The Notch Ligands, Jagged and Delta, Are Sequentially Processed by α-Secretase and Presenilin/γ-Secretase and Release Signaling Fragments*

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The cleavage of Notch by presenilin (PS)/γ-secretase is a salient example of regulated intramembrane proteolysis, an unusual mechanism of signal transduction. This cleavage is preceded by the binding of protein ligands to the Notch ectodomain, activating its shedding. We hypothesized that the Notch ligands, Delta and Jagged, themselves undergo PS-mediated regulated intramembrane proteolysis. Here, we show that the ectodomain of mammalian Jagged is cleaved by an A disintegrin and metalloprotease (ADAM) 17-like activity in cultured cells and in vivo, similar to the known cleavage of Drosophila Delta by Kuzbanian. The ectodomain shedding of ligand can be stimulated by Notch and yields membrane-tethered C-terminal fragments (CTFs) of Jagged and Delta that accumulate in cells expressing a dominant-negative form of PS or treated with γ-secretase inhibitors. PS forms stable complexes with Delta and Jagged and with their respective CTFs. PS/γ-secretase then mediates the cleavage of the latter to release the Delta and Jagged intracellular domains, a portion of which can enter the nucleus. The ligand CTFs compete with an activated form of Notch for cleavage by γ-secretase and can thus inhibit Notch signaling in vitro. The soluble Jagged intracellular domain can activate gene expression via the transcription factor AP1, and this effect is counteracted by the co-expression of the γ-secretase-cleaved product of Notch, Notch intracellular domain (NICD). We conclude that Delta and Jagged undergo ADAM-mediated ectodomain processing followed by PS-mediated intramembrane proteolysis to release signaling fragments. Thus, Notch and its cognate ligands are processed by the same molecular machinery and may antagonistically regulate each other's signaling.

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as an unusual and hitherto unrecognized mechanism of signal transduction (1). A salient example of RIP is provided by the sequential processing of certain type 1 membrane glycoproteins by A disintegrin and metalloprotease (ADAM) and then by presenilin/γ-secretase (2). Presenilin (PS) was first identified as a polytopic membrane protein bearing mutations that cause the most aggressive form of familial Alzheimer’s disease (3). Subsequently, PS was shown to be a facilitator of signaling by Notch receptors during cell fate determination in Caenorhabditis elegans (4), Drosophila (5, 6), and mammals (7). There is now compelling evidence that PS represents the active site component of γ-secretase, a multiprotein complex that affects the intramembranous cleavages of Notch, the amyloid β-protein precursor (APP), Erb-B4, E-cadherin, and several other type 1 membrane proteins (8–16).

The requirement for PS in Notch signal transduction appears to be explained by the finding that a PS-mediated cleavage within the single transmembrane domain of Notch releases its intracellular domain (NICD) to the nucleus, where it regulates transcription of target genes (17, 18). Based on the finding that APP, like Notch, is sequentially cleaved by an ADAM family protease and PS/γ-secretase, the APP intracellular domain (AICD) was recently shown to reach the nucleus (19, 20), where it can regulate transcription of target genes (21, 22). Thus, emerging data suggest that presenilins, which are ubiquitously expressed in metazoans, serve as crucial switches in the signaling of a variety of single pass transmembrane receptors.

The initiation of Notch signaling at the plasma membrane is believed to require the binding of an extracellular ligand (e.g. Delta or Jagged) to the Notch ectodomain, triggering the shedding of that domain by an ADAM protease (e.g. ADAM 10/kuz and/or ADAM 17/TACE) (23–25) and the subsequent PS-mediated intramembrane cleavage of the retained C-terminal fragment (CTF). Release of the large ectodomains of Notch, APP, and other PS substrates may remove steric hindrance on PS/γ-secretase and allow intramembrane cleavage to occur (26). Based on these findings, we postulated that numerous type 1 single transmembrane proteins that undergo ADAM-mediated ectodomain shedding are substrates of PS-mediated RIP. Because the Notch ligand, Delta, undergoes cleavage by Kuzbanian (related to mammalian ADAM 10/kuz) in flies (27), we hypothesized that another Notch ligand, Jagged, would undergo a similar ADAM-mediated ectodomain shedding to generate a suitable PS substrate in the RIP mechanism. Here, we report several lines of evidence that Delta and Jagged are...
indeed cleaved by ADAM family proteases to generate free ectodomains and CTF fragments and that this ectodomain shedding can be stimulated in a Notch-dependent manner. Further, we show that following ectodomain shedding, the Delta and Jagged CTFs undergo intramembrane cleavages by PS/γ-secretase and can thus compete with NEXT, the analogous CTF of Notch, for γ-secretase cleavage, resulting in decreased Notch signaling. We then show that the Jagged intracellular domain (JICD) can stimulate AP1-dependent gene expression and that this effect of JICD is inhibited by the Notch-derived NICD. Taken together, these findings demonstrate that both Notch and its ligands are processed by the same molecular machinery and suggest that the regulated intramembrane proteolysis of both receptor and ligand may play important, potentially competitive roles in cell signaling.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The expression plasmids for FL Jagged (pBOS-SN3T) and FL Delta (pBOS-deltaHA) of rat origin were generous gifts of G. Weinmaster. The CBF reporter construct, JH23, was a gift of S. D. Hayward, and the NAE and NICD constructs were gifts of R. Kopan. The JAE construct was generated by a series of PCRs. Starting with two independent PCRs, one insert was amplified that encoded the first 54 amino acids of rat Jagged (aa 1–28 contain the signal peptide) such that the 3′-end of the oligonucleotide would anneal with the 5′-end of a second PCR product encoding the predicted Jagged CTF (aa 1057–1220) followed by a C-terminal HA tag and stop codon. Aliquots of these two PCR products were annealed and amplified in a third PCR to generate the full-length JAE DNA insert. This PCR product was then purified and TA-cloned into the pEPF6/V5-His vector (Invitrogen), colonies were screened for orientation, and sequence was verified. The Jagged JICD and JICD-NLS plasmids were cloned by PCR amplification of the appropriate cDNA regions from the full-length plasmid encoding aa 1057–1220 and aa 1103–1220, respectively, cloned into the above mentioned vector, and then screened and sequence-verified as well.

