The Notch Ligands, Delta1 and Jagged2, are Substrates for Presenilin-Dependent "γ-Secretase" Cleavage*

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Running Title: Presenilin-Dependent Cleavage of Notch Ligands

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Summary

The evolutionary conserved Notch signaling pathway is involved in cell fate specification and mediated by molecular interactions between the Notch receptors and the Notch ligands, Delta, Serrate and Jagged. In this report, we demonstrate that like Notch, Delta1 and Jagged2, are subject to presenilin (PS)-dependent, intramembranous "γ-secretase" processing, resulting in the production of soluble intracellular derivatives. Moreover, and paralleling the observation that expression of familial Alzheimers disease (FAD)-linked mutant PS1 compromises production of Notch S3/NICD, we show that the PS-dependent production of Delta1 cytoplasmic derivatives are also reduced in cells expressing mutant PS1. These studies lead us to conclude that a similar molecular apparatus is responsible for intramembranous processing of Notch and it’s ligands. To assess the potential role of the cytoplasmic derivative on nuclear transcriptional events, we expressed a Delta1-Gal4VP16 chimera and demonstrate marked transcriptional stimulation of a luciferase-based reporter. Our findings offer the proposal that Delta1 and Jagged2 play dual roles as activators of Notch receptor signaling and as receptors that mediate nuclear signaling events via "γ-secretase"-generated cytoplasmic domains.
Introduction

Mutations in genes encoding presenilins (PS1<sup>1</sup> and PS2) cosegregate with the vast majority of pedigrees with early-onset familial Alzheimer's disease (FAD) (1). Multiple lines of evidence indicate that PS expression is essential for intramembranous "γ-secretase" processing of a number of type I membrane proteins, including the β-amyloid precursor protein (APP) (2,3), the developmental signaling receptor, Notch 1 (4,5), the receptor tyrosine kinase, ErbB4 (6), cell adhesion molecules, N- and E-cadherins (7), the LDL receptor-related protein, LRP (8), the cell surface adhesion protein, CD44 (9) and the synaptic adhesion protein, nectin-1α (10).

The Notch signaling pathway is an evolutionarily conserved signal pathway for local cell-cell communication between neighboring cells involved in cell fate determination (11). The Notch receptors undergo proteolytic processing in the trans-Golgi network by a furin-like convertase in the ectodomain (12), resulting in a mature heterodimeric receptor that accumulates on the cell surface (13). Ligand binding triggers sequential proteolytic processing within the extracellular juxtamembrane region by a member of the ADAM (a disintegrin and metalloprotease domain) family, termed TACE (TNFα-converting enzyme) (14,15), and subsequent intramembranous cleavage of this membrane-tethered derivative, termed S2/NEXT, by a PS-dependent "γ-secretase" activity (4). The resulting soluble cytoplasmic domain, termed S3/NICD (Notch intracellular domain), translocates to the nucleus and interacts with the DNA-binding proteins CSL, resulting in transcriptional activation of target genes (16,17).
Several Notch ligands have been identified in vertebrates and invertebrates, including Delta, Serrate, and Jagged, transmembrane proteins that share several structural features including a DSL (Delta, Serrate, Lag-2) domain, required for Notch binding, and multiple EGF repeats in respective extracellular domains (18,19). Like Notch, Delta is a substrate for proteolysis by a metalloprotease of the ADAM family, termed Kuzbanian (20), resulting in shedding of the ectodomain segment. The precise role of Kuzbanian-dependent proteolytic processing of Delta is not fully understood, but recent studies suggest that this event downregulates Delta-mediated Notch signaling (21). In this regard, ectodomain shedding of Jagged has not been described to date.

In this report, we demonstrate that like Notch, Delta1 and Jagged2, are subject to presenilin (PS)-dependent, "γ-secretase" processing, resulting in the production of soluble intracellular derivatives. We show that a plasma membrane-resident ~40 kDa carboxy-terminal fragment (CTF) that is presumably generated by a Kuzbanian-like activity serves as substrate for "γ-secretase", resulting in the liberation of a cytosolic, ~38 kDa CTF. We demonstrate that expression of a Delta1-Gal4VP16 chimera is capable of activating transcription of a luciferase reporter and that nuclear transactivation is abrogated by a highly potent and selective "γ-secretase" inhibitor. In parallel, we demonstrate that an ~27 kDa Jagged2 CTF is also a substrate for "γ-secretase". Our findings suggest that Delta1 and Jagged2 may play dual roles as activators of Notch receptor signaling and as receptors that mediate nuclear signaling events via "γ-secretase"-generated cytoplasmic domains. Finally, we report that expression of FAD-linked PS1 variants lead to compromised intramembranous cleavage of Delta1. These
observations mimic earlier studies showing reduced cleavage at the Notch S3 site and the APP 
"ε" site within respective transmembrane domains in cells expressing FAD-linked mutant PS1. 
Thus, we argue that a similar molecular apparatus is responsible for intramembranous cleavage 
of Notch and its ligand, Delta1.
Experimental Procedures

