Conservation of the biochemical mechanisms of signal transduction among mammalian Notch family members

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Mouse Notch1, which plays an important role in cell fate determination in development, is proteolytically processed within its transmembrane domain by unidentified γ-secretase-like activity that depends on presenilin. To study this proteolytic event, we established a cell-free Notch cleavage assay system using the membrane fraction of fibroblast transfectants of various Notch constructs with deletion of the extracellular portion (Notch ΔE). The cytoplasmic portion of Notch1 ΔE was released from the membrane upon incubation at 37°C, which was inhibited by the specific γ-secretase inhibitor, MW167, or by overexpression of dominant negative presenilin1. Likewise, other members of mouse Notch family were proteolytically cleaved in a presenilin-dependent, MW167-sensitive manner *in vivo* as well as in the cell-free Notch ΔE cleavage assay system. All four members of the mouse Notch family migrated to the nucleus and activated the transcription from the promoter carrying the RBP-J consensus sequences after they were released from the membrane. These results demonstrate the conserved biochemical mechanism of signal transduction among mammalian Notch family members.

The Notch receptor plays an essential role in cell fate determination of various lineages in a variety of organisms from nematode to higher vertebrates (1). Mammals contain four Notch members (2–6), all of which have an extracellular region containing multiple epidermal growth factor repeats and three lin12/Notch repeats, a single transmembrane region and an intracellular region containing the RAM region, six cdc10/ankyrin repeats, nuclear localizing signals, and PEST sequence. The signal transduction triggered by interaction between the extracellular region of the Notch and its ligand, Delta or Jagged/Serrate, either blocks differentiation of stem or progenitor cells (7–10) or induces differentiation into specific lineages (11–13).

In both cases, it has been shown that at least three steps of proteolytic processing events are involved in the signal transduction of mouse Notch1 (mNotch1). mNotch1 is presented at the plasma membrane as a functional heterodimer after being constitutively processed by a furin-like convertase at site 1 (refs. 14 and 15; Fig. 1*B*). The ligand binding to mNotch1 results in the sequential cleavage at sites 2 and 3 (Fig. 1*B*). Tumor necrosis factor α-converting enzyme (TACE), a member of the ADAM family, cleaves the extracellular region of C-terminal fragment of the functional heterodimeric mNotch1 at site 2 (16, 17). This leads to the intramembranous cleavage at site 3 between Gly-1743 and Val-1744 (Fig. 1*A*), and translocation of the intracellular domain (RAMIC) of mNotch1 to nucleus (18, 19). The intracellular region of mNotch1 directly interacts with RBP-J/CBF1, the mammalian homologue of *Drosophila Suppresser of Hairless*, to activate transcription of downstream target genes such as the mammalian homologues of *Drosophila Enhancer of split*, *HES-1* and *HES-5* genes (20).

Alzheimer's disease (AD) is the most common form of senile dementia. Production and accumulation of β-amyloid (Aβ), which is the principal and specific component of the neuritic plaques in brains of AD patients (21), is important in pathogenesis of AD. The amyloid precursor protein (APP), a single transmembrane protein, can undergo endoproteolytic processing at three sites, one within the transmembrane domain (α-site) by α-secretase (22, 23), another at the N terminus of the Aβ domain (β-site) by β-secretase (24–27), and the other at the C terminus of the Aβ domain (γ-site) γ-secretase. Aβ is the result of sequential cleavages of APP by β-secretase and γ-secretase (28–30).

It has been shown that site 3 cleavage of mNotch1 and γ-secretase cleavage of APP both are dependent on presenilin (PS), which is a multiple membrane-spanning protein and synthesized as a single polypeptide that rapidly undergoes endoproteolysis within the cytoplasmic loop to generate two stable fragments, NTF and CTF (31). In mammals, two PS are found, and mutations in PS, which increase the generation of Aβ, are the most common known cause of autosomal dominant familial