**Cell Culture, Transfection, and γ-Secretase Inhibitor Treatment**

Chinese hamster ovary (CHO) cells, African green monkey kidney cells (COS7), and human embryonic kidney (HEK) cells were cultured in Dulbecco’s modified essential medium (DMEM) plus 10% fetal bovine serum, penicillin, and streptomycin. The 12- and 24-h CHO cell lines were cultured as previously described (25). For transient transfections, 10 μg of plasmid DNA per 10-cm dish were incubated with LipofectAMINE 2000 (Invitrogen) and aliquoted onto cells cultured in minimal volumes of Opti-MEM (Invitrogen) medium. 4–6 h after transfection, cells were washed, returned to their appropriate growth media, and maintained for an additional 24–30 h prior to transfection. For experiments involving γ-secretase inhibitors and NAE/γ-secretase reporter constructs, cells were maintained in minimal serum media. The 24–30 h after transfection (see above) cells were lysed in passive lysis buffer (Promega) and assayed using the Dual Luciferase Assay kit (Promega) on a Wallach 1420 multilabel counter. Other luciferase measurements were normalized against the Renilla luciferase to correct for transfection efficiency.

**Notch Reporter Assay**—COS cells were plated into poly-lysine-coated 24-well plates (Becton Dickinson) at least 24 h prior to transfection. Cells were transfected with the TK-Luc vector (20 ng/well; Promega) to normalize for transfection efficiency as well as with the JH23 CBF-Luc reporter vector (75 ng/well). Minimum concentrations of NAE cDNA required for CBF-Luc expression were determined, and this concentration of NAE cDNA was held constant for all experiments (150 ng/well). In some cases, the JAE expression vector was co-transfected as well. 24–30 h after transfection (see above) cells were lysed in passive lysis buffer (Promega) and assayed using the Dual Luciferase Assay kit (Promega) on a Wallach 1420 multilabel counter. Other luciferase measurements were normalized against the Renilla luciferase to correct for transfection efficiency.

**Alkaline Phosphatase Reporter Assay for Transcriptional Signal-**

For screening experiments, COS cells were plated onto poly-lysine-coated 96-well dishes (Becton Dickinson) and grown to ~90% confluence. Cells were transfected with 200 ng of various reporter DNAs (Mercury Pathway Profiling System; Clontech) along with 400 ng of luciferase expression vector as well as with the JH23 CBF-Luc reporter vector (75 ng/well). Minimum concentrations of NAE cDNA required for CBF-Luc expression were determined, and this concentration of NAE cDNA was held constant for all experiments (150 ng/well). In some cases, the JAE expression vector was co-transfected as well. 24–30 h after transfection (see above) cells were lysed in passive lysis buffer (Promega) and assayed using the Dual Luciferase Assay kit (Promega) on a Wallach 1420 multilabel counter. Other luciferase measurements were normalized against the Renilla luciferase to correct for transfection efficiency.

**RESULTS**

**Ectodomain Shedding of Full-length Jagged and Delta**—The intramembranous cleavage of type I integral membrane proteins by PS/γ-secretase is preceded by an initial endoproteolysis just outside of the membrane that results in the shedding of the large ectodomain. For example, ADAM-mediated cleavage of the ectodomain of the Delta/Jagged receptor, Notch, occurs prior to the PS/γ-secretase-mediated release of the NICD (23–25). We hypothesized that the mammalian Notch ligands,
Jagged and Delta, also undergo these α- and γ-secretase scissions. To assess whether full-length (FL) Jagged and Delta undergo ectodomain shedding to yield appropriate PS/γ-secretase substrates, COS cells were transiently transfected with plasmids encoding either FL Jagged (Fig. 1a, left panel) or FL Delta (Fig. 1a, middle panel) with C-terminal HA tags, and cell lysates were analyzed by Western blotting. Both Jagged and Delta were expressed at high levels, and single C-terminal immunoreactive fragments were consistently identified in cell lysates and bicarbonate-washed membrane fractions at the appropriate molecular weights predicted for the respective membrane-associated CTFs. These data suggest that the ectodomains of both Jagged and Delta are shed to generate suitable candidate substrates of PS/γ-secretase (i.e. CTF Jagged and CTF Delta). Previous work in Drosophila demonstrated the Kuzbanian-dependent cleavage of Delta (27); however, there is no prior report of such α-secretase-type cleavage (or any other proteolysis) of Drosophila Serrate or its mammalian homolog, Jagged. We next performed experiments in which COS cells were transiently transfected to express Delta and co-cultured for 4 h with mock-transfected cells or COS cells transiently transfected to express Notch. Following this brief co-culture with Notch-expressing cells, densitometric analyses of the ratio of CTF-Delta to FL-Delta revealed an ~51% increase (n = 5, p < 0.05) in the levels of CTF-Delta (Fig. 1a, right panels). A more dramatic effect was observed following cotransfection with of Delta and Notch in the same cell; however, it is likely that this represents both increased ectodomain shedding of Delta and competition between CTF-Delta and the analogous NEXT fragment of Notch for γ-secretase (see below).

