Notch1 and Amyloid Precursor Protein Are Competitive Substrates for Presenilin1-dependent γ-Secretase Cleavage*

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Proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretases results in the production of a highly amyloidogenic Aβ peptide, which deposits in the brains of Alzheimer’s disease patients. Similar γ-secretase processing occurs in another transmembrane protein, Notch1, releasing a potent signaling molecule, the Notch C-terminal domain. It has been shown that both events are dependent on a presenilin-dependent protease. We now test the hypothesis that activated Notch1 and APP are competitive substrates for the same proteolytic activity in neurons. Treatment of neurons with the native Notch ligand, Delta, induces endogenous Notch1 intramembranous cleavage and diminishes Aβ production in a dose-dependent manner. Complementary experiments showed that the converse was also true. Overexpressing human APP (APP695Sw) in neurons leads to a decrease in endogenous Notch1 signal transduction, as assessed by a CBF1 luciferase transcription assay, by Notch C-terminal domain nuclear translocation in vitro and by analysis of Notch C-terminal domain generation and Notch1 staining in vivo. In summary, two complementary approaches suggest that APP and Notch1 are physiologically relevant competitive substrates for γ-secretase activity.

Amyloid peptide (Aβ)† is the major component of senile plaques in the brains of Alzheimer’s disease (AD) patients. Aβ peptide derives from the sequential proteolytic processing of a single pass transmembrane protein, the amyloid precursor protein (APP) by β- and γ-secretases (1–3). Processing of APP by these secretases is a normal physiological process. Cleavage by β-secretase produces a secreted N-terminal APPβ (sAPPβ) protein and a C-terminal C99 fragment, which can be cleaved by γ-secretase to produce the 40–42 amino acid Aβ. It has been suggested that a presenilin1 (PS1)-associated enzymatic activity is responsible for the intramembranous γ-secretase cleavage of APP (4–6). Whether PS1 is γ-secretase itself or is a critical cofactor modulating γ-secretase activity remains uncertain; however, photoaffinity labeling of PS1 by potent γ-secretase inhibitors indicates that PS1 may contain the active site of γ-secretase (7, 8).

Another single pass transmembrane receptor, Notch1, undergoes proteolytic processing upon ligand binding by an intramembraneous protease, resulting in the release and nuclear translocation of the signaling C-terminal domain of the Notch1 molecule, NICD (9–11). The C-terminal domain interacts with the CSL family of Notch effectors either on its way toward the nucleus or in the nucleus (12–16), causing activation of a cascade of gene products ultimately influencing neuronal phenotype during development (17–19) and neurite outgrowth in differentiated neurons (20, 21). Similar to APP γ-secretase cleavage, intramembranous cleavage of Notch1 depends on PS1 and PS2 proteolytic activities (11, 22). In addition, it has been shown that substrate-based difluoroketone peptidomimetics designed to block γ-secretase cleavage of APP (28, 29) also inhibit intramembranous proteolysis of Notch1 (11, 22). These data suggest that a similar PS1-dependent enzymatic activity is involved in intramembranous processing of both APP and Notch1.

In the present study we explore the possibility that APP and activated Notch1 compete for γ-secretase activity. We hypothesize that if both APP and Notch1 are processed by the same protease, then activated Notch1 may compete with APP for γ-secretase cleavage with a consequent decrease in Aβ production. Conversely, we predict that conditions that lead to elevated Aβ production (such as APP695Sw overexpression) will decrease Notch1 signaling. To test these predictions, we activated endogenous Notch by treatment of APP695Sw-overexpressing neurons with the soluble Notch1 ligand DI-Fc and showed that the level of Aβ in the conditioned medium was substantially decreased in a dose-dependent manner. In addition, we assessed the activity of the Notch1 signaling pathway in APP695Sw-overexpressing neurons by counting neurons with nuclear Notch1 staining and by measuring the transactivation of a Notch1 target gene, a CBF1 luciferase reporter. We found that both measures of Notch1 activation were significantly diminished in comparison to that observed in non-APP-overexpressing neurons prepared from control non-transgenic littermates. Finally, we examined Notch1 cellular localization in the hippocampal formation of adult APP695Sw-overexpressing mice and found a statistically significant decrease in nuclear localization of Notch1, suggesting that Notch activation is less efficient in the presence of increased APP expression in vivo. Taken together, these data support the idea that Aβ generation and Notch1 activation are competing biological processes.

