Positive and Negative Regulation of the \(\gamma\)-Secretase Activity by Nicastrin in a Murine Model*

Jinhe Li, Gregory J. Fici, Chai-An Mao, Richard L. Myers, Rongqing Shuang, Gregory P. Donoho, Adele M. Pauley, Carol S. Himes, Wenning Qin, Ismail Kola, Kalpana M. Merchant, and Jeffrey S. Nye

From the Discovery Research, Pfizer Inc., Kalamazoo, Michigan 49001

Nicastrin is a component of the \(\gamma\)-secretase complex that has been shown to adhere to presenilin-1 (PS1), Notch, and APP. Here we demonstrate that Nicastrin-deficient mice showed a phenotype that is indistinguishable from PS1/PS2 double knock-out mice, whereas heterozygotes were healthy and viable. Fibroblasts derived from Nicastrin-deficient embryos were unable to generate amyloid \(\beta\)-peptide and failed to release the intracellular domain of APP or Notch1-Gal-VP16 fusion proteins. Additionally, C- and N-terminal fragments of PS1 and the C-terminal fragments of PS2 were not detectable in Nicastrin-null fibroblasts, whereas full-length PS1 accumulated in null fibroblasts, indicating that Nicastrin is required for the endoproteolytic processing of presenilins. Interestingly, cells derived from Nicastrin heterozygotes produced relatively higher levels of amyloid \(\beta\)-peptide whether the source was endogenous mouse or transfected human APP. These data demonstrate that Nicastrin is essential for the \(\gamma\)-secretase cleavage of APP and Notch in mammalian cells and that Nicastrin has both positive and negative functions in the regulation of \(\gamma\)-secretase activity.

Alzheimer’s disease is characterized by the progressive accumulation of cerebral deposition of 39–42-amino-acid peptides, termed \(\beta\)-amyloid (\(\beta\)\(\beta\)). \(\beta\)\(\beta\) peptides are generated by consecutive proteolysis of amyloid precursor protein (APP) by \(\beta\)-secretase and \(\gamma\)-secretase (1). \(\beta\)-Secretase has been characterized as a single membrane-bound aspartyl protease (2), whereas \(\gamma\)-secretase is a membrane-bound aspartyl protease complex (3). Presenilin 1 and 2 (PS1 and PS2) are key components of the \(\gamma\)-secretase complex because double knock-out of PS1 and PS2 fails to generate \(\beta\)\(\beta\) (4, 5), and mutations of either of the two conserved critical aspartates of PS1 and PS2 dominantly reduce \(\beta\)\(\beta\) production (6–8). The other candidate components of the \(\gamma\)-secretase complex, Nicastrin (9), Aph-1a and b, and Pen-2 (10–12), also appear to be required for \(\beta\)\(\beta\) production in cell culture experiments. Nicastrin is a type-1 transmembrane glycoprotein identified as a component of the membrane-bound \(\gamma\)-secretase complex that has been shown to adhere to PS1, Notch, and APP (9, 13, 14). In Drosophila cells, Nicastrin as well as PS, Aph-1, and Pen-2 are required for Notch signaling and the \(\gamma\)-secretase cleavage of Notch and APP (10, 15–17). Nicastrin is also one of the core components of the invertebrate \(\gamma\)-secretase complex that is important for the stabilization of the Drosophila presenilin protein (10, 17). Despite an ability to functionally substitute human Nicastrin for the worm or fly gene (10, 15–17), the role of mammalian Nicastrin in the \(\gamma\)-secretase cleavage of Notch and APP is less well characterized. A reduction of Nicastrin expression in cell cultures by RNA interference reduces \(\gamma\)-secretase cleavage of APP to \(\beta\)\(\beta\) (9), but the role of Nicastrin in mammalian development is less clear. By comparison, the requirement of PS genes for the intramembranous cleavage of APP and Notch has been demonstrated not only in mammalian cells (4, 18) but also in vitro in the mouse (5, 21). Thus PS1- and PS1/PS2-deficient mice showed Notch phenotypic embryonic lethality, and their embryonic neuronal and fibroblast cultures showed reduced \(\gamma\)-secretase-mediated cleavage of APP and Notch and reduced production of \(\beta\)\(\beta\) (22–27). The goal of the present study was to elucidate the function of Nicastrin in the mouse by generating a genetic knock-out (KO) animal. Here we demonstrate that Nicastrin-deficient mice showed features similar to PS1/PS2 double KO mice, including Notch phenotypic embryonic lethality, and their embryonic neuronal and fibroblast cultures showed reduced \(\gamma\)-secretase-mediated cleavage of APP and Notch and reduced production of \(\beta\)\(\beta\) (22–27).