Characterization of the Ectodomain Shedding of Jagged—In flies, Kuzbanian is responsible for the ectodomain shedding of both Delta and Notch (25, 27). However, in mammals, it appears that ADAM 17/TACE mediates the S2 cleavage of Notch (23, 24). Mammals share a single Kuzbanian/ADAM 10 gene, whereas these are distinct but highly related genes in the fly. Therefore, we surmised that the initial cleavage of Jagged was probably mediated by either ADAM 17/TACE or ADAM 10/kuz. By preferentially inhibiting either ADAM 10 or ADAM 17, we characterized the protease responsible for cleaving the Jagged ectodomain and generating its CTF. CHO cells were transfected to express FL Jagged and treated for 24 h with either the ADAM 17 inhibitor, batimistat (10 μM), or the ADAM 10 inhibitor, TIMP-1 (5–10 nM) (Fig. 1b, top panel). Batimistat virtually prevented Jagged CTF formation, whereas TIMP-1 had very little effect. At doses previously shown to inhibit the ADAM
17-mediated (i.e. α-secretase) cleavage of APP (32), TAPI-1 (10 μM) also blocked the generation of Jagged CTF (Fig. 1b, bottom panel). Furthermore, in the presence of a γ-secretase inhibitor, the addition of the ADAM 17/TACE inhibitor, batimastat, increased FL Jagged levels while decreasing Jagged CTF, whereas TIMP-1 had no effect on FL or CTF Jagged (data not shown). Consistent with a previous report of Kuzbanian-mediated cleavage of Delta in Drosophila (27), we found that the α-secretase processing of Delta was not affected by the ADAM 17/TACE inhibitors batimastat or TAPI-1 but was decreased by the ADAM 10/kuz inhibitor TIMP-1 (data not shown).

The α-secretase processing of APP consists of both regulated and constitutive components. The regulated component is thought to be mediated by ADAM 17 and can be enhanced by phorbol ester treatment (33–35). To determine whether the cleavage of Jagged by ADAM 17 could likewise be regulated, we investigated the effect of the phorbol ester, phorbol 12-myristate 13-acetate, on endogenous APP and exogenous Jagged CTF production. A 1-h treatment with phorbol 12-myristate 13-acetate (10 μM) increased the production of both the APP CTF (C83), and the Jagged CTF (Fig. 1c). Furthermore, we detected a soluble form of Jagged ectodomain in the conditioned medium of Jagged-transfected cells that was absent in control medium (Fig. 1d). We observed greater ectodomain processing of Delta and Jagged in CHO cells than in COS cells, and we speculated that the ectodomain shedding of Delta and Jagged that generated membrane-associated CTFs could have been due to endogenous Notch expression. To determine whether Notch was present endogenously in these cells, we performed IP-Western blotting using two different rodent-specific antibodies to the Notch ectodomain, one for IP and the second for detection. We found two immunopositive bands in CHO cell lysates (presumably FL and furin-processed Notch) that co-migrated with transfected rat Notch (data not shown). This result is reminiscent of previous work in HEK cells, in which endogenous ligand was expressed at sufficient levels to induce exogenous Notch activation (23).

Detection of Endogenous Full-length and CTF Jagged Molecules in Vivo—Whereas the ectodomain shedding of Drosophila Delta has been demonstrated in vivo (27), this has not been confirmed in mammals. Furthermore, there is no report describing any proteolysis of the Serrate/Jagged family of Notch ligands. We therefore searched for evidence of processing of the Jagged ectodomain in the developing rat embryo. Jagged message levels in rat embryo rise during early development to peak at day E13 (36). We harvested whole rat embryos at this age and prepared microsomal membranes. When solubilized membranes were probed with two different antibodies to the C terminus of Jagged-1, high levels of both the full-length protein and its CTF were readily detected (Fig. 2a). We believe this to be the first demonstration of the existence of the Jagged CTF in vivo. To test our hypothesis that this endogenous Jagged CTF is further cleaved by PS/γ-secretase, we incubated E13 rat embryo membranes in a sodium citrate buffer for 4 h at 37 °C in either vehicle alone (1% Me2SO) or one of three structurally unrelated γ-secretase inhibitors. Jagged CTF was proteolytic processed into a JICD fragment of the appropriate size, and this was blocked by each of the three γ-secretase inhibitors (Fig. 2b).

PS/γ-secretase-dependent Accumulation of Jagged and Delta CTFs—Shortly after their biosynthesis, FL PS1 and PS2 are converted by endoproteolysis into NTF/CTFs heterodimers that enter into stable multimeric complexes containing nicastrin and one or more additional membrane proteins (9, 37, 38). Overexpression of FL human PS1 in CHO cells leads to quantitative replacement of the endogenous hamster PS heterodimers by exogenous human heterodimers (28). We previously generated a CHO cell line stably co-expressing human FL APP, wild type PS1, and wild type PS2 (28). This cell line (12–19) shows replacement of endogenous hamster NTF/CTFs with functional human NTF/CTFs (Fig. 3a, lane 1); the latter participate in active γ-secretase complexes that mediate cleavages of C83 and C99 to generate the p3 and Aβ peptides, respectively. An analogous cell line (2A-2) stably expresses human FL APP plus human PS1 and PS2 each bearing mutations in one of the critical intramembrane aspartate residues required for PS endoproteolysis and γ-secretase activity (28). In these 2A-2 cells, endogenous PS is replaced by the aspartyl-mutant FL PS, and there are very few or no detectable PS heterodimers (Fig. 3a, lane 2). As a result, the 2A-2 cells strongly accumulate C83 and C99 (Fig. 3b, lane 2) and fail to generate p3 and Aβ (not shown; see Kimberly et al. (28)).