**EXPERIMENTAL PROCEDURES**

**Primary Neuronal Cultures**—Primary neuronal cultures from mutant human APP695Sw-overexpressing transgenic mice, Tg 2576, (30)
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were prepared as described previously (31, 32). The neurons from individual embryos (E16-18, 10^6 cells/ml) were cultured separately, and tails of the embryos were dissected for genotyping to identify cultures from Tg (expressing human mutant APP) or non-Tg embryos. At 2–6 days in vitro (DIV), prior to treatment with Di-Fc or control medium, the growth medium in the neuronal cultures was replaced with fresh medium and Di-Fc or control medium were applied on a daily basis for 2 days.

CBF1 Luciferase Assay—The CBF1-luciferase assay was performed as described previously (11) using a CBF1 luciferase reporter construct (33) and β-galactosidase as an internal control for transfection efficiency. The assay was performed in triplicate using an LKB 1251 Luminometer.

Conditioned Medium (CM)—Conditioned medium containing a secreted form of the Notch ligand Delta, Di-Fc, was collected from cells growing in hygromycin B-free medium for 2 days prior to collection (34). Collected CM was concentrated 4–5-fold, incubated with an anti-Fluorescein antibody to precluster Di-Fc (11) and applied to primary neurons. CM from 293T cells, which do not secrete Di-Fc, served as a negative control and was prepared in a similar way as Di-Fc CM. 20, 40, or 60 μl of CM (Di-Fc or control) were applied to the primary neurons growing in 200 μl of the growth medium daily. 24 h after the last treatment with Di-Fc control CM, the total volume of neuronal conditioned medium was replaced with fresh growth medium to an equal amount in all treatments. The CM was collected for ELISA (Aβ) or Western blots (secreted APP, sAPP), and the cells were lysed to measure the total level of APP expression as well as the amounts of C-terminal (C83 and C99) APP fragments.

ELISA and Western Blots—Cell lysates and CM were adjusted to equal protein concentrations for the analyses. For Aβ ELISA we used 25 μl of tissue culture-conditioned medium. The capture antibody was 22C4, (to the C terminus of Aβ), and the detection antibody was biotinylated 6E10 (to Aβ residues 1–17) (35). For the Western blot analysis, the CM was electrophoresed on 10–20% Tricine gel for Aβ analysis or on 4–20% Tris-glycine gel for sAPP. SDS-polyacrylamide gel electrophoresis of the cell extracts was carried out on 4–20% Tris-glycine gel for total APP expression and for C-terminal fragments of APP (C83 and C99). The cells (brains) were lysed in a buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40. The immunoblotting was performed with the following anti-APP antibodies (C-terminal C99). The cells were immunoprecipitated with an anti-biotin antibody, washed with Tris-buffered saline, counterstained with Hoescht nuclei, and coverslipped using GVA mounting solution (Zymed Laboratories Inc.). The images of all neurons (confirmed by MAP2 staining) immunostained with anti-Notch1 antibody in 5–6 random visual fields per well were collected using × 40 objective (zoom 2) on a confocal microscope (Bio-Rad 1024) mounted on a Nikon Eclipse TE300 inverted microscope. Four wells per condition were examined in each experiment, and data were collected from two independent experiments. The percent of neurons containing nuclear Notch1 staining was recorded by an observer unaware of transgene status of the culture and was then calculated for APP-overexpressing and non-transgenic littermate controls. A total of 500 neurons were quantified.

