Notch1 Competes with the Amyloid Precursor Protein for γ-Secratease and Down-regulates Presenilin-1 Gene Expression*

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Presenilin 1 (PS1) is a critical component of the γ-secretase complex, which is involved in the cleavage of several substrates including the amyloid precursor protein (APP) and Notch1. Based on the fact that APP and Notch are processed by the same γ-secretase, we postulated that APP and Notch compete for the enzyme activity. In this report, we examined the interactions between APP, Notch, and PS1 using the direct γ-secretase substrates, Notch 1 Δextracellular domain (N1ΔEC) and APP carboxyl-terminal fragment of 99 amino acids, and measured the effects on amyloid-β protein production and Notch signaling, respectively. Additionally, we tested the hypothesis that downstream effects on PS1 expression may coexist with the competition phenomenon. We observed significant competition between Notch and APP for γ-secretase activity; transfection with either of two direct substrates of γ-secretase led to a reduction in the γ-cleaved products, Notch intracellular domain (NICD), or amyloid-β protein. In addition, however, we found that activation of the Notch signaling pathway, by either N1ΔEC or Notch intracellular domain, induced down-regulation of PS1 gene expression. This finding suggests that Notch activation directly engages γ-secretase and subsequently leads to diminished PS1 expression, suggesting a complex set of feedback interactions following Notch activation.

Amyloid-β protein (Aβ) is a 40–42 residue peptide believed to play a central role in the pathogenesis of Alzheimer’s disease (1). It arises from cleavage of a much larger precursor polypeptide, the β-amyloid precursor protein (APP), encoded by a gene on human chromosome 21. APP undergoes a proteolytic event by an α-secretase creating a large soluble fragment (α-APPs) and a membrane-retained carboxyl-terminal fragment (CTF) of 83 amino acids (C83). The resultant 10-kDa C83 can be cleaved by a presenilin (PS)-dependent γ-secretase to generate a small fragment called p3 precluding the formation of Aβ. In an analogous fashion, other APP holoproteins can be cleaved by another protease, β-secretase, generating a 12-kDa CTF (or C99) that is cleaved by the same γ-secretase to create Aβ. Although the generation of the different isoforms of Aβ and p3 has been extensively studied, the normal biological function of APP proteolysis remains unclear. Recent data suggest that APP could act as a cell surface receptor that mediates signaling through the release of the APP intracellular domain (AICD) (2, 3).

Notch receptor is another type I transmembrane receptor that is critically required for a variety of signaling events and cell fate decisions during embryogenesis and in adulthood (4, 5). It has been shown that the Notch1 receptor continues to be expressed in the adult brain and inhibits neurite outgrowth in postmitotic neurons (6–8). Full-length Notch is cleaved in the presence of its biological ligand Delta, and the generated Notch intracellular domain (N1ΔEC) rapidly translocates to the nucleus where it acts as a transcriptional coactivator (9, 10). Similar to APP proteolysis, Notch undergoes sequential cleavage by α- and PS-dependent γ-secretase-like activities, named S2 and S3 respectively (11). These data suggest that the same PS-dependent proteolytic complex is involved in the processing of both APP and Notch. Thus, it seems conceivable that the different substrates compete for the same protease.

We previously showed that endogenous Notch and APP are competitive substrates for γ-secretase activity; treatment of neurons with the native Notch ligand, Delta, induced a dose-dependent decrease in Aβ production, and overexpression of APP in neurons led to a decrease in Notch signaling (12). However, conflicting data were reported by Chen et al. (13) using human embryonic kidney (HEK)-293 cells overexpressing either C99 or NotchΔextracellular domain (N1ΔEC), in which no competition was observed for γ-secretase. In an effort to resolve these apparent conflicts in the present study, we examined the competition between Notch and APP under different circumstances. We postulated that if APP and Notch compete for γ-secretase activity, competition should only be observed with active Notch constructs that require a PS1-dependent cleavage, such as the truncated form N1ΔEC, but not with constructs that do not require this cleavage, such as N1ΔICD, or with forms that require ligand binding for its activation, such as full-length Notch. In this report, we extend the previous findings and show that transfection with either of two direct substrates of γ-secretase, C99 or N1ΔEC, lead to a reduction in the γ-cleaved products (N1ICD or Aβ respectively). However, surprisingly, we found that NICD also decreased Aβ production from C99. We found that this decrease in Aβ was associated with a down-regulation in the expression of PS1 gene at both the transcriptional and protein levels.
**FIG. 1.** APP C99, a direct substrate for γ-secretase, decreases Notch signaling. Transcriptional activation of a CBF1-luciferase promoter in HEK cells transfected with APP C99 or empty vector. The assay was performed in triplicate 24 h after transfection, and the results were normalized to β-galactosidase activity. Transfection with C99 leads to a decrease in the activation of the CBF1-luciferase promoter. *, p = 0.01.

