γ-Secretase-regulated Proteolysis of the Notch Receptor by Mitochondrial Intermediate Peptidase

Sheu-Fen Lee, Bhooma Srinivasan, Chantelle F. Sephton, Daniel R. Dries, Bing Wang, Cong Yu, Yun Wang, Colleen M. Dewey, Sanjiv Shah, Jin Jiang, and Gang Yu

From the Departments of Anatomy and Neurobiology, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390 and the Merck Research Laboratories, Boston, Massachusetts 02115

Notch is a transmembrane receptor that controls a diverse array of cellular processes including cell proliferation, differentiation, survival, and migration. The cellular outcome of Notch signaling is dependent on extracellular and intracellular signals, but the complexities of its regulation are not well understood. Canonical Notch signaling involves ligand association that triggers sequential and regulated proteolysis of Notch at several sites. Ligand-dependent proteolysis at the S2 site removes the bulk of the extracellular domain of Notch. Subsequent γ-secretase-mediated intramembrane proteolysis of the remaining membrane-tethered Notch fragment at the S3 site produces a nuclear-destined Notch intracellular domain (NICD). Here we show that following γ-secretase cleavage, Notch is proteolyzed at a novel S5 site. We have identified this S5 site to be eight amino acids downstream of the S3 site. Biochemical fractionation and purification resulted in the identification of the S5 site protease as the mitochondrial intermediate peptidase (MIPEP). Expression of the MIPEP-cleaved NICD (ΔNICD) results in a decrease in cell viability and mitochondria membrane potential. The sequential and regulated proteolysis by γ-secretase and MIPEP suggests a new means by which Notch function can be modulated.

EXPERIMENTAL PROCEDURES

Materials—Chemicals used included γ-secretase inhibitor N-[N-(3,5-Difluorophenylacetyl)-L-alanyl]-S-phenylglycine t-butyler ester (DAPT) (Calbiochem/EMD) and the EDTA-free complete protease inhibitor mixture tablet (Roche). Antibodies used included anti-myc 9E10 (ATCC), anti-cleaved Notch1 (Val-1744) (Cell Signaling Technology, Inc.) and anti-FLAG (Sigma). Western blot analysis was performed as described previously. The constructs NÄE and NICD in the pCS2 vector were obtained from Dr. R. Kopan (Washington University School of Medicine, St. Louis, MO) (7). Mutations in Notch were generated using site-directed mutagenesis. Mammalian Notch constructs were used as PCR templates to clone into pFastBac1 (Invitrogen). PS1^−/− mouse embryonic fibroblast (MEF) cells were obtained from Dr. B. De Strooper (Katholieke Universiteit, Leuven, Belgium).

The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2, materials and methods, and references.
**Proteolytic Activity Assay and Edman Protein Sequencing Analysis**—Biochemical analyses, proteolytic assays, and Edman protein sequencing were performed as described (8, 21, 22). Reconstituted recombinant γ-secretase was purified from SF9 cells as described (8). Mouse Notch1-derived substrates and recombinant MIPEP protein were also purified from baculovirus-infected SF9 cells. The soluble extracts (S100) from cell lines were used in activity assays with Notch substrate and reconstituted γ-secretase. S100 was obtained from cells lysed in 50 mM PIPES (pH 7.0), 150 mM KCl, 10 μg/ml leupeptin, 5 μg/ml trypsin inhibitor, 5 μg/ml Na-Tosyl-Lys-chloromethylketone, 5 μg/ml aprotinin, and 1 mM PMSF using nitrogen cavitation at 800 psi for 10 min at 4 °C. Postnuclear supernatant was collected at 800 × g, and the supernatant was centrifuged at 100,000 × g for 1 h to obtain the S100. Reconstituent FLAG/His-tagged N100 was incubated with reconstituted γ-secretase and assayed as described previously (8). For identification of substrate sequences, proteins on the membrane were stained with Amido Black, and the proteolytic fragments were excised and subjected to Edman degradation and sequenced at the Protein Chemistry Technology Center, University of Texas Southwestern Medical Center.

