A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain


*Neuronal Cell Biology and Gene Transfer Laboratory, Flanders Institute for Biotechnology (VIB4), Center for Human Genetics, KU Leuven, B-3000 Leuven, Belgium
†Zentrum Biochemie und Molekulare Zellbiologie, Abteilung Biochemie II, Universität Göttingen, B-37073, Germany
‡Division of Dermatology and the Department of Molecular Biology and Pharmacology, Washington University, St Louis, Missouri 63110, USA
§Department of Pharmaceutical Sciences, University of Tennessee, Memphis, Tennessee 38138, USA
¶Departments of Psychiatry and Genetics, Washington University School of Medicine, St Louis, Missouri 63110, USA

Signalling through the receptor protein Notch, which is involved in crucial cell-fate decisions during development, requires ligand-induced cleavage of Notch. This cleavage occurs within the predicted transmembrane domain, releasing the Notch intracellular domain (NICD), and is reminiscent of γ-secretase-mediated cleavage of β-amyloid precursor protein (APP), a critical event in the pathogenesis of Alzheimer's disease. A deficiency in presenilin-1 (PS1) inhibits processing of APP by γ-secretase in mammalian cells, and genetic interactions between Notch and PS1 homologues in Caenorhabditis elegans indicate that the presenilins may modulate the Notch signalling pathway1,2. Here we report that, in mammalian cells, PS1 deficiency also reduces the proteolytic release of NICD from a truncated Notch construct, thus identifying the specific biochemical step of the Notch signalling pathway that is affected by PS1. Moreover, several γ-secretase inhibitors block this same step in Notch processing, indicating that related protease activities are responsible for cleavage within the predicted transmembrane domains of Notch and APP. Thus the targeting of γ-secretase for the treatment of Alzheimer's disease may risk toxicity caused by reduced Notch signalling.

Notch-1 is synthesized as a type I integral-membrane protein of relative molecular mass 300,000 (M, 300K) (Fig. 1) that is cleaved by a furin-like protease in the Golgi during trafficking of Notch to the cell surface. The two proteolytic fragments remain associated to form the functional receptor3,4. Following ligand binding, Notch-1 undergoes further cleavage close to or within its transmembrane domain, releasing the NICD. The NICD translocates to the nucleus and modifies transcription of target genes through its association with CSL proteins (where CSL stands for CBF1, Su(H), Lag-1)5–7. APP is also a type I integral-membrane protein. APP is the precursor of the amyloid-β peptide, which is deposited in the brains of Alzheimer's disease patients. Amyloid-β peptide is derived from APP as a result of cleavage of APP by β- and γ-secretase, whose molecular nature remains unclear. β-Secretase cleaves the extracellular domain of APP, producing a soluble ectodomain and a membrane-associated carboxy-terminal fragment (Fig. 1). γ-Secretase catalyses an intramembranous cleavage of this membrane-associated fragment, resulting in generation of amyloid-β peptide and the production of a C-terminal fragment of APP. Cleavage of APP by α-secretase precludes production of amyloid-β peptide (Fig. 1).

The functional role(s) of these APP fragments is unknown. Genetic and biochemical studies have implicated the proteolytic processing events that lead to amyloid-β-peptide generation in the pathogenesis of Alzheimer's disease. Mutations in APP and the presenilins (PS1 and PS2) cause familial early-onset Alzheimer's disease, and presenilin mutations lead to increased processing of APP by γ-secretase in vitro and in vivo8. PS1-deficient mice show decreased γ-secretase processing of APP9 and developmental abnormalities consistent with altered Notch signalling10. Genetic interactions between the notch homologues glp-1 and lin-12 and the presenilin homologues sel-12 and hop-1 in C. elegans provide indirect evidence for the involvement of the presenilins in the Notch signalling pathway11,12. Here we identify the specific step in Notch signalling that is controlled by PS1.