EXPERIMENTAL PROCEDURES

Generation of Nicastrin Knock-out Mouse Line—A targeting vector was designed to effectively delete the N-terminal 63 amino acids of Nicastrin by replacement with an IRESlacZ/MC1neo cassette (28). This resulted in the removal of 1530 genomic bases, including a portion of exon 1 beginning 3 nucleotides upstream of the translation initiation codon and extending into intron 2. After targeted 129/SvEvBrd embryonic stem cells were verified by Southern blot using 5'-external and 3'-internal probes, male chimeras were generated by blastocyst microinjection and were mated to C57BL/6 albino females. Agouti offspring heterozygotes for the Nicastrin KO allele were used for continued backcross to C57BL/6 mice to establish a colony for subsequent experiments.

Embryonic Analysis—Midday of the plug day was designated as embryo day 0.5 (E0.5). Embryos were dissected in ice-cold phosphate-buffered saline from timed pregnant females and fixed in 4% paraformaldehyde in phosphate-buffered saline at 4°C. Whole mount in situ hybridization was performed as described (29). The template for Nicastrin in situ probe was generated by PCR using primers (5’-TGAACCTGAGGCAAGCTCATACTT-3’) and AR (5’-GGGAACTTGCGTCTGATCAGTG-3’) and subcloned into pCRII-TOPO (Invitrogen). Digoxigenin-labeled RNA probe was synthesized using the DIG RNA labeling kit (Roche Applied Science).

Embryonic Fibroblast Culture—Mouse embryonic fibroblasts were
isolated by trypsinization of separate embryos at E8–E12. Homogenous cell suspensions were maintained and expanded in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cells grown less than seven passages were used in the experiments.

**Recombinant Adenovirus and Infection of Fibroblasts—**Human APP695 (A33292) cDNA from the initiation methionine to the stop codon with the Swedish mutation (Lys at position 595 to Asn and Met-596 to Leu) was cloned into pPepAdapt10DestB, a Gateway vector with an optimized CMV promoter and Ad5 adenovirus backbone (Galapagos Genomics). The recombinant virus was produced and purified by Galapagos Genomics. Viral titer after the final propagation was 1.8 × 10¹⁰ viral particle/mL. The fibroblast cells were infected with the recombinant adenovirus with a viral titer of a multiplicity of infection of 100 in culture dishes containing 5 × 10⁴ cells/cm². Control infections were done using a recombinant adenovirus bearing GFP cDNA (Ad5.CMV-GFP, Biogen) at the same multiplicity of infection.

**Detection of Aβ in Conditioned Medium—**Conditioned medium was collected from the fibroblast cultures for 3 days with the addition of protease inhibitor mixture (Complete, Roche Applied Science) followed by cell viability assay using the MTS One Solution cell proliferation assay (Promega) for normalization of data to viable cell number. Analysis of the conditioned medium for human Aβ1–40 was performed using a liquid phase direct sandwich enzyme-linked immunosorbent assay following antibodies separately: rabbit polyclonal antiserum C8KK that was directed to the C-terminal 8 amino acids of APP695 with a dilysine motif ([37]) and an optimized CMV promoter and Ad5 adenovirus backbone (Ga-

