate and γ-secretase-generated NCT fragments. Center and bottom panels, HEK293 APPSwe/scFv double-stable cells that were transiently transfected with cDNA encoding mouse NCT were pulse-labeled for 40 min with [35S]methionine (center panel, pulse), and one set of labeled cells was further chased for 2 h (bottom panel, chase). Cell lysates were immunoprecipitated with anti-myc antibody to detect both full-length mNCT and NCT fragments. WB, Western blot. B, level of NICD detected as the percentage of that in [35S]methionine-labeled HEK293 APPSwe/vector cells during the 40-min pulse labeling. Data are the mean ± S.E. (n = 2), C. level of NICD detected as the percentage of that in [35S]methionine-labeled HEK293 APPSwe/vector cells during the 2-h chase. Data are mean ± S.E. (n = 2). D. In vitro γ-secretase activity assay of membrane preparations from HEK293 APPSwe/scFv double-stable cells with a Notch substrate. The production of NICD was measured by ELISA. Data are mean ± S.E. (n = 2).
to mature and accumulate on the cell surface, therefore limiting intramembranous processing of 

**DISCUSSION**

The γ-secretase complex, composed of PS, PEN-2, APH-1, and NCT, catalyzes the intramembranous proteolysis of over 90 membrane-tethered substrates, including APP and Notch. Although γ-secretase inhibitors have been developed that effectively block the production of pathogenic Aβ peptides in vivo, clinical trials of such agents have been terminated because of Notch-based liabilities. Other strategies under consideration include the development of γ-secretase “modulators” that inhibit processing of APP to generate Aβ peptides but spare processing of Notch (6). Both classical γ-secretase inhibitors and γ-secretase modulators appear to target PS, the catalytic center of the enzyme complex (40). Alternatively, we and others have examined the feasibility of modulating NCT function using antibody-based approaches (21–24) with the notion that NCT-specific antibodies could bind to and modulate the binding of NCT to individual substrates. The rationale for this
Nicastrin scFv Affects γ-Secretase Assembly and Trafficking

approach is the finding that the activity of γ-secretase-containing NCT subunits harbors a series of experimentally generated mutations can differentially affect the processing of APP versus Notch (41).

In this report, we examined the mechanism of action of two novel anti-NCT Fabs and offer several important insights regarding their modulation of γ-secretase function. First, we show that one of these, FabG9, binds to a conformational epitope in an amino-terminal segment of NCT contained within the region encoded by exons 1–6 and mammalian cells that stably express the scFv derivative, scFvG9, leading to lowered steady-state levels of PS, PEN-2, and mature NCT glycoforms. These results resemble the findings by Hayashi et al. (22) that described a NCT-specific scFv, termed 5201F, but we note several significant differences. First, 5201F was shown to bind to multiple regions of the NCT ECD. Expression of this antibody in mammalian cells lowered the levels of mature NCT, PEN-2, PS, and APH-1, whereas we did not observe any changes in APH-1 in cells expressing scFvG9. The levels of immature NCT are elevated in scFvG9 cells, a finding that does not parallel those seen in cells expressing 5201F, where both immature and mature forms of NCT were virtually depleted. Second, we report that, although the levels of mature NCT, PS, and PEN-2 are reduced in scFvG9 cells, treatment of these cells with the proteasome inhibitor MG132 fails to elevate the steady-state levels of these polypeptides over the treatment period. Therefore, we argue that reduced expression of the γ-secretase components is the result of enhanced degradation of these polypeptides immediately after biosynthesis. Unfortunately, the synthetic rate of γ-secretase components is extremely low (42), therefore precluding any estimation of degradation rates following synthesis of these polypeptides. Third, we demonstrate that intramembranous processing of Notch to generate NICD is reduced markedly in scFvG9 cells. Moreover, the production of the APP derivative AICD is also reduced in these cells. Most interestingly, the processing of APP to Aβ peptides appears to be unimpaired. In sharp contrast, Hayashi et al. (22) demonstrated a clear decrease in Aβ production from cells expressing the NCT-specific scFv 5201F, whereas Notch processing was not evaluated. These studies demonstrate that NCT is a feasible target to selectively modulate processing of γ-secretase substrates.

Our finding that production of AICD (37) is reduced in scFvG9 cells, whereas Aβ40 and Aβ42 peptide production appears spared, is seemingly discordant with earlier studies by Ihara and co-workers (43), which showed that AICD and Aβ peptides are produced at equimolar ratios. This has led to the view that the amino-terminal derivative generated following ε cleavage of C99, the product of BACE1 cleavage of APP, is successively trimmed to generate the termini of Aβ40 and Aβ42. However, it should be noted that these studies were conducted in cell-free assays with purified membranes and C99. On the other hand, AICD that is generated in cells and tissues is extremely difficult to detect because, like NICD, these derivatives are subject to rapid intracellular degradation. The mechanistic relationship of ε-generated AICD to γ-secretase-mediated cleavage to generate the termini of Aβ peptides is largely unclear, but several independent studies have suggested that γ and ε cleavages are independent. For example, expression of several familial AD-linked PS1 variants elevate the production of Aβ42 but fully block AICD formation (44–46). Similarly, Hecimovic et al. (50) have reported that mutations within the APP transmembrane domain that elevate Aβ42 production have no effect on production of AICD. In this regard, He et al. (47) demonstrated that a γ-secretase-activating protein increases Aβ production but appears to reduce the generation of AICD.

The mechanism by which scFvG9 reduces levels of mature NCT, PS, and PEN-2 is not fully understood. However, our demonstration that scFvG9 can be coimmunoprecipitated with immature NCT would lead us to propose that antibody binding results in unfavorable folding kinetics and/or glycosylation of a population of these molecules, leading to retention and subse-
Nicastrin scFv Affects γ-Secretase Assembly and Trafficking

FIGURE 7. Proposed model of scFvG9 binding to NCT and immature γ-secretase complexes. The single-chain fragment variable G9 is depicted as two attached small ovals (VH and VL). NCT is shown as a hammer, APH-1 is shown as a square, PS1 is shown as an irregular quadrilateral shape, and PEN-2 is shown as an oval. Our data suggest that scFvG9 binds to NCT alone or immature γ-secretase subcomplexes rather than a mature complex, leading to retention and subsequent degradation of these components in the endoplasmic reticulum.

References


Acknowledgments—We thank A. A. Kossiakoff for discussions. We also thank Jacob Becker and Maggie Scimeca for assistance with the experiments.
Nicastrin scFv Affects γ-Secretase Assembly and Trafficking