Analysis of Notch1 Cellular Localization in Vivo—The sections of five-month-old Tg 2576 and control non-Tg mouse brains were immunostained with Notch1 antibody (TC) and counterstained with DAPI to localize the nuclei of the neurons. The location of the nucleus was identified in the DAPI channel, then a “mask” was applied to the Notch1 channel and the intensity of fluorescence within the nucleus was measured using a computerized image analysis system (Bioquant, Nashville, TN). The location of neurons within the CA1 field of the hippocampus to be measured was chosen using a systematic random sampling scheme analogous to those used in stereology; ~2,000 neurons were examined using a × 100 water immersion objective. The ratio of Notch1 nuclear fluorescence to total Notch1 fluorescence in the CA1 field was compared in three Tg2576 and three non-Tg control littermate mice using Student’s t test analysis.

RESULTS

APP Competes with Notch1 for γ-Secretase—To test the hypothesis that APP competes with Notch1 for γ-secretase processing, we activated endogenous Notch1 signaling in primary neurons by treatment with different concentrations of the Notch ligand, Di-Fc, for 2 days and measured the amount of Aβ in conditioned medium. Treatment with Di-Fc, but not control CM, appeared to cause a significant dose-dependent decrease in total Aβ, as assessed by Western blot analysis (Fig. 1A). To confirm this observation, we used a sensitive and specific sandwich ELISA to quantitate total Aβ. We found consistent, correlative decreases of total Aβ after treatment with Di-Fc in a dose-dependent manner, with the highest dose leading to a decrease of about 45% (p < 0.001) in Aβ production (Fig. 1B).
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Results are representative of nine independent experiments, each performed at least in duplicate.

We next examined whether the decrease in Aβ secretion after Notch1 activation with DI-Fc was due to any effects of this treatment on APP expression, metabolism or trafficking. We performed Western blot analysis of cell lysates (for APP expression and γ-secretase cleavage) and conditioned medium (for secreted APPs and APPβs). Three different antibodies to APP were used to analyze the level of APP expression: C-terminal 13G8, N-terminal 8E5, and Aβ-specific 6E10 antibodies. There was no change in the level of total APP expression in DI-Fc-treated neurons, in comparison to that in cells treated with control medium. The result was replicated in four independent experiments (Fig. 2A).

We also analyzed whether treatment with DI-Fc alters trafficking of APP molecules to the cell surface. To label cell surface proteins we biotinylated the surface molecules of the primary neurons with EZ-Link Biotin. Cell lysates were immunoprecipitated with an anti-biotin antibody, and immunoblotted with 8E5 to detect cell surface APP. There was no difference in the amount of biotinylated APP in DI-Fc-treated neurons in comparison to that in control CM-treated cells. The result was replicated in four independent experiments (Fig. 2A).

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We next performed the converse experiment. We reasoned that if γ-secretase activity is a limiting step, overexpression of APP might inhibit the efficiency of ligand-induced Notch1 cleavage/nuclear translocation/signal transduction by competing with Notch1 for the γ-secretase activity. Thus, we compared the amount of endogenous Notch signaling in response to its physiological ligand, Delta, in primary neuronal cultures derived from APP695Sw-overexpressing mice and non-transgenic littermate controls. Neurons in which Notch1 is activated show a predominantly nuclear pattern of staining reflecting translocation of the C-terminal proteolytic product, NICD, to the nucleus ((21); Fig. 3, A and B). The percent of neurons with nuclear-activated Notch1 was reduced by more than 50% (p < 0.001) in neurons from APP695Sw transgenic mice compared with that in neurons from non-transgenic littermates (Fig. 3C). Interestingly, the total level of Notch1 immunoreactivity in APP695Sw-overexpressing neurons was slightly higher than that in normal control littermates, perhaps reflecting diminished processing (Fig. 3, A and B).