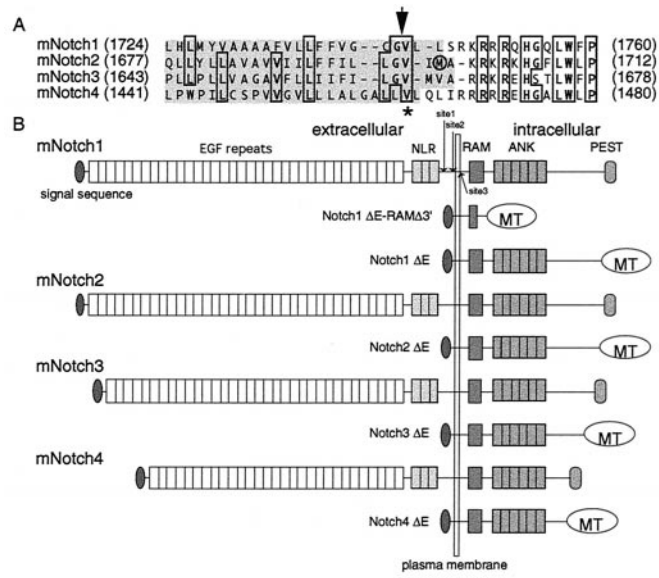

Fig. 1. (*A*) The alignment of the amino acid sequences of mNotch 1, mNotch2, mNotch3, and mNotch4 around the intramembrane cleavage site of mNotch1 (site 3). Numbers indicate positions of amino acid residues. Conserved residues are boxed. Dotted area, transmembrane region; arrow, the cleavage point of mNotch1; asterisk, the conserved valine; circled, Met-1699 of mNotch2. (*B*) Schematic representation of full-length, ΔE, and ΔE-RAM of Notch family. Sites 1, 2, and 3 are sequential cleavage sites. NLR, Notch/lin12 repeats; ANK, cdc10/ankyrin repeats; MT, 5xMyc tag.

Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid; APP, amyloid precursor protein; PS, presenilin; PNS, postnuclear supernatant.

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Alzheimer's disease (32–34). Deficiency of PS or substitution of either of its two conserved aspartates, predicted to be located within its transmembrane domain, leads to significant reduction of not only site 3 cleavage of mNotch1 but also γ-secretase cleavage of APP (35–39). γ-Secretase inhibitors block not only γ-secretase cleavage of APP but also site 3 cleavage of mNotch1 (36). Moreover, it was reported that PS could *associate* with either APP or mNotch1 (40).

Because prevention of production or accumulation of Aβ has been thought to be the target for the treatment of AD, a γ-secretase inhibitor is one of the drug candidates for AD. But its effect on mNotch1 as well as APP implicates that it would cause side effects in patients by inhibition of Notch1 signaling, for example on hematopoiesis. It has been thought that resolving this problem would make γ-secretase inhibitors more promising as medication for AD.

Although four mouse Notch family members (mNotch1, mNotch2, mNotch3, and mNotch4) are structurally similar (41), the signaling systems of mNotch2, mNotch3, and mNotch4 are not well understood. Here, we report that involvement of γ-secretase-like activity and PS in the cleavage system is conserved among four members of the mouse Notch family. These facts indicate that side effects by γ-secretase inhibitors should be carefully examined for possible interference of a wide range of biological activities of all Notch family members.

Materials and Methods

Construction of Plasmids. mNotch1 ΔE (M1727V)/pCS2$^+$ was described previously (42). mNotch1 ΔE-RAMΔ3′ (residues Ile-1704–Ala-1808), mNotch2 ΔE (residues Ser-1660–Glu-2154), mNotch3 ΔE (residues Phe-1623–Arg-2150), and mNotch4 ΔE (residues Leu-1421–Arg-1788) were generated by PCR based on the published sequences (3, 43, 44). The signal sequence of mNotch1 and 5xMyc tag were fused to 5′ and 3′ ends, respectively, of all ΔE and mNotch1 ΔE-RAMΔ3′. All of them were cloned into pEF-BOSneo mammalian expression vector. The intracellular region (RAMIC) of mNotch1 (residues Arg-1747–Lys-2531)/pEF-BOSneo and RAMIC of mNotch4 (residues Gln-1465–Asn-1964)/pEF-BOSneo were described previously (41, 45). RAMICs of mNotch2 (residues Lys-1701–Ala-2352) and mNotch3 (residues Lys-1669–Leu-2304) were generated by PCR and cloned into pEF-BOSneo. All PCR-generated fragments were confirmed by sequencing. PS-1 D257A/pcDNA3.1 Zeo has been described (35). pGa981–6, containing the hexamerized 50-bp EBNA2RE of the EBV TP-1 promoter in front of the minimal β-globin promoter of pGa50–7 luciferase reporter plasmid, and pCMX-LacZ containing the β-galactosidase gene driven by the CMV promoter have been described (41, 46).