We first investigated whether the absence of PS1 interferes with the normal expression of Notch, as has been suggested13. We used brain extracts from PS1-deficient (PS1−/−) mouse embryos at embryonic day (E) 14. As expected, no expression of the amino-terminal or C-terminal fragments of PS1 was found (Fig. 2a and ref. 4). Moreover, in agreement with our previous results obtained from study of neuronal cultures14, we observed an accumulation of endogenous mouse APP C-terminal fragments in the same brain extracts, indicating deficient γ-secretase processing of APP when PS1 is absent (Fig. 2a, lower arrow). These fragments are predominantly 'stubs' produced by α-secretase, because endogenous mouse APP is not a good substrate for β-secretase (owing to the presence of amino-acid substitutions between the mouse and the human sequences at the β-secretase site)15,16. In the same homogenates, we detected normal steady-state levels of furin-processed Notch-1 C-terminal fragments, indicating that PS1 is not required for Notch biosynthesis or cleavage by furin within the Golgi (Fig. 2b).

We conclude that PS1−/− mouse cells produce normal amounts of mature (furin-cleaved) Notch 1 protein. The similarities between the proteolytic processing of Notch and APP are striking, especially the final proteolytic events that release the cytoplasmic domains, raising the possibility that PS1 might mediate the intramembranous proteolytic cleavage of Notch-1 to...
release NICD^7–9. As endogenous NICD levels in cells and tissues are biochemically below the level of detection^7, we took advantage of two well-characterized Notch constructs, mNotchΔE and NICV, that have been used to demonstrate the relationship between processing of and signalling through Notch-1 (refs 7, 13). The mNotchΔE construct codes for the transmembrane and intracellular domains of murine Notch-1 and undergoes a single proteolytic cleavage to release NICD. This cleavage resembles cleavage of endogenous Notch-1 in all respects^7,11. The NICV construct encodes the intracellular domain of Notch-1 without the membrane anchor, producing a protein identical to NICD. NICV has been used as a control for the fate of NICD in PS1^−/− cells^12. Both constructs have part of their C-terminal domain replaced with six consecutive Myc-epitope tags^7. We cloned these constructs into recombinant Semliki forest virus (SFV) vector, to enable their expression in primary cultures of neurons^4. The expression and subcellular localization of mNotchΔE were similar in wild-type and PS1^−/− cells, as was also the case for NICV (Fig. 3). However, NICD protein produced from NICV was immunoprecipitated from 100 μg mouse brain extract using IC antisera, an anti-Notch-1 antisera^5 and stained with the monoclonal antibody Tan20 (ref. 6) (6% SDS–PAGE). TMIC identifies the furin-generated mature Notch-1 fragment.

To assay directly the role of PS1 in mNotchΔE cleavage, we performed pulse-chase experiments and analysed the generation of NICD. Within 30–60 minutes after labelling with ^35S-methionine, NICD was first detected in PS1^+/+ cultures, NICD continued to accumulate throughout the 120-minute chase period (Fig. 4a, b; ratio of NICD to total mNotchΔE present at the start point of the chase). In contrast, NICD production was greatly reduced in PS1^−/− neurons (Fig. 4a, b). As the PS1^−/− embryos tend to yield fewer cells than their wild-type littermates^4, we analysed twice the amount of PS1^+/+ cell extracts. Only residual amounts of NICD were detected in PS1^+/+ neurons (Fig. 4c), even when the mNotchΔE signal was stronger than in the PS1^−/− cell extracts. This difference could not be explained by a higher turnover rate of NICD in PS1^−/− neurons, as NICD produced from control NICV constructs showed the same stability in both PS1^+/+ and PS1^−/− cells (Fig. 4d).

To test the generality of this observation in another, non-neuronal, cell type, we transiently transfected PS1^+/+ fibroblasts with mNotchΔE plasmids. Although NICD was produced in PS1^+/+ cells, it was hardly detectable in PS1^−/− cell extracts (Fig. 4e, extract). Co-immunoprecipitation of NICD with its nuclear partner, Flag-tagged CSLRBP3 (ref. 7), confirmed the existence of very low amounts of NICD in the PS1^−/− cells (Fig. 4e). As residual amounts of both NICD and amyloid-β peptide can be detected in PS1^−/− cells, it is possible that the PS1 homologue PS2 (expressed in PS1^−/− cells; K.C. and B.D.S., unpublished observations), can partially compensate for the loss of PS1 function. We conclude that NICD production is decreased markedly in PS1^−/− cells.