**TABLE I**

| Condition | n   | Ap40 mean | p Compared to empty vector | Ap40 mean | p Compared to empty vector |
|-----------|-----|-----------|-----------------------------|-----------|-----------------------------|
| Empty     | 46  | 100       | ns                          | 100       | ns                          |
| N1FL      | 17  | 92.9      | ns                          | 104.6     | ns                          |
| NAEc      | 51  | 81.4      | p < 0.001                   | 77.5      | p < 0.001                   |
| NICD      | 51  | 86.6      | p < 0.001                   | 81.6      | p < 0.001                   |

* Total number of wells for each condition.

**FIG. 2.** Transfection with NAEc attenuates the downstream signaling effects of the APP-Gal4 construct. HEK cells were cotransfected with APP-Gal4, Fe65, and NAEc or empty vector and measured transactivation of transcription from cotransfected Gal4-dependent reporter plasmid expressing luciferase. Results were normalized to β-galactosidase activity. As expected, transfection with Fe65 induced an increase in the ability of APP-Gal4 to activate transcription compared with that of an empty vector. Cotransfection with NAEc led to a marked decrease in the transactivation induced by APP-Gal4. *, p < 0.01.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Full-length Notch1 (N1FL) and two truncated forms of Notch1 (NAEc and NICD) cloned into pBos vectors (generous gift of Gerry Weinmaster, UCLA), were used in the transfection experiments. NAEc contains the cytoplasmic portion and the membrane-spanning region of the molecule (5374–7836 bp), and NICD contains the active signaling portion of the Notch1 molecule (5476–7836 bp). Cells were transfected with human full-length APP or with a deletion mutant containing only the C99 cloned into a pSecTag2B vector (In Vitrogen) and with β-galactosidase as an internal control for transfection efficiency. Transfection with an empty pBos vector served as a negative control for each experiment.

**Cell Culture Conditions and Transient Transfection**—N2a mouse neuroblastoma or HEK293 cells were cultured in OPTI-MEMI with 5% fetal bovine serum at 37 °C with 5% CO₂ in a tissue culture incubator. Transient transfection was performed using Superfect reagents (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The cells were plated onto 12-well plates 1 day before transfection. The day of transfection, 1.5 μg of plasmid DNA was dissolved in 75 μl of Dulbecco’s modified Eagle’s medium, and 7.5 μl of Superfect reagent was added. The mixture was left for 5–10 min at room temperature, and 400 μl of growth medium was added to the mixture. Then, 475 μl of conditioned medium collected 24 h after transfection. The capture antibody was BNT77 (directed against Aβ1–40), and the detection antibody was horseradish peroxidase-conjugated BA27 for Ap40 (directed against Aβ1–40) and horseradish peroxidase-conjugated BC05 for Aβ42 (directed against Aβ1–40) (20). The antibodies were a generous gift of Takeda Pharmaceuticals, Japan. For the Western blot analysis, the cell lysates were adjusted to equal protein concentrations and electrophoresed in 10–20% SDS-polyacrylamide Tris-glycine gels for β1-40 expression. The immunoblotting was performed with the PS1 loop antibody (1:300, Chemicon), mouse actin (1:500), and APP C8 antibody (1:500, a gift of D. J. Selkoe), followed by detection with a horseradish peroxidase-
conjugated secondary antibody. Bands on films were quantitated using Odyssey software (LI-COR Biosciences, Lincoln, NE), and the values were normalized to actin expression.

RNA Extraction and Real-time Quantitative PCR—RNA was extracted from cells 24 h after transfection using TRIzol reagent (Invitrogen), chloroform, and precipitated with isopropyl alcohol. RNA was purified using the RNeasy mini-kit (Qiagen), and 5 μg of total RNA was used to synthesize cDNA in a 20-μl reaction using the SuperScript First-Strand Synthesis System for reverse transcriptase-PCR (Invitrogen). Mouse PS1 gene was amplified by using the primers 5′-ATCTAAGGCACAGGCCGA-3′ and 5′-TTGGTAAGGCCTCAGCAACC-3′. GAPDH was used as an internal control gene to normalize for the amount of RNA. Real-time PCR was performed in an iCycler iQ Thermal Cycler (Bio-Rad). 1 μl of cDNA was added to 25 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Each sample was run in triplicate, and analysis of relative gene expression was done by using the 2^-ΔΔCt method (21). Briefly, the relative change in gene expression is calculated by subtracting the threshold cycle (ΔCt) of the target gene (PS1) from the internal control gene (GAPDH). Based on the fact that the amount of cDNA doubles in each PCR cycle (assuming a PCR efficiency of 100%), the final fold-change in gene expression is calculated by using the following formula: relative change = 2^-ΔΔCt (21).

