Murine Notch Homologs (N1–4) Undergo Presenilin-dependent Proteolysis*

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Notch proteins are type I cell surface receptors of short range signals exchanged between cells. Notch (N1–N4 in mammals) is used repeatedly throughout development (1) and in the adult (2) to determine cell fate selection. In adult mammals, anomalous Notch signaling has been linked to neoplasia (N1, N2, and N4), stroke (N3), and possibly schizophrenia (N4) (3–5). The biochemically characterized γ-secretase substrates from each receptor translocate to the nucleus and interact with the transcriptional regulatory protein CSL. All four Notch proteins display presenilin-dependent transactivating potential on a minimal promoter region as well as normal activity of these genes.

In this study, we sought to determine whether N2, N3, or N4 NEXT mimics undergo intramembranous proteolysis in a manner analogous to N1 and whether the cleavage site is conserved. We observe that all presenilin-dependent proteolysis at a conserved transmembrane position to produce NICD fragments that are post-translationally modified. All NICD peptides translocate to the nucleus and interact with CSL proteins. Additionally, all activate a simple reporter containing multiple CSL binding sites in a presenilin-dependent manner.

EXPERIMENTAL PROCEDURES

Notch Constructs—All Notch ΔE constructs were amplified from plasmids using PCR. Primers were created to introduce flanking ClaI sites to the amplified products. ClaI-digested PCR products were inserted into ClaI-digested pCS2+/N1ΔE, which removes the N1 intracellular domain and leaves the N1 signal peptide. N2ΔE (1660–3146 amino acids of the full-length N2) was amplified from a N2 plasmid (a gift from Dr. Y. Hamada) using primers mN2–2 (5’GGATCCGATTCCTACCCCTAGTGTCGTTCG3’) and mN2–2 (5’CCGATCCGATTCACCTGAGACGAGAAGGAGCTGGAGGACGAGAAG3’). Both fragments were digested with ClaI and EcoRV, and a three-piece ligation with the backbone vector was conducted. N4ΔE (1421–1864 amino acids) was amplified from the Int-3 plasmid, a gift from Dr. J. Kitajewski, using 5’ACAATCGATACCTGAGACGAGAAGGAGCTGGAGGACGAGAAG3’ and 5’GCGATCCGATTCACCTGAGACGAGAAGGAGCTGGAGGACGAGAAG3’. Stratagene QuikChangeTM site-directed mutagenesis kit was utilized to create N2ΔE/M1697L and N3ΔE/M1697L. Primers utilized for N2ΔE/M1697L were 5’GGGGGTCATCCTGGCCAAGCGGAAGC3’ and 5’CCGATCCGATTCACCTGAGACGAGAAGGAGCTGGAGGACGAGAAG3’. Stratagene QuikChangeTM site-directed mutagenesis kit was utilized to create N2ΔE/M1697L and N3ΔE/M1697L.

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§The abbreviations used are: DSL, Delta, Serrate, lag-2; NEXT, Notch extracellular truncation; PCR, polymerase chain reaction; CSL, CBF1, Su(H), Lag-1; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; NIDC, Notch intracellular domain; CIP, calf intestinal phosphatase; APP, amyloid precursor protein.
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GCCCACAGGGCCAGAAGAACCCACACTGGTAACTATAATGGGGCGAGGGCT-3

and mN4-15 (5'-GTGGTGAGGGCAGGGGAGCT-3'). The resulting PCR fragments were double-digested with BamHI and ClaI and introduced into a derivative of pCS2.

Transfections and Assays—For the presenilin cells, 2.5 x 10^6 cells were plated in 1 ml of the media (DMEM, 10% fetal bovine serum (Sigma), 100 units/ml penicillin/streptomycin (Life Technologies, Inc.)) per well of a 12-well plate. Each well was transfected with 200 ng of 4xCSL-luciferase reporter, 100 ng of Notch, 20 ng of pcS2±/β-gal, and the remaining DNA up to 1 μg with pcS2± vector. The DNA mixture was transfected into MEF cells using FuGene (Roche Molecular Biochemicals). Cells were re-fed with medium after 16 h and lysed 48 h after transfection in 200 μl of buffer (100 mM KPO4 buffer, pH 7.8; 0.2% Triton). 20 μl of lysate was used to determine β-galactosidase concentration to normalize for transfection efficiency. These assays were performed per the Tropix Galacton chemiluminescent substrate protocol. 50 μl of lysate incubated with luciferin assay buffer (30 mM Tricine, pH 7.8; 3 mM ATP, 15 mM MgSO4; 10 mM dithiothreitol; 0.2 mM CoA; 1 mM EGTA, pH 7.5) and incubated (30 min, 4°C) with 50 μl of 0.25 M CaCl2 and 500 μl of 2x BBS (10 min, 23°C). Cells were re-fed with appropriate medium 16 h later.