The site of cleavage of Notch-1, releasing NICD, may occur at an analogous position to the site of cleavage of APP by γ-secretase^14, N-terminal to the RKRRRQ stop-transfer signal. This apparently

Figure 2 Processing of APP and Notch-1 in PS1^−/− mouse brain and MEFs. Proteins were detected in western blots. a, Brain-membrane extracts (4–20% SDS–PAGE, 50 μg per lane) were reacted with APP675-695, an anti-serum specific for the APP C terminus, or with an anti-serum specific to the PS1 N terminus (PS1NTF). Arrows identify PS1NTF and a mouse APP fragment generated by a-secretase-mediated processing (mouse APP α-stubs). b, Notch-1 was immunoprecipitated from 100 μg brain extract using IC antisera, an anti-Notch-1 antisera and stained with the monoclonal antibody Tan20 (ref. 6) (6% SDS–PAGE). TMIC identifies the furin-generated mature Notch-1 fragment. c, MEF cell extracts were reacted with mN1A, a monoclonal antibody against Notch-1 (4–20% SDS–PAGE, 50 μg per lane).

Figure 3 Nuclear transport of the intracellular domain (NICD) of Notch-1. Neurons were transfected with SFV driving expression of mNotchΔE or NICV (which is identical to the NICD), fixed and immunostained. Red fluorescence indicates Myc-tagged mNotchΔE (upper panels) or NICD fragment (lower panels). Nuclear import of NICD is not disturbed in the PS1^−/− neurons. The green fluorescence in the lower panels indicates immunoreactivity towards calnexin, revealing the ER. The presence of uninfected neurons in the lower panels shows the specificity of the anti-Myc staining. +/+ PS1^+/+ neurons; –/– PS1^−/− neurons.
intramembranous cleavage is unusual and occurs in only one other known protein, the sterol-regulatory-element-binding protein (SREBP)13 (Fig. 1). However, mutants deficient in SREBP cleavage show no abnormality in generation of amyloid-β peptide, indicating that the enzyme that mediates SREBP cleavage may not be closely related to γ-secretase14,15. No effects on SREBP processing were observed in PS1+/− cells (data not shown). In contrast, our results from PS1+/− cells show that PS1 is important for cleavage of both Notch-1 and APP. To determine whether the enzyme responsible for NNICD release has similar pharmacological properties to γ-secretase, we compared the effects of protase inhibitors on processing of Notch-1 and APP, selecting inhibitors that have been shown previously to block γ-secretase15,18. These inhibitors also interfere with mNotchΔE processing (Fig. 5 and refs 7, 13). MDL 28170, which is a peptide aldehyde calpain inhibitor, was the first inhibitor shown to interfere with γ-secretase6, and MG132, another peptide aldehyde, also inhibits γ-secretase. However, MG132 (and possibly MDL 28170) are relatively broad-spectrum protease inhibitors that inhibit the proteasome. We ruled out the possibility that inhibition of the proteasome is involved in the observed effects of MDL 28170 and MG132 on Notch-1 processing by using lactacystin, a specific proteasome inhibitor, which has no effect on NICD generation (Fig. 5 and E.H.S. and R.K., unpublished observations). Likewise, lactacystin does not affect γ-secretase-mediated cleavage of APP17.

To further explore the apparent similarity between γ-secretase and the Notch-1 protease, we used the γ-secretase inhibitor MW167, a difluoro ketone peptide analogue designed on the basis of the primary sequence of the site of cleavage by γ-secretase in APP17. This compound inhibited, in a concentration-dependent manner, γ-secretase-mediated cleavage of human APP expressed in neurons using recombinant SFV (Fig. 6), leading to the accumulation of APP C-terminal stubs and the inhibition of amyloid-β peptide secretion into the medium. As both α- and β-secretase-mediated protease fragments appeared in the cells (α- and β-stubs, Fig. 6) and as secretion of the APP ectodomain into the medium was unaffected (data not shown), neither α- nor β-secretase enzymatic activity is inhibited by MW167 (see also ref. 17). Processing of NotchΔE was inhibited by MW167 at concentrations similar to those that inhibit APP processing (Fig. 6). The estimated half-maximal inhibitory concentration (IC50) for MW167 (10 μM) in NotchΔE processing is nearly identical to its IC50 for APP17, MW167 is also a potent inhibitor of cathepsin D23; however, using a series of related difluoro ketone analogues, we found that the effect of MW167 on APP processing is not a consequence of cathepsin-D inhibition24. The three inhibitors (MG132, MDL28170 and the chemically different MW167) all inhibit NotchΔE processing and γ-secretase-mediated cleavage of APP to similar extents. Although neither γ-secretase nor the enzyme that cleaves mNotchΔE has been purified yet, our results are consistent with the hypothesis that these two proteolytic activities are closely related.