**Biochemical Identification of MIPEP**—HeLa S100 was subjected to ammonium sulfate precipitation. Precipitated proteins were resuspended in 20 mM PIPES, 150 mM KCl, 10% glycerol, 1 mM DTT plus protease inhibitors and chromatographed over a size exclusion column Sephacryl S300 (Pharmacia). Fractions (1 ml) were collected from the column and assayed for S5 site proteolysis. Fractions that exhibit γ-secretase-dependent cleavage activity were pooled and buffer-exchanged against 20 mM PIPES, 50 mM KCl, 10% glycerol, and 1 mM DTT plus protease inhibitors and passed successively over a Hi-Trap S and Hi-Trap Q (Pharmacia). The flow-through fraction was loaded on ω-aminoacyl-agarose (Sigma) (1 × 5 cm) at 20 mM PIPES, 50 mM KCl, 10% glycerol, and 1 mM DTT plus protease inhibitors. Protein fractions were eluted using a 40-ml gradient from 300 mM to 1 M KCl. The active fractions from ω-aminoacyl-agarose were pooled and chromatographed directly over a 1 × 5 cm hydroxyapatite type 1 (Bio-Rad) and eluted. Protein fractions were dialyzed against the wash buffer before subjecting the sample for γ-secretase-dependent protease activity assay. The peak of activity was found to elute at 150 mM sodium phosphate (pH 8). The protein fraction containing the activity was subjected to mass spectrometry at the Protein Chemistry Technology Center, University of Texas Southwestern Medical Center. Purification of mitochondria using sucrose gradient fractionation was performed according to standard procedures (23, 24).

**MIPEP Knockdown**—Cell culture and RNAi experiments were done as reported (22, 25). MIPEP (5′GCCGGGAUCCGGCCCGAATT’3’ and 5′CGUGCAGAGGGUAUAATAATT’3’), presenilin-1 (5′GGUCCACUUCGUAUGCUGGT’3’), or scrambled sequence (5′GAUCAGGGAUCCC-AUGGCTT’3’) siRNA (Dharmacon) were transfected into mammalian cells using oligofectamine. Scramble oligo duplex, FMR-8, duplex and MIPEP sense oligo were also used as controls for the experiments, but only the data from the scrambled oligo duplex is shown because the data obtained from these controls were similar. More details of the biochemical and cellular methods are described in the figure legends when appropriate. Representative data are presented for experiments performed a minimum of three independent times.

**Cell Viability Assay**—HeLa cells were maintained in DMEM supplemented with 10% FBS. Equal numbers of cells were transfected with cDNA constructs using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 h post-transfection and distributed in triplicates into 96-well plates. Cell viability was determined at 40 h post-transfection using MTS reagent (Promega). Absorbance was read at 490 nm, and data were normalized to cells expressing empty vector and plotted as % cell viability (mean ± S.D.).

## RESULTS

**Proteolysis of Notch at the S5 Site by a Novel Peptidase**—We had previously established an *in vitro* assay whereby recombinant γ-secretase purified from insect SF9 cells coexpressing mammalian presenilin, nicastrin, Aph-1, and Pen-2 faithfully proteolyzes a Notch substrate (N100) at the S3 site, generating a proteolytic fragment beginning with Val-1744 (NICD’ (Fig. 1A) (8, 22). In this study, we utilized this assay to search for novel factors that regulate γ-secretase activity or its Notch products in mammalian cell extracts. As expected, soluble protein extracts themselves did not contain γ-secretase activity (data not shown). However, addition of the soluble extracts to the *in vitro* Notch cleavage assay in the presence of recombinant γ-secretase produced the expected NICD’ fragment and another robust and faster-migrating proteolytic fragment referred to as ΔNICD’ (Fig. 1B). To identify the nature of the protease responsible for this secondary cleavage, the *in vitro* Notch cleavage assay was repeated with HeLa soluble extracts and recombinant γ-secretase and in the presence of several types of protease inhibitors in the assay. NICD’ production by recombinant γ-secretase was inhibited by γ-secretase inhibitor DAPT not but by dimethyl sulfoxide, the divalent cation chelators EGTA and EDTA, or the Roche EDTA-free protease inhibitors mixture known to inhibit proteases like chymotrypsin, papain, and trypsin (Fig. 1B, top left panel). Addition of HeLa soluble extract, on the other hand, generated the ΔNICD’ fragment in samples that produced NICD’ except for that treated with EDTA and ECTA (Fig. 1B, top right panel). These observations suggest the existence of a novel γ-secretase- and divalent cation-dependent activity responsible for generating ΔNICD’ *in vitro*.