**Western Blot Analysis—**The fibroblast cell cultures at the last day of collection from the fibroblast cultures for 3 days with the addition of protease inhibitor mixture (Complete, Roche Applied Science) followed by cell viability assay using the MTS One Solution cell proliferation assay (Promega) for normalization of data to viable cell number. Analysis of the conditioned medium for human Aβ1–40 was performed using a liquid phase direct sandwich enzyme-linked immunosorbent assay following antibodies separately: rabbit polyclonal antiserum C8KK that was directed to the C-terminal 8 amino acids of APP695 with a dilysine motif ([37]) and an optimized CMV promoter and Ad5 adenovirus backbone (Ga-

**RESULTS**

**Generation of Nicastrin-deficient Mice—**Nicastrin-deficient mice were generated by homologous recombination (see “Experimental Procedures”). No obvious abnormalities were observed in Nicastrin −/− mice. Nicastrin −/− embryos began to show somite segmentation defect at embryonic day 8.5 (E8.5) and died before E10.5 (Fig. 1a). This phenotype is similar to that of PS1 −/−/PS2 −/− double KO mice (5, 21) but more severe than PS1 −/− or Notch1 −/− single KO mice (23–26) and has been proposed to be a result of disturbed Notch signaling (23, 26). The expression of Nicastrin mRNA detected by in situ hybridization was ubiquitous throughout the wild type embryo at E10.5 (data not shown), similar to PS1 expression during early development (27).

**Lack of Aβ Production in Nicastrin −/− Fibroblasts—**To study the γ-secretase activity in Nicastrin-deficient cells, we derived fibroblasts from the embryos of Nicastrin +/+, +/−, and −/− genotypes at E8–E12 (see “Experimental Procedures”). Cells with equivalent passage numbers were infected with recombinant adenovirus expressing either GFP or human APP bearing the Swedish familial Alzheimer’s disease mutations (APPSw, Lys at position 595 to Asn and Met-596 to Leu) (31). GFP expression levels after GFP adenovirus infection in cells of all three genotypes were comparable (data not shown). The levels of Aβ1–40 and 1–42 in the conditioned medium were assessed over the course of 3 days of culture, and cell viability and cell extracts were examined on the last day (see “Experimental Procedures”). The conditioned medium of Nicastrin +/− and Nicastrin −/− fibroblast cultures expressing APPsw showed accumulation of both human Aβ1–40 and 1–42, whereas conditioned medium of Nicastrin −/− cultures showed no detectable human Aβ1–40 or 1–42 (<25 pg/ml) (Fig. 2, a and b). In contrast, APPsw holoprotein expression in the lysates from these cells was comparable between cultures of the different genotypes (Fig. 3). In addition, we were able to measure the production of secreted endogenous mouse Aβ1–40 in Nicas-

**Altered γ-Secretase Activity in Nicastrin KO Mice**

**FIG. 1. Phenotype of Nicastrin knock-out mice.** a, representative embryos with the different genotypes from a single litter at day E8.5. The Nicastrin −/− embryos showed abnormally segmented somite pairs as compared with the normal somite pairs in the Nicastrin +/− embryos (arrows). b, representative whole mount in situ hybridization result showing Nicastrin mRNA expression throughout E8.5 embryos.

---

*J. Li, G. J. Fici, C.-A. Mao, R. L. Myers, R. Shuang, G. P. Donoho, A. M. Pauley, C. S. Himes, W. Qin, I. Kola, K. M. Merchant, and J. S. Nye, unpublished data.*
generated by the β-secretase cleavage of APP, in contrast to Nicastrin+/− and Nicastrin−/− cell lysates that showed minimal levels of APP-CT99 (Fig. 3). Taken together, these results demonstrate that Aβ production was abolished in cells lacking Nicastrin, similar to the results from the PS1/PS2 double KO embryos (23, 24, 32–35).