Cell Culture and Inhibitor Treatment

Mouse neuroblastoma N2a cells stably expressing mouse Delta1 and NIH 3T3 cells stably expressing human Jagged2 with a C-terminal myc-epitope tag, were maintained in 200 μg/mL G418 (Invitrogen) and 2.5 μg/mL puromycin (Clontech), respectively. γ-secretase inhibitor treatments were for 16 hrs with 2 μM of L-685,458 (22).

Transfections

Mouse N2a cells constitutively expressing myc-epitope Swedish APP695 (23) were cotransfected with 10 μg of PS1 cDNAs and 100 ng of pIREShyg (Clontech) and selected with 400 μg/mL hygromycin. Hygromycin-resistant colonies were further screened with 400 μg/mL of zeocin to generate a stable "pool" of ~100-200 colonies.

Antibodies and Western Blot Analysis

Cells were lysed in immunoprecipitation (IP) buffer containing detergents and protease inhibitors as described (24). Solubilized proteins were fractionated by electrophoresis on SDS-polyacrylamide gels and electrophoretically transferred to PVDF membranes (Bio-Rad). Membranes were blocked, then probed with primary antibodies and horseradish peroxidase-coupled secondary antibodies (Pierce). Myc-tagged Delta1 and Jagged2 derivatives were detected using monoclonal myc specific antibody, 9E10. Polyclonal antibody, PS1NT (25), was used to detect full-length PS1 and PS1 N-terminal fragment (NTF). β-Tubulin was detected by
anti-β-Tubulin antibody (Sigma). Bound antibodies were visualized by enhanced chemiluminescence (ECL) detection system (PerkinElmer Life Science).

**Cell Surface Biotinylation**

Cells were grown to near confluence in a 10-cm dish, and subject to cell surface biotinylation 0.5 mg/mL sulfosuccinimidobiotin (Sulfo-NHS-SS-biotin, Pierce) essentially as described (24). Cells were then lysed in IP buffer and biotinylated proteins were captured with streptavidin-agarose beads (Pierce).

**Luciferase Reporter Assay**

To generate a construct encoding the Delta1-Gal4VP16 fusion protein, the primer pairs, 5’-CCATCGATTTAAGAAGCTACTGTCTTCTATC-3’ and 5’-CCATCGATCACCGTCCTCGTC AATTCC-3’ were incubated with pMst-GV-APP (26) in a PCR. The PCR product was inserted between the Delta1 C-terminus and myc sequences. 0.4 µg of the resulting Delta1-Gal4VP16-myc construct was cotransfected the Gal4 reporter plasmid (pG5E1B-luci) (26), and 50 ng of a control plasmid encoding Rellina luciferase into HEK293 cells. Cells were harvested 48 h after transfection and luciferase activities were determined using Dual-Luciferase Reporter Assay System (Promega) following manufacture’s instructions. Values shown are the averages from triplicate experiments for each condition.
Results

The family of Notch receptors and Notch ligands are type I integral membrane proteins. It is now well-established that Notch and the Notch ligand, Delta1, are substrates for processing by metalloproteases of the TACE/ADAM family (14,15,20), resulting in shedding of respective ectodomains. In the case of Notch, TACE cleavage generates a membrane-tethered derivative, termed S2/NEXT, that is the substrate for intramembranous proteolysis by a presenilin-dependent "γ-secretase" activity (4). This cleavage event, termed S3 cleavage, occurs between amino acids 1743 and 1744 (16); the P1 valine residue is indispensable for S3 cleavage and subsequent nuclear signaling activity (16). Intramembranous cleavage at the S3 site results in the generation of a soluble, cytoplasmic derivative of Notch, termed S3/NICD that is a transcriptional coactivator. Intrigued by the finding that Delta1 undergoes ectodomain shedding, and the presence of valine residues at analogous positions within the transmembrane domains of Delta1 and the Jagged2 (Fig. 1A), we asked whether these Notch ligands may be substrates for "γ-secretase" cleavage, as well.