One possible explanation for our observations is that an unknown factor in the soluble extract modulates γ-secretase activity through reconstitution with the presenilin, nicastrin, Aph-1, and Pen-2 γ-secretase complex to differentially process Notch substrate at separate sites. Alternatively, a novel protease might be responsible for processing Notch after a prerequisite γ-secretase cleavage event. To distinguish between these two possibilities, N100 was preincubated with recombinant γ-secretase to generate NICD’ prior to the addition of inhibitors or HeLa extract. In the absence of the extract, the inhibitors had no effect on the preincubated N100 and its proteolytic product NICD’ (Fig. 1B, bottom left panel). Addition of HeLa extract to the preincubated substrate generated the ΔNICD’.
proteolytic product that was sensitive to inhibition by EGTA and EDTA but not by DAPT or general protease inhibitors (Fig. 1B, bottom right panel). Moreover, the production of NICD' and ΔNICD' was markedly reduced in lysates from presenilin-deficient MEF cells (PS1-/-, Ref. 6) but was restored when recombinant γ-secretase was added back (Fig. 1C). Together, these observations suggest that the ΔNICD'-generating activity is metalloprotease-dependent and regulated by, but distinct from, γ-secretase.

We have previously shown that NICD' produced in our in vitro γ-secretase assay starts from 1744VLLSR... (8), the physiological S3 site cleavage of Notch (27, 28). Similarly, Edman protein sequencing revealed that the first five N-terminal amino acids of ΔNICD' are 1752RQHGQ... (Fig. 1A), indicating that the novel peptidase activity cleaves NICD between Arg-1751 and Arg-1752, hereafter referred as the S5 site, to remove eight amino acids from the N terminus of NICD. To confirm that we have indeed identified the correct proteolytic site, we replaced both arginine residues that constitute the S5 site with lysine residues (ΔNICD'). Moreover, the production of NICD' after incubation with recombinant γ-secretase was restored when lysine 1749 was replaced by histidine (K1749H) because K1749H is thought to be important for Notch being a proper γ-secretase substrate in cells (27, 29). None of the mutations in ΔNICD' from either the K1749H or the wild-type Notch substrate interfered with the generation of NICD' after incubation with recombinant γ-secretase-cleaved product, indicating that mutation of the lysine residue did not hinder the ability of the novel peptidase to cleave Notch in the

FIGURE 1. Notch is proteolyzed by a novel metallopeptidase activity in a γ-secretase-dependent manner. A, summary of the Notch proteolytic fragments analyzed in this study. The ligand-independent, membrane-tethered Notch trimmed of its extracellular domain (NΔE) consists of a transmembrane domain (TM) and an intracellular cytoplasmic domain (NICD). The cytoplasmic region includes a putative mitochondrial targeting signal (MTS), a RBP-Jκ/CBF1-associated module (RAM) domain, four known or putative nuclear localization signals (NLS), and a carboxyl-terminal PEST domain. NΔE is cleaved at the S3 site between Gly-1743 and Val-1744 by γ-secretase to generate NICD. A second γ-secretase-dependent cleavage occurs at the S5 site between Arg-1751 and Arg-1752 to generate ΔNICD. N-terminal Edman protein sequencing confirmed the presence of these three equivalent Notch fragments in our in vitro cleavage assay: N100, a Notch transmembrane substrate harboring Val-1711-Glu-1809 of mouse Notch-1 with a carboxyl-terminal FLAG/His tag. NICD', the proteolytic fragment of N100 with Val-1744 as the first amino acid and 8 amino acids from the N terminus of NICD. To confirm that we have indeed identified the correct proteolytic site, we replaced both arginine residues that constitute the S5 site with lysine residues (ΔNICD'). Moreover, the production of NICD' after incubation with recombinant γ-secretase was restored when lysine 1749 was replaced by histidine (K1749H) because K1749H is thought to be important for Notch being a proper γ-secretase substrate in cells (27, 29). None of the mutations in ΔNICD' from either the K1749H or the wild-type Notch substrate interfered with the generation of NICD' after incubation with recombinant γ-secretase (Fig. 1D). Addition of the peptidase activity from soluble HeLa extract produced similar level of ΔNICD' from either the K1749H or the wild-type Notch substrate, suggesting that mutation of the lysine residue did not hinder the ability of the novel peptidase to cleave Notch in the...
MIPEP Proteolyses Notch