We assumed that these dominant negative effects of the aspartate mutations would prevent the cleavage of other PS substrates. Indeed, the 2A-2 cells show defective Notch intramembrane cleavage and NICD signaling (39). To further test this assumption, we first ruled out potential differences in ADAM-mediated ectodomain shedding between the 12-19 and 2A-2 lines by quantifying the proteases that specifically mediate the APP, Notch, Delta, and Jagged ectodomain cleavages, ADAM 17/TACE and/or ADAM 10/kuz. The levels of both the zymogens and the mature enzymes were unaffected by the PS1 and PS2 aspartate mutations (Fig. 3a). Therefore, accumulation of potential PS/γ-secretase substrates in the 2A-2 cell line...
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**Fig. 3.** The CTFs of the Notch ligands, Delta and Jagged, accumulate in a PS/γ-secretase-dependent manner. A, CHO cells stably co-expressing human wild-type (12-19) or dominant-negative aspartyl mutant (2A-2) isoforms of both PS1 and PS2 were probed for any differences in proteases that serve as α- and γ-secretases. Bicarbonate-washed microsomes were blotted for the PS1 NTF/CTF heterodimers (γ-secretase), and for the immature and mature forms of ADAM 10 and ADAM 17 (α-secretases). The faint band immediately above the position of the PS1 NTF in the 2A-2 cells is a background band, not PS1 NTF; note that there is also no detectable PS1 CTF in these cells. B, the effect of stably over-expressing aspartyl mutant PS1 and PS2 on the levels of APP CTFs (C83 and C99) was assessed by blotting with monoclonal antibody 13G8. *, a band corresponding to a probable APP CTF dimer. C, 12-19 and 2A-2 cells were transiently transfected to express FL Jagged (A) or FL Delta (B). Cells were collected 36 h later, and lysates were blotted with 12CA5 (to the C-terminal HA tag). D, 12-19 and 2A-2 cells were transiently transfected to express FL Jagged and treated with vehicle alone or the γ-secretase inhibitor, III-31-C, for 16 h. Lysates were collected, and the levels of Jagged CTF were determined by 12CA5 Western blotting (top panel). Levels of the APP γ-secretase substrates C83 and C99 were determined by blotting the same lysates with APP C-terminal antibody 13G8 (bottom panel).

should be attributable to the loss of function of this protease. 2A-2 cells transiently transfected with FL Jagged or FL Delta showed accumulation of the Jagged and Delta CTFs, compared with levels obtained in simultaneously transfected 12-19 cells (Fig. 3c). Both lines were then transfected with FL Jagged (Fig. 3d) or FL Delta (not shown) and treated for 12 h with vehicle alone or 500 nM III-31-C, a well-characterized, cell-permeant PS/γ-secretase inhibitor (IC_{50} = 250 nM). As reported previously (9), treatment with III-31-C substantially augmented the levels of the APP CTFs, C83 and C99, although not as strongly as the PS1 and PS2 aspartate mutations did (Fig. 3d, lower panel). In accord, we observed an inhibitor-mediated increase in the Jagged CTF in the 12-19 cells that was not as great as the elevation caused by the aspartate mutations in the 2A-2 cells (Fig. 3d, upper panel). III-31-C had a modest additional effect on the levels of APP C83/C99 and also PS1 with the γ-secretase inhibitor, III-31-C, for 16 h. Lysates were collected, and the levels of Jagged CTF were determined by 12CA5 Western blotting (top panel). Levels of the APP γ-secretase substrates C83 and C99 were determined by blotting the same lysates with APP C-terminal antibody 13G8 (bottom panel).

Complex Formation of PS with the CTFs of Jagged and Delta—We previously reported that small amounts of FL APP and, in particular, C83 and C99, can be co-immunoprecipitated with PS (29, 30). Furthermore, the levels of such complexes of PS with C83 and C99 are markedly enhanced in cells lacking γ-secretase activity (29). We therefore investigated whether antibodies to human PS1 could immunoprecipitate the FL Jagged and Delta proteins and their CTFs. Relying on conditions previously used for the successful co-precipitation of PS1 with FL APP as well as C83 and C99 and also PS1 with the Notch membrane-anchored CTF (i.e. NαE, an ectodomain-truncated form of Notch) (29, 30, 41, 42), FL Jagged and Delta as well as their respective CTFs were efficiently co-precipitated by the PS1 antibody X81 in both the 12-19 and 2A-2 cell lysates, whereas the preimmune serum of X81 did not bring down any form of the Notch ligands (Fig. 4a).

In agreement with our previous finding that co-immunoprecipitation of PS with C83 and C99 is more efficient in PS aspartate mutant than wild type cells (29), the co-precipitation of the Jagged CTF with the PS1 antibody was also greater in the 2A-2 than 12-19 cells (Fig. 4a). Analysis of the co-precipitation of PS1 with FL Delta and its CTF revealed similar results; FL Delta was effectively co-precipitated from both 12-19 and 2A-2 cell membranes, and more Delta CTF was recovered from the latter cells (Fig. 4b). Similar experiments were performed after co-expressing neprilysin, an integral membrane protein not predicted to be a substrate of γ-secretase, and there was no evidence of co-IP with PS (data not shown).

The 12-19 and 2A-2 cells used in these experiments stably
overexpress wild type or mutant PS1 and PS2, and FL Jagged and Delta were transiently expressed. Therefore, we asked whether X81 could co-precipitate the substrates at endogenous levels of both presenilin and substrate. Mouse primary mixed cortical cultures were lysed under similar conditions in 1% Nonidet P-40, co-precipitated with X81 and probed for Delta. A small amount of FL Delta was detected that was absent in the X81 preimmune precipitate (Fig. 4c).