We first examined stable N2a cells that constitutively express myc-tagged mouse Delta1 harboring a carboxyl-terminal, myc-epitope tag. Western blot analysis revealed the presence of full-length ~117 kDa Delta1-myc and a prominent ~40 kDa Delta1 carboxyl-terminal fragment (D-CTF1), that presumably represents the membrane-tethered fragment generated following metalloprotease cleavage within the ectodomain (20,21) (Fig. 1B, lane 1). In addition, we observed low levels of an ~38 kDa CTF (D-CTF2) at steady-state (Fig. 1B, lane 1).
Importantly, the ~38-kDa D-CTF2 derivative fails to accumulate in cells treated with a highly potent and selective "γ-secretase" inhibitor, L-685,458 (Fig. 1B, lane 2), findings strongly suggesting this fragments is generated by "γ-secretase". To further establish that production of D-CTF2 is PS-dependent, we transiently expressed Delta1-myc in N2a cells that constitutively express either wild-type human PS1 or a dominant negative human PS1 variant that harbors the D385A mutation (24); intramembranous processing of Notch1 is abrogated in cells expressing PS1 D385A (24). As we had observed in cells treated with the "γ-secretase" inhibitor, D-CTF2 failed to accumulate in cells expressing PS1 D385A (Fig. 2B, lane 4). Collectively, these data strongly suggest that Delta1 is a substrate of PS-dependent, "γ-secretase" cleavage.

We then examined the processing of the Notch ligand, Jagged2, in an NIH-3T3 cell line that stably expresses human Jagged2 harboring a carboxyl-terminal myc-epitope tag. Western blot analysis revealed the presence of ~170 kDa full-length Jagged2 and an ~25 kDa Jagged2 carboxyl-terminal fragment (J-CTF2) (Fig. 1C, lane 1). However, the ~25 kDa CTF2 was eliminated in cells treated with the γ-secretase inhibitor, L-685,458, and interestingly, a new ~27 kDa J-CTF1 accumulates under these conditions (Fig. 1C, lane 2). These findings suggest that J-CTF1 is constitutively processed by a "γ-secretase"-like activity, to generate J-CTF2. To establish that production of J-CTF2 is PS-dependent, we transiently expressed Jagged2-myc into N2a cells that express the PS1 D385A mutation (24). As we had observed in cells treated with the "γ-secretase" inhibitor, the production of J-CTF2 is eliminated, and J-CTF1 now accumulates (Fig. 1C, lane 4). These results indicate that Jagged2 is also a substrate of PS-dependent "γ-secretase" cleavage.
In order to identify the subcellular site(s) at which the D-CTF2 and D-CTF1 derivatives of Delta1-myc accumulate, we treated N2a cells that constitutively express Delta1-myc with the membrane-impermeant, biotinylation reagent, sulfosuccinimidobiotin at 4°C. Biotinylated, cell surface polypeptides were recovered from detergent-solubilized lysates using streptavidin-conjugated agarose, and captured proteins were subject to Western blot analysis with the myc-specific, 9E10 antibody. In cells expressing Delta1-myc, we observed biotinylated full-length Delta1-myc and ~40 kDa D-CTF1 (Fig. 2A, lane 5). However, the ~38 kDa D-CTF2 was not recovered by immobilized streptavidin, despite the presence of the fragment in detergent lysates (Fig. 2A, lane 2). Hence, D-CTF2, like the Notch S3/NICD derivative is not present at the cell surface, but presumably present in the cytosol. Moreover, D-CTF2 failed to accumulate in cells treated with the "γ-secretase" inhibitor, as expected, and streptavidin only recovered both full-length and ~40 kDa D-CTF1 from detergent lysates of these inhibitor-treated cells (Fig. 2A, lane 6). These observations strongly suggest that the soluble, cytoplasmic domain of Delta1 is generated following sequential cleavage of full-length Delta1 species by the concerted action of Kuzbanian-like metalloprotease(s) and a PS-dependent "γ-secretase".

It is now clear that the soluble intracellular domains of Notch and APP that are generated by "γ-secretase" are translocated to the nucleus and serve as transcriptional coactivators (16,17,26). To test the possibility that D-CTF2 could be transported to nucleus, and exhibit nuclear signaling activity, we generated cDNA encoding a Delta1-Gal4VP16 fusion protein and transfected this construct into human embryonic kidney 293 (HEK293) cells together with the reporter plasmid, pG5E1B-luciferase (26). Compared to cells expressing the pG5E1B vector,
expression of Delta1-Gal4VP16 fusion protein stimulated transcription by approximately 70-fold (Fig. 2B). Notably, transactivation of the reporter plasmid by the Delta1-Gal4VP16 fusion protein was markedly inhibited upon treatment of cells with the γ-secretase inhibitor (Fig. 2B). These results suggest that intracellular domain of Delta1 that is generated by PS-dependent "γ-secretase" cleavage can be transported into nucleus, findings that raise the possibility that the cytosolic derivative of Delta1 may play a role in nuclear signaling events.