We have shown that efficient processing of Notch-1 to produce NICD requires PS1 and is inhibited by γ-secretase inhibitors. We showed this by using the mNotchΔE construct, a reliable model for intracellular processing of Notch-1 (ref. 7). Furthermore, the expression of endogenous Notch-1, its maturation by a furin-like enzyme, and the translocation of NICD to the nucleus are not affected by PS1 deficiency. Notch-1 and APP both exhibit a genetic
Methods

The generation and characterization of PS1-/- mice, the generation of neuronal cell cultures, the use of recombinant SFV, metabolic labelling, cell extraction and double immunoprecipitation were done as described13,14. For pulse-chase experiments, cells from one embryo were seeded in a 12-well or 4-well cell culture plate (Nunc) pretreated with polylysine and serum. After infection with the appropriate recombinant SFV11,12, neurons were pulse-labelled for 30 min with 200 µCi 35S-methionine per ml and, for the indicated time in normal medium. Cells were processed as before2. Every lane (Figs 4a, c, d, 5, 6) shows the material obtained from one well, and signals were compared between littermate embryos. MG132 (Calbiochem), MDL 28170 (provided by B. Cordell) and MW167, each dissolved in dimethylsulphoxide (DMSO), were added at the end of the pulse labelling to the indicated final concentrations. DMSO in the cell culture was 1%.

Mouse brain was homogenized in 5 mM Tris–HCl, pH 7.4, 250 mM sucrose, 1 mM EGTA, 5 mM EDTA, 1 µg ml-1 pepstatin A and 100 µl-1 aprotinin, and centrifuged at 500g for 10 min. The resulting supernatant was centrifuged at 100,000g for 60 min. The pellet was resuspended in PBS and protein concentration was measured (Biorad protein assay).

We plated 5 × 105 mouse embryonic fibroblasts (MEFs) immortalized with SV40 large T antigen in 100-mm plates and transfected them by the CaPO4/BBS method17 with both Flag–CSL18,19 and mNotchE. After lysis in co-immunoprecipitation buffer containing 200 mM KCl, 10% of the sample was used for crude extract analysis. The remainder was co-immunoprecipitated using anti-Flag antibody. The washed pellet was resuspended in 50 µl Laemmli lysis buffer and resolved by 6% SDS–PAGE, transferred to nitrocellulose membrane and visualized using 9E10 anti-Myc antibody20. For immunocytochemistry, neurons cultured on glass coverslips were fixed in 4% paraformaldehyde and permeabilized in ice-cold methanol and acetone. Antibodies were diluted in blocking buffer and cover slips were mounted in Mowiol (Calbiochem). Cells were analysed using a MRC1024 confocal microscope (Biorad).

The complementary DNAs coding for mNotchE and NcY19,21, antiserum APF675-695 (ref. 12) used to immunoprecipitate the APP C-terminal stubs, and antiserum APF597-612 (ref. 12) used to immunoprecipitate amyloid-β peptide have been described. We used mNotchE in which Met 1727 was mutated to Val to prevent alternative translation initiation. NICD production from mNotchE is unaffected by this mutation, as shown previously21. B19/2 was raised against amino acids 30–44 of PS1. IC-antiserum against the cytoplasmic domain of murine Notch-1 (ref. 15) was provided by A. Israël and used to immunoprecipitate endogenous Notch-1 from brain extracts. Rat monoclonal antibody Tan20 (provided by S. Artavanis-Tsakonas) was used for immunodetection of precipitated Notch-1 (ref. 6). mNIA1, a monoclonal antibody specific to the cytoplasmic domain of Notch-1 (provided by L. Milner), was used for detection of mouse Notch-1 from MEF SDS extracts. Calnexin antisera and the anti-Myc monoclonal antibody 9E10 were provided by A. Helenius and J. Cremers.

Received 5 November 1998; accepted 5 February 1999.