Identification of the S5 site peptidase as MIPEP—We next developed a six-step biochemical fractionation procedure to purify the S5 site peptidase activity from HeLa extract (Figs. 2, A–C). The activity was purified 1863-fold (Fig. 2B) and replotted in A as activity (solid line). Fractions of high ∆NICD'-generating activity (hatched bar) were pooled and subjected to further purification as detailed in C. D, purified recombinant MIPEP (lanes 4–6), BSA, MEK3, βRgs7, or HeLa extract was incubated in the presence (lanes 1–4 and 6–7) or absence (lane 5) of recombinant γ-secretase and subjected to the in vitro Notch cleavage assay for 6 h using the Notch N100 substrate. E, 100 ng (lanes 1, 4, 7, and 10) or 500 ng (lanes 2, 5, 8, and 11) purified recombinant MIPEP or 500 ng βRgs7 (lanes 3, 6, 9, and 12) was incubated in the absence (lanes 1–3 and 7–9) or presence (lanes 4–6 and 10–12) of γ-secretase and subjected to the in vitro Notch assay for 0 h or 20 h using either the Notch N100 substrate or a recombinant Notch substrate that begins with Val-1744 (NICD').

Identification of MIPEP as the Notch S5 Site Peptidase—We next developed a six-step biochemical fractionation procedure to purify the S5 site peptidase activity from HeLa extract (Figs. 2, A–C). The activity was purified 1863-fold (Fig. 2C), and the resultant products were subjected to mass spectrometric analysis. Three tryptic peptides (LNTNVDLYQSLQK, HYQCTGQPLPK, GSLEAGIR) matched the MIPEP (GI 14602871, EC3.4.24.59), an oligopeptidase that belongs to the M3 zinc metallopeptidase family. Peptides for programmed cell death 6 interacting protein (DAFDKGSGGSVK, SVIEQGGIQTVDQLIKGI; GI 22027538) and RNA-dependent helicase (SSQSSQFSGIGR, ELAQVQVQVADDYGK; GI 3122595) were also identified and were not characterized further, as they are not predicted to have peptidase activity. We tested whether recombinant MIPEP can generate the ∆NICD' fragment seen with HeLa soluble extracts. Full-length recombinant MIPEP protein was purified from S9 cells using the baculoviral expression system. Recombinant MIPEP, in the presence or absence of reconstituted recombinant γ-secretase, was then included in the in vitro Notch cleavage assay. In the presence of recombinant γ-secretase, MIPEP, but neither BSA, MAP kinase kinase 3 (MEK3), nor a regulator of G-protein signaling molecule (βRgs7), generated a proteolytic fragment that comigrated with the ∆NICD' fragment seen in an assay with HeLa extract (Fig. 2D). As with HeLa extracts (Fig. 1B), MIPEP did not generate ∆NICD' from the N100 in the absence of γ-secretase and is not dependent on γ-secretase when purified NICD' was added as a substrate (Fig. 2E). These data indicate that recombinant MIPEP is responsible for producing ∆NICD' from NICD.

To further verify that MIPEP is responsible for generating ∆NICD', we utilized small interfering RNA (siRNA) to examine how reducing endogenous levels of MIPEP affects the S5 site cleavage activity. HeLa cells transfected with MIPEP siRNA duplex but not oligofectamine alone, sense oligo, or presenilin 1 (PS1) siRNA duplex showed specific reduction in the mRNA level of MIPEP (Fig. 2F). Although both the NICD' and ∆NICD' proteolytic fragments were present in assays performed with control cells, knockdown of MIPEP caused the level of ∆NICD' fragment to decline significantly (Fig. 2G).
Recently, Perumalsamy et al. (17) found Notch in the mitochondrial fraction of cellular lysates. Having shown that NICD can be further processed by the mitochondrial protease MIPEP in vitro and in vivo, we next asked whether, too, could detect NICD and/or the upstream protease γ-secretase in mitochondria. We were able to detect MIPEP, γ-secretase-cleaved NICD (α-Val-1744), NCT, and the mitochondria marker HSP60 in mitochondria but not in the non-mitochondria postnuclear supernatant of HeLa cell lysates (Fig. 3, A and B). Furthermore, these mitochondria preparations (but not the supernatant) were able to generate NICD’ and ΔNICD’ in our in vitro Notch assay (Fig. 3B).

Having demonstrated that γ-secretase-generated NICD’ is an immediate substrate of MIPEP activity in vitro and that the necessary components for ΔNICD’ generation can be found in mitochondria, we next examined whether MIPEP-mediated Notch cleavage occurs in intact cells. This proved to be rather difficult as 1) by the N-end rule, alternate forms of the Notch intracellular domain can be extremely labile (127, 30)), 2) removal of eight N-terminal residues (~1 kDa) between the S3 and S5 site cleavage products does not result in a substantial gel shift in the ~100 kDa Notch intracellular domain, and 3) we were unable to generate specific antibodies that can differentiate the eight residues between S3 and S5 site cleavage products.