To confirm that Notch1 signaling was indeed down-regulated in APP-overexpressing neurons we also measured activation of a Notch1 downstream transcription factor, CBF1, in the neuronal cultures. We co-transfected neurons prepared from control and APP-overexpressing transgenic mice with a CBF1 luciferase reporter construct (33) and β-galactosidase, an internal control for transfection efficiency, and measured luminescence caused by DI-Fc treatment 24 h post-transfection. There was a 50% reduction (p < 0.001) in CBF1 luciferase activity in neurons prepared from transgenic embryos overexpressing APP695Sw in comparison to that in control, non-transgenic neurons (Fig. 3D).

DISCUSSION

There is a striking similarity between the proteolytic processing of the AD-related transmembrane protein APP and the Notch1 receptor. The latter is an important protein involved in cell-fate decisions during development (14, 16, 42) but continues to be expressed in the adult brain (43), with effects on...
neuronal plasticity (20, 21) and on glial (oligodendrocyte) differentiation (34). Both proteins undergo extracellular proteolytic event(s), which precede an intramembraneous cleavage. The last processing event appears to be carried out by an identical proteolytic activity, because both APP and Notch1 cleavage are highly dependent on PS1 activity, both are blocked by several APP specific γ-secretase inhibitors (5, 11, 22), and both are affected by dominant negative PS1 mutations (5, 11).

We now address the question of whether APP and Notch1 compete for the same protease in a physiologically relevant way. We used two complementary approaches. 1) We compared the amount of γ-secretase processing of APP (by analyzing the secretion of Aβ into the conditioned media) after activation of endogenous Notch1, and 2) we compared the response of the Notch1 signaling pathway to stimulation with a physiological ligand in neurons overexpressing human mutant APP (APP_{695Sw}) and in normal, non-transgenic neurons from littermate controls.

Knowing that Notch1 becomes a γ-secretase substrate only when activated with its ligand Delta, we used increasing doses of the ligand to modulate the amount of one γ-secretase substrate (Notch1) leaving the amount of a competitive substrate...
(APP) constant. We found that stimulation of the endogenous Notch1 receptor results in a significant decrease in the level of total Aβ secretion in a dose-dependent manner. Control experiments showed that the effect was not mediated by any effect on APP production or trafficking, because our data show no effect of Notch1 activation on the level of expression of total APP, cell surface APP, or α- and β-secretase products. These results are in accord with data in another system showing that APP processing by α- and β-secretases is independent of PS1γ-secretase activity (6). Moreover, whereas Aβ production was significantly impaired, there was only a subtle difference, not statistically significant, in the amount of C83 and C99 APP CTF(s) in the cell lysates as detected by Western blot analysis 24 h after the last application of DI-Fc. The discordance between Aβ production and the accumulation of APP C-terminal fragments is similar to that observed for neuroblastoma cell lines stably transfected with PS1 deletion mutants (41). Recent data on manipulation of nicastrin, a presenilin interactor, showed changes in Aβ production without alteration of C83/C99 levels (40), also suggesting that Aβ production can be dissociated from C83/C99 changes, especially in the circumstances where

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We do not rule out the possibility that there may be other substrates for γ-secretase besides APP and Notch1 or that there could be other molecules involved (such as nicastrin, for example) that modulate the γ-secretase cleavage of various substrates. There also could be indirect effects of Notch activation on APP processing or on Aβ clearance. However, our experiments clearly demonstrate that activation of Notch by its native ligand specifically resulted in diminished Aβ, without major changes in APP amount or subcellular distribution. Similarly, overexpression of APP specifically affected Notch cleavage/nuclear translocation/signaling but did not significantly change the level of Notch expression or its subcellular distribution. These observations strongly suggest that Notch and APP are competitive substrates for γ-secretase activity.

The complementary experiment is also consistent with the hypothesis that ligand-activated Notch1 competes with APP for γ-secretase activity. Using both morphological (nuclear translocation) and physiological (CBF1 transactivation) quantitative assays we found that endogenous Notch1 signaling was significantly diminished in primary neurons overexpressing APP. In addition, analyzing nuclear (activated) Notch1 staining in the brain of APP overexpressing mice revealed that this effect can be detected in vivo. We suggest that an excess of APP shifts the enzymatic activity “equilibrium” toward APP processing and thus diminishes Notch1 from being cleaved and from activating the Notch signaling pathway; i.e. that activated Notch1 and APP are competitive substrates for γ-secretase processing.