PS1/γ-secretase-mediated Cleavage of the Jagged CTF and Nuclear Translocation of Its Cytoplasmic Domain—The cleavage of APP and Notch by PS1/γ-secretase releases their intracellular domains (AICD and NICD) into the cytoplasm. Both fragments are believed to complex with other cytoplasmic proteins, traffic to the nucleus, and affect gene expression, although by different mechanisms. It appears that AICD requires a binding partner, the transcriptional co-activator Fe65, to gain entry into the nucleus. In contrast, NICD has two consensus NLS motifs C-terminal to the Notch transmembrane domain that are thought to mediate its nuclear entry. Both Jagged and Delta contain basic amino acid stretches within their respective intracellular domains (Fig. 5a), and these putative NLS sequences are conserved among the principal Notch ligands of flies, mice, and humans. AICD and NICD are highly labile proteins that are rapidly degraded and thus very difficult to detect with standard biochemical techniques.

To increase the levels of NICD generated by PS1/γ-secretase, previous studies have used an ectodomain-truncated form of Notch in which the signal peptide was placed adjacent to an N-terminally truncated Notch sequence. This construct (N/H9004E) codes for a type 1 transmembrane protein with a very short ectodomain that undergoes intramembranous cleavage by PS1/γ-secretase in a constitutive, ligand-independent manner. Transient transfection with this construct yields a readily detectable NICD that translocates to the nucleus (17, 43). We therefore designed a similar plasmid encoding the Jagged signal peptide immediately followed by the complete membrane-anchored CTF of Jagged, beginning at the predicted ADAM cleavage site and ending with a C-terminal HA tag (J/H9004E; Fig. 5b). Transfection with J/H9004E resulted in the expression of a membrane-anchored JICD protein of appropriate molecular weight in both COS and CHO cells (Fig. 6a). Furthermore, transfection with JAE resulted in JICD production only in cells with intact PS1/γ-secretase activity (i.e. the COS and the 1219 CHO cells). The 2A-2 CHO cells did not generate JICD (Fig. 6a). Treatment with the γ-secretase inhibitor, Compound E (3 nM), virtually

Fig. 4. The Notch ligands, Jagged and Delta, co-immunoprecipitate with PS. A, 12-19 and 2A-2 cells were transiently transfected to express FL Jagged, and their lysates were precipitated with the PS1 NTF-specific antiserum, X81, or the corresponding preimmune serum (pre). B, 12-19 and 2A-2 cells were transfected to express FL Delta and subjected to X81 co-IP as in A. C, primary mouse cortical neurons were cultured for 14 days and then solubilized in 1% Nonidet P-40 lysis buffer. Lysates were precipitated with X81 or its preimmune serum and probed with the Delta CTF-specific antibody, sc-12531.

Fig. 5. Primary structures of some of the known γ-secretase substrates. A, alignment of the transmembrane domains (boldface type, underlined) of human (H) APP, Notch, and ErbB4 as well as rat (R) Jagged and Delta. The putative NLS sequences are highlighted in red. The start sites of our recombinant JICD (Val1087) and JICD_NLS (Ser1183) constructs are shown in green (Fig. 5b). B, schematics of the membrane anchored JAE protein as well as the soluble JICD and JICD_NLS proteins. Amino acids in red represent the putative N-terminal residues of the predicted JICD fragment.

Fig. 6. Regulated Delta and Jagged proteolysis and signaling.
eliminated JICD production in COS cells transfected with JΔE (Fig. 6b).

Moreover, the membrane-anchored ΔΔE accumulated in the presence of the inhibitor, as expected. For these experiments on the processing of ΔΔE, we constructed two additional expression vectors (Fig. 5b). A recombinant JICD construct used the intramembranous V1087 as the initial amino acid for two reasons. First, ΔΔE and ΔΔΔE are generated from JICD with either JICD or JICD ΔNLS; the latter form lacks the basic amino acid stretches believed to represent an NLS (Fig. 5b). Following cell fractionation, the JICD fragment was markedly enriched in the nuclei of the JICD transfectants, whereas it was dramatically reduced in the nuclei and abundant in the cytosol of the JICD ΔNLS transfectants (Fig. 6c). The type 1 proteins contain basic amino acid stretches adjacent to the cytosolic face of the plasma membrane that are often considered stop-transfer sequences needed for proper orientation within the lipid bilayer. However, in the case of Jagged/JICD, we found that this stretch of basic amino acids is also necessary for the nuclear accumulation of the γ-secretase-derived JICD fragment (Fig. 6c). However, future work will be required to determine whether this putative NLS can also confer nuclear localization to an irrelevant protein, confirming its function as a bona fide NLS.

Ligand Processing Interferes with Notch Signaling at Multiple Levels—During the establishment of lateral inhibition, both the Notch receptor and its ligands are initially expressed in the same cell. Because the ectodomain shedding of both Notch and its ligands can be stimulated through ligand-receptor interactions and both result in the generation of suitable γ-secretase substrates, we sought to determine whether ligand CTF in a cell would compete for γ-secretase cleavage, reduce NICD production, and thus inhibit Notch signaling. First, we established the minimum amount of NΔE cDNA required to activate Notch reporter system (46). Then we titrated increasing concentrations of JΔE cDNA and quantified their effects on Notch signaling. In all experiments, we co-transfected the TK-Luc vector that constitutively expresses a variant of luciferase to control for transfection efficiency. Treatment with a γ-secretase inhibitor resulted in a marked reduction in HES activity, confirming the γ-secretase-dependent nature of our Notch reporter system (data not shown). We found that co-transfection with JΔE resulted in a linear dose-dependent inhibition of NΔE signaling (Fig. 7a), whereas co-transfection of vector only or HA-dynamin (another soluble, HA-tagged protein) had no effect (Fig. 7a). Surprisingly, co-transfection of NΔE and the soluble JICD fragment also caused a modest inhibition of Notch signaling, although not as great as the membrane-anchored form of Jagged did (Fig. 7a). To extend these results, we also investigated the effects of an APP-derived γ-secretase substrate (C99) on NΔE signaling. In accord with the findings for JΔE, co-transfection with C99 resulted in a dose-dependent inhibition of NΔE signaling, but in this case, AICD, the soluble γ-secretase product, had no effect.