Immunoprecipitations—3 μg of Notch DNA were transfected into 100-mm dishes of 293 cells. Lysates immunoprecipitated to enrich for Notch proteins were sheared 42 h post-transfection with 26 gauge needles and spun to remove cellular debris. Lysates were incubated overnight with 1 μg of 9E10 ascites antibody (Sigma) and bound to protein A-beads for 2 h followed by washing and addition of Notch DNA to be immunoprecipitated into a T7E1 ligase. The DNA mixture was incubated into MEF cells using FuGene (Roche Molecular Biochemicals). Cells were re-fed with medium after 16 h and lysed 48 h after transfection in 200 μl of buffer (100 mM KPO4 buffer, pH 7.8; 0.2% Triton). 20 μl of lysate was used to determine β-galactosidase concentration to normalize for transfection efficiency. These assays were performed per the Tropix Galacton chemiluminescent substrate protocol. 50 μl of lysate incubated with luciferin assay buffer (30 mM Tricine, pH 7.8; 3 mM ATP, 15 mM MgSO4; 10 mM dithiothreitol; 0.2 mM CoA; 1 mM EGTA, pH 7.5) and incubated (30 min, 4°C) with 50 μl of 0.25 M CaCl2 and 500 μl of 2x BBS (10 min, 23°C). Cells were re-fed with appropriate medium 16 h later.

Results

Presenilin-dependent Proteolysis Forms a CSL-associated, Nuclear NICD for All Notch Homologs—Truncated N1 proteins lacking an extracellular domain (N1ΔE) mimic the N1ΔE fragment generated as a result of ligand binding (21) are cleaved at Val1744 (S3) releasing the N1NICD fragment; these proteins are constitutively active (19). To assess whether N2, N3, or N4 NEXT mimics, analogous to N2 and N4 oncogenic proteins, undergo S3 cleavage and are thus capable of forming NICD in vivo, similarly constructed Notch proteins were compared in parallel biochemically. N2ΔE, N3ΔE, and N4ΔE were constructed to closely resemble N1ΔE. Each of the ΔE constructs include the N1 leader peptide and ~20 amino acids of the extracellular domain, the transmembrane domain and extend intracellularly to the region N-terminal to the PEST region, ending with a hexameric Myc tag (Fig. 1A).

To determine whether a stable NICD-like fragment was present in cells expressing Notch ΔE proteins, Western blot analysis was performed. As shown in Fig. 2A, extracts from 3T3 cells transfected with each of the four Notch ΔE constructs contain an uncleaved ΔE polypeptide of the expected size. In addition, a faster migrating band is observed in all lanes. Similar results were obtained in HEK293 and CHO cells (Fig. 4). It has been previously demonstrated that the formation of smaller peptides from N1ΔE can result from initiation of translation at an alternative methionine within the transmembrane domain (22). To prevent generation of NICD-sized fragments due to alternative translation from methionines abutting the putative S3 cleavage site in N2 and N3 (Fig. 1B), these amino acids were mutated to leucine (N2ΔE(F18528L) and N3ΔE(F18528L)). Cells expressing N2ΔE(F18528L) and N3ΔE(F18528L) produced the same pattern of uncleaved ΔE and NICD-like fragments (Fig. 2B). Therefore, cells expressing any of the four truncated Notch proteins produce, in addition to the full-length ΔE protein, a stable NICD-like fragment, most likely by proteolysis.

To confirm that a precursor/product relationship exists between all uncleaved ΔE polypeptides and NICD-like fragments, metabolic pulse-chase and immunoprecipitation experiments were performed demonstrating that despite the low degree of amino acid conservation within the transmembrane domain, all Notch ΔE constructs produce a NICD fragment (Fig. 3, A and B). In addition, several higher molecular mass bands were also observed in cells expressing N2ΔE and N3ΔE (Fig. 3B), suggesting...
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that N2NICD and N3NICD fragments may be post-translationally modified more extensively than N1NICD and N4NICD. Indeed, a similar shift in mobility was detected with N2IC and N3IC proteins after labeling (Fig. 3B). Immunoprecipitation of Notch proteins from orthophosphate (32PO4)-labeled cells unequivocally demonstrate that NICD proteins are phosphorylated (Fig. 3, C–F). Incubating immunoprecipitated proteins in the presence of CIP only affects the mobility of N2 peptides (Fig. 3, D and F), suggesting either that additional modifications are present on N3 or that the phosphates on N3NICD cannot be removed by CIP.