Finally, and intrigued by the finding that cells expressing FAD-linked PS1 mutants enhance production of pathogenic Aβ42 peptides (1), but exhibit impaired processing within the transmembrane domains of Notch and APP that liberate NICD and AICD, respectively (27-29), we analyzed processing of Delta1-myc in pools of N2a cells that constitutively express human wild-type PS1 or the PS1 ΔE9, M146L, E280A, or C410Y FAD variants. Compared to N2a cells expressing wild-type human PS1 (Fig. 3A, lane 1), in which Delta1-myc was processed to D-CTF1 and low levels of D-CTF2, as described above (Fig. 1B), the production of the D-CTF2 fragment is reduced in most cell lines expressing FAD-linked PS1 variants, albeit with only a modest effect in the M146L cells (Fig. 3A, lanes 2-5, quantified in Fig. 3B), this despite comparable levels of expression of human PS1 (Fig. 3A, lower panel). Taken together with the curious observation that expression of FAD-linked PS1 variants leads to reduced processing at the Notch S3 and APP "e" sites, our findings that production of the D-CTF2 derived from Delta1 is also reduced by expression of mutant PS1 argues that a similar, if not identical, molecular apparatus is involved in substrate recognition and intramembranous processing of these functionally divergent membrane proteins.
Discussion

A wealth of evidence has emerged to support a role for PS in intramembranous "γ-secretase" processing of a host of type I membrane proteins. Intrigued by the similarity in the amino acid sequence of the transmembrane domains of Notch and its ligands, Delta and Jagged, we hypothesized that these ligands might also serve as substrates for "γ-secretase". In the present report, we confirm our prediction and offer several insights relevant to the molecular apparatus responsible for intramembranous processing of Delta1 and Jagged2 and the potential functional significance of this processing event.

First, we demonstrate that the production of cytosolic derivatives of Delta1 and Jagged2, termed DICD and JICD, respectively, are inhibited either by a highly potent, and selective transition-state isostere of "γ-secretase" activity, or by expression of the dominant-negative D385A PS1 mutant. Thus, the Notch ligands, Delta1 and Jagged2, are novel substrates of PS-dependent "γ-secretase" processing.

Second, and in view of earlier demonstrations that FAD-linked PS1 mutations impair cleavage at the Notch S3 and APP "ε" sites that lead to production of S3/NICD and AICD (27-29), respectively, we assessed the effects of FAD-linked PS1 variants on "γ-secretase" processing of Delta1. We show that the generation of DICD is impaired in most of cell lines stably expressing 4 independent FAD-linked PS1 mutants. Most interestingly, the relative levels of reduction in DICD parallel the reported effects on S3/NICD production (29); expression of the
C410Y variant has the most pronounced effect, while the M146L variant has only a modest effect on "γ-secretase" processing. Thus, we argue that the molecular apparatus involved in production of NICD, AICD and DICD are similar, if not one in the same.

Third, and in view of earlier conclusions that the intracellular domains of Notch (S3/NICD) and APP (AICD) are transcriptional coactivators (16,17,24), we hypothesized that "γ-secretase"-generated DICD could be translocated to the nucleus and activate transcription of a reporter gene. Our present results support this prediction. Despite the strengths of these observations, the factors responsible for translocating DICD into the nucleus is not known. For the APP derivative, AICD, a cytosolic adaptor protein, Fe65, promotes nuclear translocation (24). For Notch1, three putative nuclear localization signals (NLS) are present within the ICD, and these may serve as recognition motifs by members of the importin/karyopherin alpha and beta receptors involved in nuclear import. Similarly, the Delta1 intracellular domain contains two putative NLSs, \textbf{PDRKRPE} at amino acids 686-692, and \textbf{RKR} at amino acids 688-691, while the Jagged2 intracellular domain contains putative NLSs \textbf{RKRR} at amino acids 1107-1110, and \textbf{KRRK} at 1108-1111. Hence, it is conceivable that DICD and JICD may be imported via the classical importin pathway, but further mutagenesis studies of the putative NLSs will be required to validate this hypothesis.