RESULTS

APP C99, a Direct Substrate for γ-Secretase, Decreases Notch Signaling—We postulated that if APP and Notch compete for γ-secretase, we should observe a reduction in Notch signaling after transfection with APP C99. HEK cells were cotransfected with NΔEC and APP C99 or an empty vector; CBF1 luciferase activity in the lysates was measured 24 h after transfection as a measure of Notch signaling. The results were normalized to β-galactosidase expression levels. We observed a marked reduction (63%, p < 0.01) in CBF1 luciferase activity in cells transfected with APP C99 compared with empty vector (Fig. 1). A statistically significant reduction (compared with an empty vector) in the endogenous Notch signaling was also seen in cells transfected with NΔEC (data not shown).

Transfection with Activated Notch Constructs Leads to a Decrease in Aβ Production—To test the hypothesis that Notch1 and APP are competitive substrates, we transfected N2a cells with N1FL and the two truncated forms of Notch1 (NΔEC and NICD) and measured Aβ production using ELISA in the con-
ditioned media 24 h after transfection. We reasoned that, in the absence of ligand, N1FL would have minimal effect, that NΔEC, the truncated membrane-bound form of Notch that requires PS1-dependent cleavage, would lead to a reduction in Aβ levels if it competed with APP for γ-secretase, and that NICD, which does not require γ-secretase, would not alter Aβ levels. N1FL had only a small effect on Aβ production, perhaps because of activation by endogenous ligands (Table I). However, both truncated forms of Notch (NΔEC and NICD) led to a decrease in Aβ production.

We also examined the effects of cotransferring the direct substrate NΔEC on the ability of APP-Gal4 to be cleaved by γ-secretase, releasing the Gal4-AICD fragment which activates a Gal4-dependent, artificially expressed luciferase plasmid (2). It has been shown that the presence of the Fe65 adaptor protein is a requisite for the stabilization of the AICD fragment and its subsequent nuclear translocation for luciferase activation (2, 22). To readily test any competitive effects of NΔEC on downstream activation by APP-Gal4, we cotransfected HEC cells with APP-Gal4, pG5E1B-luc, Fe65, and NΔEC or an empty vector. We observed an expected increase in the ability of APP-Gal4 to stimulate transcription when Fe65 is cotransfected, as compared with an empty vector control (Fig. 2). In addition, we found that NΔEC attenuated this phenomenon, decreasing the downstream signaling effects of the Fe65-stabilized AICD generated from the artificially expressed Gal4-dependent luciferase reporter plasmid. This finding is consistent with the reduction in Aβ40 and Aβ42 levels observed after transfection with NΔEC.

To confirm that Notch1 signaling was indeed activated by the transfection with the truncated forms of Notch, we measured activation of a Notch downstream transcription factor, C-promoter binding factor 1 (CBF1), in the cell lysates. We cotransfected N2a cells with a CBF1 luciferase reporter construct (18, 19) and β-galactosidase as an internal control for transfection efficiency, and we measured luminescence 24 h after transfection. As expected, we found that NICD and NΔEC led to a robust increase in CBF1 luciferase activity in comparison to an empty vector (Fig. 3A).

Next, we examined whether the decrease in Aβ secretion after transfection with truncated Notch1 constructs was due to any effects of the transfection on APP expression or processing.

**Table II**

Transfection with NICD downregulates endogenous PS1 gene expression

N2a cells were transfected with NΔEC, NICD, or an empty vector and expression of endogenous PS1 gene was measured using real-time quantitative PCR. The results were normalized to GAPDH expression as a control for RNA amount and data was normalized using the 2^(-ΔΔCt) method (21). The values are expressed as a change in PS1 expression compared to an empty vector. Three to five independent experiments were performed, each run in triplicate.

| Condition       | Change in expression (mean ± SD) | p value |
|-----------------|----------------------------------|---------|
| Empty vector (N = 5) | 100                 | -       |
| NΔEC (n = 5)    | 83 ± 18              | ns*     |
| NICD (n = 5)    | 58 ± 13              | <0.01   |
| C99 (n = 3)     | 131 ± 35             | ns      |

*ns, not significant.

**Fig. 4.** [Truncated Notch1 constructs down-regulate presenilin 1 expression.](#) A, Western blot analysis of the expression of PS1 in N2a cells transfected with the Notch1 constructs full-length, NΔEC, NICD, or an empty vector. B, protein bands were quantified using Odyssey software, and values from four different blots are represented in the figure.
We performed Western blot analysis of cell lysates transfected with empty vector, N\AE C, or N\ICD for full-length APP expression and APP CTFs generation. Although after quantification we did not detect any significant change in the expression of any of these fragments, we observed a small non-significant increase in the expression of the APP CTFs after transfection with N\AE C (110.3\% compared with an empty vector, Fig. 2B).