We therefore tested whether reducing the MIPEP level by siRNA would impair S5 site proteolysis of Notch, thereby resulting in an increased level of the substrate, NICD. Detecting NICD in cells is feasible with the anti-cleaved Notch-1 (Val-1744) antibody that specifically recognizes the amino terminus of the S3 site proteolyzed Notch intracellular domain. Taking advantage of this antibody, we cotransfected HeLa cells with various Notch constructs (empty vector, NΔE, or NΔE V1744K, each containing a C-terminal 6x-myc tag) and siRNA (no oligo, MIPEP sense, or MIPEP or PS1 duplex), permeabilized, and stained the cells with anti-cleaved Notch-1 (Val-1744) or control (anti-c-myc) antibodies, and sorted the cells by FACS analysis. In HeLa cells expressing NΔE, knockdown of PS1 resulted in a dramatic loss of α-Val-1744 staining (Fig. 3C, center panel). Moreover, mutation of Val-1744 to lysine resulted in a dramatic loss in α-Val-1744 staining (Fig. 3C, right panel), thus confirming that this analysis specifically detects Notch that has been cleaved by γ-secretase. Knockdown of MIPEP (and, therefore, inhibition of further processing) resulted in an increase in the amount of cleaved Notch, suggesting a substrate-product relationship in cells.

**DISCUSSION**

In this study, we identify a novel proteolytic event of Notch at the new S5 site: ligand-induced proteolysis of the Notch receptor at the S2 and S3 sites (by TACE and γ-secretase, respec-
The connection between Notch proteolysis and a mitochondrial protease is unexpected and warrants further investigation. There is growing evidence for Notch-mediated cellular events at the mitochondria (15–20), and Perumalsamy et al. (17) recently showed that NICD localizes to the mitochondrial fraction. We note, however, that despite best efforts, cellular studies using a technique such as mitochondrial fractionation can be complicated by contamination of the samples with the extracellular and intracellular environments (1, 36).

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Regulation of Notch signaling and the diverse physiological outcomes are dependent on the context of specific cells and their extracellular and intracellular environments (1, 36). Notch has previously been implicated in context-dependent cell death events (37–40). Depending on cellular context, Notch-mediated transcription can be either pro- or antiapoptotic (41, 42). For example, activated Notch in T-cells promotes tumorigenic properties and protects against T-cell receptor-mediated apoptosis (43, 44). However, Notch enhances apoptosis in malignant B-cells and the HeLa cervical cancer cell line in a dose-dependent manner (45). It is therefore possible that the γ-secretase-regulated processing of Notch at the newly identified S5 site by MIPEP may contribute to the molecular switching mechanism for the context-dependent decisions of different cell fates, including cell death. In this study, we show that ΔNICD negatively affects cell viability and mitochondria membrane potential. It has been suggested that NICD localizes to the mitochondria and acts as a cellular sensor to block staurosorine-induced apoptosis (17). In the context of our study, cellular cues may induce MIPEP cleavage of Notch as a terminal signal. Moreover, several studies have found that Notch and γ-secretase can regulate levels of mitochondrial proteins, including MIPEP, to impact mitochondrial function (18, 46), suggesting the existence of a feedback loop of Notch and MIPEP regulation via γ-secretase. The role of MIPEP in the context of apoptotic cellular cues and the possibility of a feedback loop will need to be addressed in the future.

Our findings raise the possibility that MIPEP is a modulator of Notch signaling. Analysis of MIPEP knockdown in Drosophila supports this hypothesis (supplemental Fig. S2). Mechanistically, MIPEP could modulate Notch signaling by trimming the first eight residues of NICD in mitochondria, resulting in an alternative Notch intracellular fragment. It also removes several key N-terminal residues of the RAM sequence, a domain thought to trigger allosteric changes in the Notch transcriptional activation complex for the derepression of transcription (47–50). It is possible that N-terminal trimming affects the compartmentalization, trafficking, stability, or quantity of the active Notch species and that MIPEP-mediated processing represents a regulatory mechanism to produce the right amount of bioactive Notch intracellular domain at the right time under the right condition. Although provocative, the unexpected implication of a mitochondrial peptidase in a key intrinsic cell signaling pathway will advance our understanding of regulated and sequential proteolysis (51, 52) and expand the roles of mitochondria in cell regulation (53–57).
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