The relationships among APP, PS1-related γ-secretase activity, and Notch1 are complex and in some ways have appeared to be contradictory, but may be clarified in the context of our current studies. PS1 familial AD (FAD) mutations are gain-of-function in terms of APP processing resulting in increased production of (especially) Aβ42 peptide (44–46). However, FAD mutations in PS1 show partial loss-of-function in terms of Notch1 function as assessed by measuring the generation of the C-terminal Notch1 signaling domain (NICD) (24, 47), Notch1 signaling (CBF1-luc activation assay, Ref. 48), neurite outgrowth (31), or rescue of the sel-12 mutant phenotype in Caenorhabditis elegans (49–51). PS1 mutations that increase Aβ production the most resulted in elimination of Notch1 proteolysis (47). In contrast, PS1 deficiency (6, 27) or aspartate to

**FIG. 4. Analysis of Notch1 signaling in vivo in APP695Sw-overexpressing mice.** A, Western blot analysis of NICD generation (inset) reveals that the ratio of the NICD band (*) to the furin-cleaved band (**) is significantly decreased in the brains of APP-Tg mice compared with that in control, non-Tg littermates (graph). The NICD lane represents cells transfected with NICD portion of the Notch1 molecule as a control (mean ± S.D.; n = 3, *, p < 0.02; Student’s t test). B, the intensity of nuclear Notch1 immunoreactivity in the neurons of the CA1 area of the hippocampus is represented as a ratio of photon counts in the nuclei to the photon counts in the CA1 area. Control mice show higher level of nuclear Notch1 than APP-Tg mice (mean ± S.D.; n = 2,000 neurons, **, p < 0.001; Student’s t test).

**FIG. 5. Schematic representation of the competition between APP and Notch1 for PS1-dependent protease activity.** A, control cell. B, activation of Notch1 cleavage/signaling after binding of Notch ligand, Delta, leads to a decreased APP processing. C, overexpression of APP695Sw results in a decreased Notch1 cleavage/signaling. D, are FAD mutations in PS1 “gain-of-function” in terms of APP processing, and loss-of-function in terms of Notch intramembranous cleavage/signaling?
FAD mutations could be interpreted as altering the relative agents could differentially affect APP and Notch1 processing by (TG2576) transgenic mice. Dr. D. and Women’s Hospital, Boston, MA) for Notch1 antibody. Because Notch1 expression diminishes markedly in another system did not alter Aβ production but inhibited NICD generation from a constitutively active Notch1 construct, Ref. 52. Similarly, Petit et al. (53) have described γ-secretase inhibitors that diminish Aβ without altering Notch cleavage (53).

Based on our current data we propose that Notch1 and APP are competitive substrates for γ-secretase activity. If so, PS1 FAD mutations could be interpreted as altering the relative affinities of γ-secretase for APP and Notch1 to favor APP over Notch1 (Fig. 5). This also predicts the possibility that some agents could differentially affect APP and Notch1 processing by γ-secretase, e.g. by inhibiting access of one or the other substrate, providing a possible explanation for the recent results of Petit et al. (53). This formulation (Fig. 5) predicts that relative levels of Notch1 and APP expressed in cells, as well as the degree to which Notch1 is activated by ligand, could impact Aβ synthesis. Because Notch1 expression diminishes markedly with age (54), there may be a subtle increase in Aβ production with age as a result of the release of APP/γ-secretase (PS1) from this substrate competition. However, the present study (using APP-overexpressing mice) and the fact that mice heterozygous for PS1/H9253 α-amyloid production/Notch signaling (although substitution at Asp-257/H9252/erozygous for PS1/H11002 and the fact that mice het-

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