Truncated Notch1 Constructs Induced Down-regulation of the Presenilin 1 Gene—We tested the hypothesis that the unexpected effects on A\beta secretion produced by N\ICD, which does not require PS1-dependent cleavage, were associated with changes in PS1 expression. RNA was extracted from N2a cells transfected with N\AE C, N\ICD, or an empty vector, and the expression of endogenous PS1 gene and GAPDH as a control gene were analyzed by using quantitative real-time PCR. We found that N\ICD and, to a lesser extent, N\AE C down-regulated the expression of the endogenous PS1 gene (Table II). Transfection with N\ICD and N\AE C was associated with an \~40 and \~20\% reduction in PS1 expression, respectively. To investigate whether other \gamma-secretase substrates also have an effect on gene expression, we analyzed PS1 expression in N2a cells transfected with another direct substrate, C99. However, we did not observe a significant change compared with an empty vector.

Finally, we examined the expression of endogenous PS1 at the protein level after transfection with Notch1 constructs. Western blot analysis of the cell lysates for endogenous PS1 expression showed that both N\AE C and N\ICD were associated with a reduction in PS1 CTF expression compared with transfection with an empty vector (Fig. 4, A and B). In accordance with the quantitative PCR results, we failed to observe a change in PS1 protein expression in cells transfected with C99.

DISCUSSION

We showed previously that activation of endogenous Notch in primary neurons with Delta ligand caused a dose-dependent decrease in total A\beta secretion (12). Furthermore, the opposite experiment confirmed that overexpression of APP in neuronal cultures derived from APP \textsuperscript{695Sw}-overexpressing mice led to a decrease in total A\beta secretion produced by NICD, which does not require PS1-dependent cleavage, which strongly suggests that the same \gamma-secretase complex is responsible for the processing of both substrates. The existence of multiple substrates that are cleaved by the same \gamma-secretase raises the possibility of competition among them for the enzymatic activity. However, there are conflicting reports regarding this issue. For instance, Chen \textit{et al.} (13) did not observe competition between Notch and APP for \gamma-secretase in HEK cells transfected with N\AE C. To examine this issue in greater depth and to extend our previous studies, we analyzed the effect of the immediate \gamma-secretase substrates (N\AE C and C99) on A\beta production and Notch signaling.

We found that transfection of cells with direct \gamma-secretase substrates led to a decrease in the formation of the \gamma-cleaved products from the alternative substrate. Transfection with C99, the direct substrate of \gamma-secretase, induced a marked reduction in Notch signaling, assessed by a CBP1 luciferase transcription assay. On the other hand, transfection of cells with N\AE C, a truncated form of Notch that requires a \gamma-secretase cleavage, induced a significant decrease in A\beta production measured with a sensitive and specific sandwich ELISA and in APP-\textit{Gal4} transcriptional activation. The effects on A\beta were not due to a change in APP expression, although a small non-significant increase in APP CTFs was seen after transfection with N\AE C. These results confirm and extend the idea that, under several different experimental conditions in intact cells, substrates compete with each other for \gamma-secretase.

However, we also unexpectedly observed that transfection with N\ICD, the active signaling portion of the Notch1 molecule that does not require \gamma-cleavage, also down-regulated A\beta production. This decrease was consistent and associated with an \~40\% down-regulation in endogenous PS1 gene expression detectable at both the transcriptional and the protein level. However, we cannot readily demonstrate whether the knock-down of the PS1 gene is responsible for the decrease in A\beta seen in our experiments. The effects of knocking-down presenilin 1 have been evaluated by obtaining lines heterozygous for PS1 \textsuperscript{\textast } (26–28), by chronic pharmacological treatment with \gamma-secretase inhibitors (29), or by RNA interference techniques (30, 31). Mice with total absence of PS1 (PS1 \textsuperscript{\textendash }) die late in embryogenesis and exhibit defects in axial skeleton and cerebral hemorrhages (32). The complete absence of PS1 in neuronal cultures derived from PS1 \textsuperscript{\textendash } mouse embryos inhibits the normal cleavage of APP, compromises the maturation of a tyrosine kinase receptor, and produces a dramatic drop in A\beta production (26–28). On the other hand, mice heterozygous for PS1 present a normal phenotype (32, 33), and the effects on APP processing in cell lines derived from these heterozygous mice embryos seem to be weak (26). However, in an \textit{in vitro A\beta} generation assay, knock-down of PS1 using RNA interference led to an almost complete abolition of A\beta production (31). Although the effects of knocking down PS1 remain uncertain, our results suggest that the Notch pathway exerts some control on PS1 gene regulation that may ultimately affect APP processing. It will be interesting to learn if NICD also regulates other components of the \gamma-secretase complex. These data suggest that two types of phenomenon coexist in the regulation of \gamma-secretase, competition between substrates and downstream effects on gene regulation. These results depict a novel and more complex feedback relationship between \gamma-secretase and its substrates.

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