Next, we transiently transfected COS cells with secreted alkaline phosphatase (SEAP) reporter constructs that are driven by various transcriptional enhancer elements, and these cells were then co-transfected with either vector alone or JICD or JICD ΔNLS. Conditioned media were collected 48 h post-transfection and analyzed for SEAP activity. The pTAL negative control vector contains only a TATA box upstream of the SEAP gene and serves as a control for background alkaline phosphatase activity, whereas the pSEAP positive control vector contains an SV40 promoter upstream of the SEAP gene to allow for robust constitutive alkaline phosphatase secretion. Of the eight candidate enhancer elements we screened, only the API element produced a clear and consistent increase in SEAP activity in response to JICD co-expression (Fig. 7b). JICD ΔNLS produced slightly less response than did intact JICD (Fig. 7b).

Fig. 6. PS/γ-secretase dependent generation of JICD and the nuclear localization of recombinant JICD. A, COS cells and FS12–19 and 2A-2 CHO cells were transiently transfected with JΔE and homogenized in 1% SDS lysis buffer. B, COS cells were transfected with ΔΔE and cultured with the γ-secretase inhibitor, Compound E, for vehicle alone for 16 h. Sister COS cultures transfected with recombinant JICD were run as a sizing control in the far right lane. C, COS cells were transfected with either JICD or JICD ΔNLS. Cytosol (C) and nuclei (N) were prepared in equal volumes, and aliquots were separated on a 14% Tris-Glycine gel and probed with 3F10 (upper panel). *, a nonspecific nuclear protein detected with the 3F10 antibody, demonstrating equal protein loading. As a control, the same cytosolic and nuclear fractions of the JICD-transfected cells were probed for the protein kinase CdK5 as a cytosolic marker and for the DNA-binding protein histone H1 as a nuclear marker (lower panels).

PS 12-19 and 2A-2 cells were each transiently transfected with JΔE, and both cell lines were fractionated to enrich for nuclei (19, 45). A protein corresponding to the predicted size of JICD was detected in the washed nuclear fraction of the 12–19 cells but not in that of the aspartate mutant 2A-2 cells (Fig. 6c). Next, we transfected COS cells with plasmids expressing either JICD or JICD ΔNLS; the latter form lacks the basic amino acid stretches believed to represent an NLS (Fig. 5b). Following cell fractionation, the JICD fragment was markedly enriched in the nuclei of the JICD transfectants, whereas it was dramatically reduced in the nuclei and abundant in the cytosol of the JICD ΔNLS transfectants (Fig. 6c). The type 1 proteins contain basic amino acid stretches adjacent to the cytosolic face of the plasma membrane that are often considered stop-transfer sequences needed for proper orientation within the lipid bilayer. However, in the case of Jagged/JICD, we found that this stretch of basic amino acids is also necessary for the nuclear accumulation of the γ-secretase-derived JICD fragment (Fig. 6c). However, future work will be required to determine whether this putative NLS can also confer nuclear localization to an irrelevant protein, confirming its function as a bona fide NLS.
Transfection with the AP1 reporter construct alone served as the base line for additional quantitative experiments that clearly confirmed the activation of AP1-mediated transcriptional activity by JICD in COS cells (Fig. 7c). Importantly, these results occurred independently of cell type, since both the increase in AP1 activity by JICD and the lack of response with cAMP-response element and six other enhancer elements were also observed in both CHO and HEK cells (data not shown).

A recent study demonstrated that NICD is capable of repressing AP1 activation, using a similar reporter system (48). Therefore, we examined whether NICD could have an antagonistic effect on the AP1-mediated activation produced by JICD. Indeed, co-expression of NICD with either JICD or JICD_{NLS} almost completely blocked the AP1 activation caused by each Jagged fragment alone (Fig. 7c). These data suggest that JICD can selectively stimulate AP1 activation in a manner that is not entirely dependent upon nuclear entry and that is opposed by the effects of NICD.

### DISCUSSION

Here, we report the discovery that the Notch ligands, Jagged and Delta, like Notch itself, undergo ectodomain processing by ADAM family proteases and subsequent cleavage by PS/γ-secretase to release potential signaling fragments. In view of the fact that four previously reported PS/γ-secretase substrates, APP, Notch, ErbB4, and E-cadherin, each generate intracellular fragments that are implicated in signal transduction in the nucleus, we asked whether the JICD is also liberated by PS/γ-secretase and translocates to the nucleus. Our results suggest that the regulated intramembrane proteolysis of the Notch ligands has implications for Notch signaling that partly involve the function of the soluble JICD.

We assigned several criteria to a potential PS/γ-secretase substrate, and the Notch ligands meet all of them. First, it was previously shown that the ectodomain of Drosophila Delta is cleaved by Kuzbanian, a protease related to mammalian...
Regulated Delta and Jagged Proteolysis and Signaling

Regulated Ectodomain Cleavage of Jagged—We found that the mammalian Jagged-1 protein is subject to an S2 (Notch)-like or β-secretase (APP)-like cleavage that is consistent with ADAM 17/TACE-like activity. This was established via pharmacological inhibition and phorbol ester stimulation of ADAM 17 cleavage, two methods previously shown to alter the ADAM 17/TACE-mediated shedding of APP (33–35, 49). We demonstrated ectodomain processing of Jagged in vitro following transfection with Jagged expression constructs and observed significant levels of the Jagged CTF in the developing rat embryo. Furthermore, low levels of a novel Jagged ectodomain fragment are secreted into the medium of Jagged-expressing cells and are absent in control medium. This protein migrates on SDS-PAGE gels at a smaller apparent $M_r$ than would be predicted for the entire Jagged ectodomain, suggesting that it may be subject to degradation in the conditioned medium. It is also possible that, like Notch in fly cells, the ectodomain is not secreted directly into the medium but is endocytosed by adjacent cells, which could explain the low levels of ectodomain we observed in medium. We find that the S2-like cleavage of rat Delta appears to be mediated by an ADAM 10/kuz-like activity, consistent with the report that Drosophila Delta is shed by Kuzbanian (27). Taken together, these data demonstrate that both families of mammalian Notch ligands undergo the same type of ectodomain processing as the Notch receptor itself. These initial findings supported the possibility that the Notch ligands could also be β-secretase substrates.