**Altered γ-Secretase Activity in Nicastrin KO Mice**

**FIG. 2.** Altered Aβ production in Nicastrin knock-out embryonic fibroblast cultures. The fibroblasts were infected with recombinant adenovirus expressing APPsw. As shown in a and b, Aβ1–40 (a) and Aβ1–42 (b) in the conditioned medium of fibroblast cultures of the three Nicastrin genotypes were measured, and the values were normalized by cell viability measured by MTS One Solution cell proliferation assay (see “Experimental Procedures”). All the measurements were done using the same conditioned medium. The data were from a representative experiment as mean values of three cultures. ND, non-detectable. As shown in c, mouse endogenous Aβ1–40 in the conditioned medium were measured and normalized as described in a. As shown in d, Nicastrin−/− fibroblasts were infected with recombinant adenovirus expressing APPsw and then transfected with Nicastrin cDNA and control vector pcDNA3 followed by the measurement of Aβ1–40 in the conditioned medium as described in a. Student’s t test was used to compare wild type and heterozygotes values. NCT, Nicastrin.

**FIG. 3.** Accumulation of CT99 in Nicastrin−/− embryonic fibroblasts. The fibroblasts were infected with recombinant adenovirus expressing APPsw or GFP. Protein lysates from the fibroblasts of each Nicastrin genotype were analyzed by Western blot (see “Experimental Procedures”). The data were from a representative Western blot using lysates from the same cell cultures for Aβ measurement described in the legend for Fig. 2.

**FIG. 4.** Altered γ-cleavage of CT99 and Notch in Nicastrin−/− embryonic fibroblasts. Reporter assays for γ-cleavage of CT99 and Notch were performed by co-transfection of the cells of the different genotypes with Notch1IC-G4VP16 or CT99-G4VP16 and UAS-Luc reporter firefly luciferase followed by luciferase assays (see “Experimental Procedures”). The corrected luciferase activity was defined as the ratio of the values of firefly luciferase activity and the values of the control reporter renilla luciferase activity in each assay. The data were from a representative experiment as mean values of three measurements for CT99 and six measurements for Notch in each genotype culture (see “Experimental Procedures”).

**FIG. 5.** Destabilization of PS-NTF and -CTF and accumulation of full-length PS in Nicastrin−/− embryonic fibroblasts. Protein lysates from the fibroblasts of each Nicastrin genotype were analyzed by Western blot (see “Experimental Procedures”). As shown in a, the levels of endogenous PS1 NTF and CTF were measured using the same blot shown in Fig. 3 and antibodies recognizing PS1 NTF and CTF. As shown in b, endogenous PS2 CTF level was measured using an antibody recognizing full-length PS2 and the CTF. As shown in c, endogenous full-length PS1 and the NTF and CTF were measured using an antibody recognizing full-length PS1 and the NTF and poorly recognizing the CTF. The data were from a representative Western blot.

**Reduced Intramembraneous Cleavage of APP-CT99 and Notch1IC Fusion Proteins in Nicastrin−/− Fibroblasts**—To evaluate further the effect of Nicastrin-deficiency on the intramembraneous γ-secretase activity, we measured the γ-secretase-mediated cleavage of APP-CT99 and Notch1IC.
fused with Gal4-VP16 (G4VP16) (32, 36, 37) in Nicastrin-deficient cells. These reagents provide a quantitative measurement of intracellular release and nuclear access of G4VP16 as measured by the activation of UAS-luciferase. Cells of the three genotypes were co-transfected with CT99-G4VP16 or NicastrinIC-G4VP16 together with UAS-Luc reporter followed by the measurement of firefly luciferase activity. These values were divided by the activity of a control thymidine kinase renilla luciferase reporter that was included in each experiment. The corrected luciferase activity elicited by both CT99 and NicastrinIC in Nicastrin+/− cells was dramatically reduced as compared with the wild type and heterozygotes cells (Fig. 4). Taken together, these results demonstrate that Nicastrin is required for the intramembranous cleavage and release of the intracellular domains of APP and Notch in mammalian cells.