Presenilin is required for activity and nuclear access of Notch in Drosophila

Gary Struhl† & Iva Greenwald†

Departments of *Genetics and Development, and †Biochemistry and Molecular Biophysics, and ‡Howard Hughes Medical Institute, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA

Presenilins are membrane proteins with multiple transmembrane domains that are thought to contribute to the development of Alzheimer’s disease by affecting the processing of β-amyloid precursor protein1. Presenilins also facilitate the activity of transmembrane receptors of the LIN-12/Notch family2–5. After ligand-induced processing, the intracellular domain of LIN-12/Notch can enter the nucleus and participate in the transcriptional control of downstream target genes6–9. Here we show that null mutations in presenilin abolish Notch signal transduction and prevent its intracellular domain from entering the nucleus. Furthermore, we provide evidence that presenilin is required for the proteolytic release of the intracellular domain from the membrane following activation of Notch by ligand.

β-Amyloid precursor protein (β-APP) is a transmembrane protein that travels by way of the endoplasmic reticulum and Golgi to the cell surface and undergoes proteolytic processing (reviewed in ref. 1). A set of β-amyloid (Aβ) peptides are generated from β-APP by proteases known as the β- and γ-secretases. The β-secretase cleavage occurs in the extracellular domain and the heterogeneous γ-secretase cleavages in the transmembrane domain. Dominant mutations in either of two human Presenilin genes appear to cause Alzheimer’s disease by increasing the amount of the Aβ42 (43) fragment that is produced. A null allele of mouse Presenilin 1 appears selectively to reduce γ-secretase activity10. These observations indicate that presenilin either stimulates the activity of γ-secretase, or is itself a component of γ-secretase10.

LIN-12/Notch proteins act as transmembrane cell-surface receptors for intercellular signals during development. It has been proposed that signal transduction involves cleavage and transport of the intracellular domain to the nucleus (reviewed in ref. 11), and results from experiments in Drosophila12–16 and mammalian cells strongly support this idea, indicating that cleavage occurs in or near the transmembrane domain. In mammalian cells, at least one

Figure 1 Presenilin activity is required for Notch activity. a, Hunchback (Hb) expression marks neuroblasts. Notch activity normally prevents more than one cell in a proneural cluster from segregating as a neuroblast. In PS− embryos, clusters of neuroblasts form instead of single neuroblasts, generating longitudinal bands, rather than lines, of neuroblasts. b, Single-minded (Sim) expression marks ventral midline cells10. Notch activity normally promotes differentiation of midline cells. In PS− (and N+) embryos, relatively few of these cells arise, in contrast to wild-type embryos where they form a continuous two-cell-wide column along the midline. c, Wing expression in Drosophila embryos, clusters of neuroblasts form instead of single neuroblasts, generating longitudinal bands, rather than lines, of neuroblasts. d, Wing expression is shown dorsally, just after completion of germ-band extension. e, Wing expression is shown dorsally, just after completion of germ-band extension. f, Wing expression is shown dorsally, just after completion of germ-band extension. g, Wing expression is shown dorsally, just after completion of germ-band extension. h, Wing expression is shown dorsally, just after completion of germ-band extension. i, Wing expression is shown dorsally, just after completion of germ-band extension. j, Wing expression is shown dorsally, just after completion of germ-band extension. k, Wing expression is shown dorsally, just after completion of germ-band extension. l, Wing expression is shown dorsally, just after completion of germ-band extension. m, Wing expression is shown dorsally, just after completion of germ-band extension. n, Wing expression is shown dorsally, just after completion of germ-band extension. o, Wing expression is shown dorsally, just after completion of germ-band extension. p, Wing expression is shown dorsally, just after completion of germ-band extension. q, Wing expression is shown dorsally, just after completion of germ-band extension. r, Wing expression is shown dorsally, just after completion of germ-band extension. s, Wing expression is shown dorsally, just after completion of germ-band extension. t, Wing expression is shown dorsally, just after completion of germ-band extension. u, Wing expression is shown dorsally, just after completion of germ-band extension. v, Wing expression is shown dorsally, just after completion of germ-band extension. w, Wing expression is shown dorsally, just after completion of germ-band extension. x, Wing expression is shown dorsally, just after completion of germ-band extension. y, Wing expression is shown dorsally, just after completion of germ-band extension. z, Wing expression is shown dorsally, just after completion of germ-band extension.