Generation of the Soluble JICD by PS/γ-Secretase—Recent evidence has demonstrated that the predominant ICDs generated by the intramembrane cleavage of both APP and Notch begin at a valine residue close to the cytosolic face of the transmembrane domain (17, 44). Therefore, we chose an analogous valine (Val1087) present within the Jagged transmembrane domain as the start site of our recombinant JICD (Fig. 5b). However, in both COS and CHO cell lines, we detected an endogenously generated JICD from the constitutively cleaved JAE that migrated slightly slower than our recombinant JICD-like fragment. This result suggests that either the γ-secretase cleavage site of Jagged is slightly N-terminal to the cleavage site of both APP and Notch or that some post-translational modification is responsible for the difference in migration. Interestingly, a portion of JICD generated from JAE remained membrane-associated (not shown), similar to what has been found after hypotonic cell lysis with portions of NICD and AICD.2 Future experiments will address the question of whether there are multiple γ-secretase cleavage sites, as has been shown for APP and Notch, and where the cleavage sites occur within the ligand transmembrane domains.

After the completion of this work, we became aware of very recently published data consistent with some of the observations in the current report. A study conducted in insect cells demonstrated the Notch-induced ectodomain shedding of Drosophila Delta (51), consistent with our findings in mammalian systems. In addition, pharmacological and genetic evidence was described suggesting that mammalian Delta1 and Jagged2 are processed by γ-secretase (52). However, this report did not include a characterization of the α-secretase processing of either protein, which is a prerequisite for downstream γ-secretase cleavage; nor was there information provided regarding protein-protein interactions between these substrates and components of the γ-secretase complex. The current data provide important insights into the protease activities that mediate the α-like and γ-secretase cleavages of both rat Delta1 and Jagged1. Further, we demonstrate both activities from embryonic tissue without relying on substrate overexpression. Finally, we provide data suggesting an APP1-mediated signaling role for Jagged.

The Impact of Ligand Proteolysis on Notch Signaling—Although expression of Delta or Serrate is required for activation of Notch in vivo, there is a paradoxical observation that overexpression of Delta or Serrate does not result in Notch overactivation but rather reduces Notch signaling (55–64). In one such study, high expression of Delta or Serrate correlated with a decreased sensitivity to receiving a Notch signal (64). Importantly, the cells that had ectopic overexpression of Delta or Serrate but could not receive a Notch signal were still able to stimulate Notch in adjacent cells, demonstrating that the ligands were present on the cell surface and functioned in receptor stimulation. Therefore, it is unlikely that the ectopically expressed ligand interfered directly with Notch; rather, some other interaction resulted in the inhibition of Notch signaling. Whereas this observation has been made in many ways in vivo, the mechanisms responsible for the Notch down-regulation are not known. Our finding that the Notch ligands undergo regulated intramembrane proteolysis raises several possible mechanisms by which ligand expression levels might modulate Notch signaling, as follows.

Several studies have examined the soluble Delta ectodomain for Notch activating potential, and most concluded that it was not an efficient Notch activator (65, 66). Indeed, a recent report confirms that purified Delta ectodomain has no activity in several cell-based assays of Notch signaling, consistent with the previous studies (67). These authors suggest that the ectodomain cleavage of Delta serves to limit the amount of ligand available at the cell surface to activate Notch. In this regard, our study makes the novel observation that the ectodomain processing of Delta can be stimulated by interaction with Notch. Therefore, if Notch and Delta interact under nonideal conditions (e.g. Notch and/or the ligand are not prepared for endoproteolysis, which is necessary for NICD signaling), the interaction could result in premature cleavage of the Delta ectodomain, thus reducing the pool of full-length Delta available for subsequent Notch activation.

Such abortive α-secretase cleavage of ligand is not the only role that ligand proteolysis could play in modulating Notch activation. Another possibility is that the resultant Delta and Jagged CTFs may compete with the Notch S2 cleavage product, NEXT, for γ-secretase processing. Such substrate competition

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2 W. T. Kimberly and D. J. Selkoe, unpublished data.
would be particularly relevant early during lateral inhibition, when both Notch and its ligands are expressed in the same cell; competition for γ-secretase would decrease the ability of a stimulated cell to release NICD. Importantly, such physical competition has already been established between Notch and another γ-secretase substrate, APP. In primary cultured neurons, endogenous Notch and APP compete for γ-secretase, indicating that changes in the levels of one substrate affect the processing of another, even when both are expressed at endogenous levels (39). These data further suggest that the protease can be saturated, which is not unexpected, given that PS/γ-secretase levels are very tightly regulated by limiting cellular co-factors (68). However, the ability of multiple substrates to compete with one another for γ-secretase cleavage and its effects on signaling remain to be confirmed in vivo. Further work will be needed to demonstrate the effects of competition in a developing organism.