In any event, our results offer the suggestion that PS-dependent "γ-secretase" processing of Delta1 or Jagged2 and production of DICD and JICD may play roles in activating nuclear transcriptional events. While the significance of these findings in relation to cell-
intrinsic versus cell-extrinsic aspects of Notch signaling remains to be determined, the proposal that the Notch ligands, Delta1 and Jagged2 may perform roles both as ligands and receptors that directly participate in transcriptional activation warrants further investigation.
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1Abbreviations used are:

PS, presenilin; FAD, familial Alzheimer disease; APP, β-amyloid precursor protein; LRP, LDL receptor-related protein; EGF, epidermal growth factor-like; LNR, Lin Notch repeat; ADAM, a disintegrin and metalloprotease domain; NEXT, Notch extracellular truncation; NICD, Notch intracellular domain; CTF, C-terminal fragment; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis; NTF, N-terminal fragment; TM, transmembrane; DICD, Delta1 intracellular domain; JICD, Jagged intracellular domain; AICD, APP intracellular domain; Aβ, β-amyloid; NLS, nuclear localization signal
Figure Legends

**FIG. 1.** PS-dependent γ-secretase cleavage of Notch ligands.

(A) Amino acid sequences of TM domain and intracellular juxtamembrane region in Notch1 and Notch ligands, Delta1 and Jagged2, are presented. The box represents the predicted TM domain. Mouse Notch1 is cleaved in a PS-dependent manner at S3 site (arrow) before Valine 1744 (16). Arrowheads point to valine residue(s) within the Delta1 and Jagged2 transmembrane domains.

(B) N2a cells stably expressing myc-tagged mouse Delta1 were incubated in the absence (lane 1) or the presence (lane 2) of γ-secretase inhibitor, L-685,459. Additionally, we transiently transfected N2a pooled cells stably expressing human wild-type (WT) PS1 (lane 3) or PS1 D385A variant (lane 4) with cDNA encoding Delta1-myc. The bands corresponding to ~117 kDa full-length, ~40 kDa C-terminal fragment (CTF1), and ~38 kDa CTF2 of Delta1, are indicated (upper panels). The bands corresponding to ~45 kDa full-length PS1 and ~29 kDa PS1 NTF, are also indicated (lower panel). Molecular markers (in kDa) are shown at right.

(C) NIH-3T3 cells stably expressing myc-tagged human Jagged2 were incubated in the absence (lane 1) or the presence (lane 2) of L-685,458. We also transfected N2a stable pools expressing PS1 WT (lane 3) or PS1 D385A (lane 4) with cDNA encoding Jagged2-myc. The band corresponding to ~170 kDa full-length Jagged2 and ~27 kDa CTF1, and ~25 kDa CTF2 of Jagged2 derivatives are indicated with arrows.

**FIG. 2.** Characterization of Delta1 intracellular domain (DICD).

(A) Cell were incubated in borate buffer alone (lanes 1 and 4) or borate buffer containing
sulfosuccinimodobiotin (lanes 2, 3, 5, and 6) at 4°C. Biotinylated surface proteins were captured with streptavidin-agarose beads and detected with myc-specific, 9E10 antibody. One-twentieth of the lysates used for precipitation were loaded for comparison (lanes 1-4).

(B) HEK293 cells were cotransfected with cDNAs including Gal4VP16 reporter plasmid, Delta1-Gal4VP16 fusion construct, and an internal control plasmid encoding Renilla luciferase. Values normalized by Renilla luciferase activity to standardize transfection efficiency, were shown as the averages (±SEM) of triplicate samples.

**FIG. 3.** Effects of FAD-linked PS1 mutants on Delta1 γ-secretase cleavage.

(A) N2a pooled cells stably expressing human wild-type (WT) PS1 (lane 1), FAD-linked PS1 ΔE9 mutant (lane 2), M146L (lane 3), E280A (lane 4), or C410Y (lane 5), were transiently transfected with cDNA encoding Delta1-myc. Delta1 (upper panel) and PS1 (lower panel) derivatives were visualized by immunoblotting with 9E10 and PS1NT, respectively.

(B) The CTF2 (DICD) / CTF1 ratios of Delta1 were determined by quantifying band intensities using Densitometry (Molecular Probe) and normalized to the PS1 WT value of 100%.
FIG. 1 Ikeuchi and Sisodia
**Fig. 2** Ikeuchi and Sisodia
FIG. 3 Ikeuchi and Sisodia
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