**Failure of Cells to Accumulate Mature PS Proteins in Nicastrin+/− Fibroblasts**—To examine the role of Nicastrin in stabilizing the mammalian presenilin complex, we measured levels of endogenous PS1 and PS2 protein levels by Western blot analysis using antibodies recognizing PS1 NTF and CTF. PS1-NTF and -CTF and PS2-CTF levels were detected in the Nicastrin+/+ and Nicastrin+/− cells but were undetectable in the Nicastrin−/− cells (Fig. 5a). Similarly, PS2 CTF was detected in the Nicastrin+/− cells but was undetectable in the Nicastrin−/− cells using an antibody recognizing PS2 holoprotein and the CTF (Fig. 5b). These results were consistent with the previous observations using an RNA interference approach (11). Furthermore, full-length PS1 was undetectable in the Nicastrin+/− cells but was observed in small amounts in the Nicastrin−/− cells (Fig. 5c). These results demonstrate that Nicastrin is essential for the accumulation or stabilization of mature PS1 fragments and suggest a role of Nicastrin in regulating the abundance of full-length PS1 as well. In contrast to PS1, full-length PS2 was not detected in the Nicastrin−/− cells. Although these data are consistent with differences between the respective roles of Nicastrin on PS1 and PS2 cleavage and/or stability, the relatively weak sensitivity of the antibody to full-length PS2 limits our ability to make such a strong conclusion.

**DISCUSSION**

The data presented here demonstrate that cells derived from Nicastrin−/− mice fail to produce Aβ and fail to release the intracellular domain of APP-CT99 and Notch1, similar to cells from PS1/PS2 double KO mice (5, 21, 23, 24, 32–35). Phenotypically, Nicastrin−/− embryos also resemble PS1/PS2 double KO embryos in displaying somitic abnormalities, indicating severe Notch pathway defects. These findings provide direct evidence of an essential role for Nicastrin in both murine development and the mammalian γ-secretase cleavage of APP and Notch, in agreement with a recent report (38).

Surprisingly, we also observed that the relative levels of endogenous mouse and human Aβ1–40 and 1–42 produced by Nicastrin+/− cells were higher as compared with Nicastrin+/+ cells. This relative increase in Aβ was observed both in neuroblastoma cells (data not shown) and in fibroblast cultures (Fig. 2) from Nicastrin heterozygous animals. These observations reveal that a partial reduction of Nicastrin expression from diploid to haploid levels stimulates Aβ production. Since Nicastrin is a component of the γ-secretase complex (9, 13, 14), the most logical explanation is that the partial deficiency of Nicastrin abrogates a weak inhibitory effect upon the γ-secretase directly, whereas a complete deficiency (or near deficiency, see Ref. 9) disrupts the γ-secretase function entirely. This finding is in contrast to the recent studies (38) that demonstrate a linear gene dose effect in Aβ secretion. The discrepancy may be related to several differences in experimental procedure including the strategy in generating the Nicastrin-null mice and Aβ measurement. Further studies are required to better understand regulation of Aβ production by Nicastrin. Prior work in *Drosophila* and mammalian cells treated with small interfering RNA has revealed only an essential or positive role for Nicastrin in the γ-secretase activity. However, a fuller examination of negative regulation by Nicastrin of the γ-secretase remains to be performed.

The present studies showing a positive and negative role of Nicastrin in the γ-secretase may fit with prior studies showing mutations in Nicastrin that decrease Aβ production (e.g. ΔS12–369) and others that increase it (e.g. D336A/Y337A) (9). Since the results presented here predict that a total and a partial loss of Nicastrin function would give rise to decreased and increased Aβ production, respectively, the published Nicastrin alleles may represent various levels of Nicastrin hypo-function along this continuum. Exactly how the mutated domains of Nicastrin exert their effects upon the γ-secretase remains to be established. In any case, a fuller appreciation of the complexity of the functional role of Nicastrin in γ-secretase regulation, and ultimately Aβ production, will be essential in designing any therapeutic strategy that utilizes Nicastrin as its target.