Additional evidence that NEXT and the Delta and Jagged CTFs can compete for γ-secretase comes from an in vitro study in which co-expression of Notch and Delta in the same cell resulted in a phenotype opposite of that observed following co-culture of independent pools of Notch and Delta expressing cells (69). In these co-transfection experiments, the expression of Delta resulted in a phenotype that was Notch-hypomorphic, suggesting that the expression of both Notch and its ligand in the same cell attenuates Notch signaling, as has been suggested in vivo (64). We modeled this competition by investigating the effects of JΔE expression on Notch signaling from the membrane anchored NAE in a CBF-luciferase reporter system (46). We found that membrane-bound JΔE inhibited CBF-luciferase activity in a dose-dependent manner (Fig. 7a, left panel). Similarly, the APP γ-secretase substrate, C99, also inhibited NAE-derived reporter activity in a dose-dependent manner, whereas the soluble APP product, AICD, had no effect (Fig. 7a, right panel). Unexpectedly, the soluble JICD does possess some Notch-inhibitory activity, although not as potent as JΔE (Fig. 7a, left panel), whereas empty vector and an irrelevant soluble HA-tagged protein had no effect. This direct effect of JICD on Notch signaling was also observed when we used soluble NICD rather than NAE as the mediator of CBF-luciferase reporter activity (data not shown), supporting a direct interaction between the ICDDs of Jagged and Notch as regards the regulation of the CBF complex. Our data are entirely consistent with 1) the observations of several groups that ligand overexpression inhibits Notch function in vivo (discussed above) and 2) the finding that ligand and Notch co-expression blocks the Notch-induced inhibition of neurite outgrowth in a cellular model of Notch function (69). These various experiments do not establish whether NICD and JICD can interact physically or rather that JICD somehow inhibits the association of NICD with its transcriptional activating complex, a question that requires further study.

Apparent Signaling Capacity of the C-Terminal Domain of Jagged—Several of the previously identified γ-secretase products have been shown to traffic to the nucleus and directly affect gene expression through association with a transcriptional activating complex. We found that JICD can translocate to the nucleus as well, and therefore we asked whether a JICD-Gal4 fusion protein would drive expression of a Gal4-luciferase vector. While an AICD-Gal4 control construct drove luciferase activity as reported (21), JICD-Gal4 was inactive (data not shown). Therefore, we examined the ability of JICD to alter gene expression through a candidate-based screen of several known enhancer elements. The results showed that JICD consistently stimulated AP1-mediated reporter expression in intact cells, whereas several other broad spectrum enhancer elements, including calcium response element, serum response element, and heat shock element, were completely unaffected (Fig. 7). The selective effect of the soluble JICD protein on AP1 activation was observed in three different cell types (COS, CHO, and HEK), in all of which cAMP-response element and several other enhancers were not JICD-responsive. It appears that the activation of AP1 by JICD may not absolutely require nuclear entry, because a JICD mutant that lacked the putative NLS still increased AP1 reporter activity. These data are particularly interesting given the emerging evidence that Notch function extends beyond the ability of NICD to affect gene expression through an association with suppressor of hairless (e.g. see Refs. 70–72). An emerging principle from these in vivo and in vitro studies is that Notch has an inhibitory effect on the AP1 modulator, Jun n-terminal kinase. Indeed, a recent report directly demonstrates that NICD is a repressor of AP1-driven gene expression in mammalian cells (48). These findings, along with other reports, suggest that some aspects of Notch signaling in vivo involve AP1 proteins (73, 74). Consistent with this work, we have now found that AP1 stimulation by JICD is abrogated by NICD. Our data support the conclusion that Jagged, too, has a role in signaling via the AP1 system.

Consistent with this activation of AP1 by cytoplasmic JICD, the C terminus of human Jagged1 has been shown to contain a PDZ-ligand domain that is capable of binding the putative Ras effector, AF6 (75). AF6 is homologous to the Drosophila canoe, a protein also involved in Notch signaling (76). It has recently been shown that overexpression of Jagged1 transforms RKE cells and that the integrity of the PDZ-ligand motif is required for this effect (47). Furthermore, these investigators found that Jagged expression resulted in increases in Jagged1, Delta1, and Notch3 mRNA levels but not those of Notch1, -2, or -4, demonstrating a specific effect on gene expression. These data were also confirmed by a luciferase reporter construct driven by the Jagged1 promoter. Further, the authors found that Delta-1, -2, and -4 share the same PDZ-ligand motif with Jagged1, whereas Jagged2 and Delta3 do not, suggesting that this difference may be important to the heterogeneity of Notch ligand effects in various tissues. How the binding of this motif to AF6 plays a role in cellular transformation by Jagged1 or in the AP1 activation by JICD remains to be seen. In our experiments, we studied the rat homolog of Jagged1, which has 100% conservation of this C-terminal PDZ-ligand motif. The fact that we have now demonstrated that Jagged1 undergoes intramembranous cleavage to release a soluble ICD that reaches the nucleus makes these recent observations of Jagged’s potential for signaling even more compelling. The functional effects of Jagged RIP and signaling on both Notch-dependent and Notch-independent signaling await further elucidation.

Clinical Implications of the Multiple Substrates of PS/γ-Secretase—Because preselin is emerging as a ubiquitous and highly conserved molecular switch for signaling by certain type 1 transmembrane receptors, it may be difficult to therapeutically target individual substrates of γ-secretase (e.g. APP). Growing evidence has implicated the Notch signaling pathway in adults in both hematopoeisis and disease. Loss-of-function mutations within human Jagged-1 are associated with Alagille syndrome, and aberrant Notch/DSL signaling is thought to be involved in some forms of myeloid leukemia (53, 54, 77, 78). Therefore, a better understanding of the biochemical processing and downstream targets of the Notch-DSL signaling cascade as well as the roles of both ligand and receptor in cell growth and differentiation in health and disease could provide novel protein targets for combating abnormal cell proliferation or degeneration. Nevertheless, it remains likely that efforts to selectively block the γ-secretase cleavage of a specific substrate