As N1NICD formation is dependent on presenilin, we sought to determine whether presenilin proteins are required for proteolysis of all murine Notch proteins. Previous experiments have demonstrated that presenilin1 preferentially binds to both presenilin1 and presenilin 2 proteins after labeling (Fig. 3B). Immunoprecipitation of Notch proteins from orthophosphate (32PO4)-labeled cells unequivocally demonstrate that NICD proteins are phosphorylated (Fig. 3, C–F). Incubating immunoprecipitated proteins in the presence of CIP only affects the mobility of N2 peptides (Fig. 3, D and F), suggesting either that additional modifications are present on N3 or that the phosphates on N3NICD cannot be removed by CIP.

Collectively, these results demonstrate that presenilin is involved in converting all truncated Notch proteins to NICD. Experiments conducted in Drosophila suggest that transmembrane proteins can undergo presenilin-dependent processing if the extracellular domain has been truncated within a particular size range (29), suggesting a lack of cleavage site specificity.

We therefore wished to determine whether Notch proteins are cleaved at an equivalent position to N1V1744. As the N4 transmembrane domain is the most divergent from N1 (Fig. 1B), N4NICD was purified and sequenced. The N-terminal amino

Fig. 1. Notch protein structure. A, the number of EGF repeats varies slightly (32–36) and the very C-terminal region is the most highly divergent area between the homologs. The known N1 cleavage sites (S1–S3) are depicted. Domains included in the constructs utilized are illustrated. B, the transmembrane domain area. The putative S3 cleavage site is marked in blue. Methionines mutated in N1LAE/M1672L, N2LAE/M1672L, and N3LAE/M1663L constructs are marked in red. EGF, epidermal growth factor repeats; LNR, Lin/Notch repeats; TM, transmembrane domain; S1,2,3, proteolytic sites (1,2,3); NLS, nuclear localization signal; NCR, Notch cytokine response domain; TAD, transactivation domain; MT, Myc tag.

Fig. 2. All murine Notch molecules form a NICD fragment that preferentially interacts with CSL. In this and all subsequent experiments N1LAE refers to N1LAE/M1672L. A, two fragments are detected from Notch ΔE constructs, the faster migrating one preferentially co-immunoprecipitates with CSLRBP3-Myc antibodies. B, N2 and N3 methionine mutants form NICD fragments that interact with CSLRBP3-Myc, N2LAE/M1672L, N3LAE/M1663L constructs, the faster migrating one preferentially co-immunoprecipitates with CSLRBP3-Myc antibodies. Equivalent amounts of the lysates were run on SDS-PAGE gels and blotted with a -Myc antibody. For these and subsequent gels, different exposures of the same gel were scanned. C, Notch ΔE constructs generate tagged proteins that are clearly detected in 3T3 cell nuclei by -Myc antibodies.
mimics) serve as markers. N1IC, N2IC, and N3IC appear to be modified after 60 min (asterisk). C, N1 and N4 ΔE proteins are phosphorylated. Notch proteins were labeled with 32PO4, and immunoprecipitated. D, CIP treatment of [35S]methionine-labeled N2 protein indicates that N2 is phosphorylated. E, N2, N3, and N4 IC proteins are phosphorylated. An aliquot of cells expressing 32PO4-labeled N2IC, N3IC, or N4IC proteins were blotted with α-Myc (left side). These same samples were also immunoprecipitated and visualized by autoradiography (right side). F, N3IC is phosphorylated. CIP and 32PO4-labeled proteins were run on the same gel, separated to better visualize the fragments by autoradiography and the images subsequently spliced together. In the above experiments, methionine mutants for N1, N2, and N3 ΔE constructs were used. Additionally, proteins were immunoprecipitated with an α-Myc antibody; immunoprecipitations with an irrelevant antibody were negative (C and D and data not shown).

Once N1 has undergone cleavage at the cell surface, the non-membrane tethered N1ICD fragment translocates to the nucleus where it interacts with CSL proteins to regulate transcription of target genes. Immunocytochemical analysis revealed Notch nuclear staining in cells expressing membrane bound N2ΔE/M1663L, N3ΔE/M1663L, or N4ΔE (Fig. 2C). Additionally, co-immunoprecipitation studies demonstrate that CSL proteins preferentially interact with nuclear N1ICD fragments rather than the uncleaved N1ΔE polypeptide (19, 30). In contrast to the enrichment for the NEXT-like fragments by presenilin co-immunoprecipitation (Fig. 4, A and B), CSL-RBP5-FLAG co-immunoprecipitants enrich for the NICD fragments produced from cells expressing N2, N3, and or N4 ΔE constructs (Fig. 2, A and B). The preference of CSL for the low molecular weight NICD may suggest that non-modified NICD (Fig. 3, C–F) accumulates preferentially in the nucleus or that phosphatases are active during co-immunoprecipitation procedure.