As noted previously, the abundance of mature PS1 and PS2 fragments is dramatically reduced in Nicastrin deficiency. We also observed a small increase in the abundance of PS1 holoprotein in Nicastrin knock-out cells (Fig. 5). At present, it is impossible to discern whether the enhancement of PS1 holoprotein is a consequence of its increased stability or results from a partial suppression of endoproteolysis of PS1. Recent studies have uncovered a role for Pen-2 in the cleavage of PS holoprotein (19), and by comparison, the deficiency of Pen-2 results in a more robust enhancement in the abundance of PS1 holoprotein than that found in Nicastrin-null cells (19). Since the endoproteolysis of PS proteins, also known as the presenilinase, is also generally dependent upon γ-secretase activity (20), it would be logical to observe some decline in the rate of PS endoproteolysis in the absence of a protein that is required for the γ-secretase complex.

**Acknowledgments**—We thank Ross Francis, Daniel Curtis, and members of the Exelixis team for helpful discussions.

**REFERENCES**

1. Walter, J., Kaether, C., Steiner, H., and Haass, C. (2001) *Curr. Opin. Neurobiol.* 11, 885–890
2. Vassar, R., and Citron, M. (2000) *Neuron* 27, 419–422
3. Sisodia, S. S., and St George-Hyslop, P. H. (2002) *Nat. Rev.* 3, 281–290
4. Herreman, A., Serneels, L., Anwari, W., Collen, D., Schoonjans, L., and De Strooper, B. (2000) *Nat. Cell Biol.* 2, 461–462
5. Zhang, Z., Nadeau, P., Song, W., Donoviel, D., Yuan, M., Bernstein, A., and Yankner, B. A. (2000) *Nat. Cell Biol.* 2, 463–469
6. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) *Nature* 398, 513–517
7. Steiner, H., Duff, K., Capell, A., Romig, H., Grim, M. G., Lincoln, S., Hardy, J., Yu, X., Picciano, M., Fechteler, K, Citron, M., Kopan, R., Pesold, B., Keck, S., Baader, M., Tomita, T., Iwatsubo, T., Baumeister, R., and Haass, C. (1999) *J. Biol. Chem.* 274, 28669–28673
8. Kimberly, W. T., Xia, W., Rahmati, T., Wolfe, M. S., and Selkoe, D. J. (2000) *J. Biol. Chem.* 275, 3173–3178
9. Yu, G., Nishimura, M., Araujo, S., Levitan, D., Zhang, L., Tandon, A., Song, Y. Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebshold, R., Farrer, L. S., Surbl, S., Bruni, A., Fraser, P., and St George-Hyslop, P. (2000) *Nature* 407, 48–54
10. Francis, R. Francis, R., McGrath, G., Zhang, J., Ruddy, D. A., Sym, M, Aepfelb, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M. C., Parka, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R. D., Ruble, C., Nye, J. S., and Curtis, D. (2002) *Dev. Cell* 3, 1–20
11. Edschafer, D., Winkler, E., Haass, C., and Steiner, H. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99, 8666–8671
12. Steiner, H., Winkler, E., Edschafer, D., Prokop, S., Basset, G., Yamasaki, A., and Selkoe, D. J. and Haass, C. (2002) *J. Biol. Chem.* 277, 39062–39065
13. Edschafer, D., Kimberly, W. T., Ostaszewski, B. L., Yu, W., Diehl, T. S., Selkoe, D. J., and Wolfe, M. S. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99, 2720–2725
14. Chen, F., Yu, G., Arawaka, S., Nishimura, M., Kawarai, T., Yu, H., Tandon, A., Supala, A., Song, Y. Q., Rogaeva, E., Milman, P., Sato, C., Yu, C., Janus, C., Lee, J., Song, L., Zhang, L., Fraser, P., E., and St George-Hyslop, P. (2001) *Nat. Cell Biol.* 3, 751–754