These results establish biochemically that presenilin-dependent γ-secretase is the predominant S3 proteolytic activity in cultured cells. To determine whether any truncated Notch protein activate a CSL-dependent reporter in the absence of presenilin proteins, MEF cells derived from embryos heterozygous for presenilin1 and presenilin2 or from littermates deficient for both genes were used to compare the CSL-dependent activity of truncated Notch proteins (Fig. 5). In the absence of presenilin, the activity is similar to that of the 4xCSL reporter alone. We conclude that no alternative protomae exists in these cells capable of releasing sufficient NICD protein to elicit activity.

**DISCUSSION**

Regulated Intramembranous Proteolysis May Be a Common Feature of Notch Signaling—Upon ligand binding, the full-length N1 receptor is cleaved by a metalloprotease just extracellular to the transmembrane domain (S2 cleavage); the resulting cleavage product, NEXT, is then processed to form NICD. Western blot analysis demonstrates that a NICD-like fragment is produced from all NEXT-like, truncated Notch proteins (Figs. 2 and 3). While such fragments can be the result of alternative translation-initiation (22) or translation from a cryptic internal ribosome entry site (31), we demonstrate unequivocally that proteolysis is responsible for NICD production as mutating methionine residues in the N2 and N3 transmembrane domains do not impact NICD production (Figs. 2B, 3, 4). Pulse-chase analyses confirm that a precursor/product relationship exists between all Notch ΔE and NICD-like peptides (Fig. 3). Post-translational phosphorylation of all Notch proteins was also observed (Fig. 3), and N2 and N3 modifications have been previously noted (32, 33). Although the Notch ΔE constructs used in this paper include the analogous S2 cleavage site (Fig. 1B), we do not detect any S2 cleavage products by N-terminal sequencing. This is consistent with the model suggesting that S2 cleavage is only necessary in molecules containing an inhibitory extracellular domain and that molecules with a short extracellular domain can be directly processed to form NICD (21, 29).

Protein sequencing data confirm that the cleaved peptide bond in N4 occurs at a position equivalent to the known N1 S3

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2 M.T. Saxena, J.S. Mumm, and R. Kopan, unpublished observations.
N1, N2, and N3 methionines were used.

Open circles

...cleavage site (N4V1463, N1V1744, Fig. 1B). This conservation is intriguing as N1 and N4 share only 24% identity within the transmembrane domain (Fig. 1B). The fact that intramembranous Notch processing is highly dependent on this conserved valine (Fig. 4F, Refs. 19 and 34) is in stark contrast to the apparent lack of cleavage site specificity demonstrated for amyloid precursor protein (APP), the first known substrate of the presenilin-dependent enzymatic complex, γ-secretase (35, 36). We only detect the proteolytic product from N1AE and N4AE by N-terminal sequencing. It is worth noting that while we measure the release of a C-terminal fragment by γ-secretase, γ-secretase activity on APP was assayed by measurements of released N-terminal fragments. Recent sequencing of the N terminus of an APP C-terminal fragment identified a presenilin-dependent cleavage site in APP at Val50, a position equivalent to S3 in Notch (37). Three of five γ-secretase substrates are now known to be cleaved at an equivalent position (N1, N4, APP). It is however possible that the low-level proteolysis observed in Notch S3 valine mutants results from cleavage at position equivalent to APP Aβ40. Alternatively, residual proteolysis may occur at the mutant S3 site. Isolation of N3AE N-terminal stubs will reveal if such additional proteolytic fragments exist, perhaps resulting from rare cleavage events in the middle of the Notch transmembrane domain as in APP (36). This possibility remains untested.

It has been suggested that many transmembrane proteins can be cleaved by a presenilin-dependent activity (29). Ectopic expression of chimeric, membrane tethered proteins containing a potent transcription activator in Drosophila demonstrated that different transmembrane domains can all be cleaved by a presenilin-dependent activity as long as they lack an extensive extracellular domain and have the appropriate conformation. However, even with this sensitive assay, the authors reported a decline in proteolysis when the conserved valine in Drosophila Notch is mutated. It remains to be determined how many other proteins are natural substrates for presenilins and whether their proteolysis depends on specific sequences at the cleavage site. Finally, the observation that all Notch proteins are dependent on presenilins for proteolysis supports the hypothesis that the more severe phenotype of the murine presenilin 1/presenilin 2 nulls is due to a loss of all Notch activity (7, 8).

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