Cell Culture and Transfection. Cells (293T) were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine and penicillin/streptomycin; 293T cells were transfected with PS-1 D257A/pcDNA3.1 Zeo (35) or pcDNA3.1 Zeo to obtain DA-20 and TZ-2, respectively, and selected by 400 μg/ml Zeocin. They were maintained in DMEM containing 10% FCS, 2 mM L-glutamine, penicillin/streptomycin, and 400 μg/ml Zeocin. The cells were transfected by calcium phosphate method using CellPhect (Amersham Pharmacia).

***In Vitro* Notch Cleavage Assay.** All procedures were conducted at 4°C unless otherwise mentioned. Cells that were transiently transfected with 10 μg of ΔE or ΔE-RAMΔ3′ plasmids on 10-cm plates were washed twice with ice-cold PBS and harvested 48 h after transfection. After the brief centrifugation, the cells were resuspended in 1 ml of 20 mM Hepes, pH 7.5, and 0.25 M sucrose and Dounce homogenized mildly with the B (loose) pestle for 15 strokes. Homogenized cells were centrifuged at 700 × *g* for 10 min. The resulting postnuclear supernatant (PNS) was centrifuged at 2,000 × *g* for 30 min to remove mitochondria in the pellet, followed by another centrifugation at 12,000 × *g* for 30 min to precipitate the membrane fraction in the pellet. This isolated membrane fraction was suspended in 120 μl of a reaction buffer (30 mM Tris·HCl, pH 7.0/150 mM NaCl/10% glycerol), and a 20-μl aliquot was used for each reaction. The suspended membrane was incubated with or without a protease inhibitor for 10 min on ice, followed by incubation at 37°C or 4°C for 2 h. The reaction was terminated by placing samples on ice and adding 20 μl of 2 × SDS sample buffer (200 mM Tris·HCl, pH 6.8/4% SDS/20% glycerol/10% 2-mercaptoethanol). The cleaved fragments were analyzed by Western blot. For the hypertonic wash of the membrane, the membrane was diluted with the buffer containing 1 M NaCl and placed on ice for 15 min. The diluted or washed membrane was centrifuged at 100,000 × *g* for 1 h at 4°C using a TLA100.2 rotor (Hitachi, Tokyo), and the pellet was used for the *in vitro* Notch cleavage assay.

Western Blot Analysis. Cells seeded on 3.5-cm-well plates were transfected with 1 μg of ΔE or ΔE-RAMΔ3′ plasmid per well. Transfected cells were harvested 48 h after transfection, added 200 μl of RIPA buffer (150 mM NaCl/1% Nonidet P-40/0.5% SDS/50 mM Tris·HCl, pH 7.5/1 mM PMSF), and placed on ice for 30 min. Whole cell lysate was obtained by centrifugation at 12,000 × *g* for 15 min. Whole cell lysate mixed with 2 × SDS sample buffer was boiled for 5 min, separated by SDS/PAGE, and transferred onto a PVDF filter. After blocking with PBS containing 1% skim milk, the filter was incubated with 9E10, an anti-Myc monoclonal antibody, for 1 h at room temperature, washed with PBS containing 0.1% Tween 20, and incubated with the horseradish peroxidase-conjugated anti-mouse IgG antibody (Organon Teknika–Cappel) for 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL plus detection system, Amersham Pharmacia).

Luciferase Assay. Cells seeded on 3.5-cm-well plates were transfected with 0 to 2 μg of ΔE or RAMIC/pEF-BOSneo of mNotch1–4, together with 0.2 μg of pGa981–6 and 0.2 μg of pCMX-LacZ. Different amounts of expression plasmids were supplemented with vector plasmid DNA to make an equivalent amount of total DNA in each sample. Cells were harvested around 40 h after transfection, and luciferase activities in the cell extracts were measured according to the manufacturer's instruction (Toyo Ink, Japan) in a Berthold luminometer (LumatLB9501). Luciferase activities were normalized by LacZ activities in each sample. Each experiment was carried out more than twice, and the representative results are shown as the average of triplicate values with standard deviations. The standard for fold activities is the activity of the sample transfected with 0 μg of ΔE or RAMIC/pEF-BOSneo of mNotch1–4.

Results

ΔE Constructs and Site 3 Cleavage of mNotch Family Members. It was reported that deletion of the extracellular region (ΔE) of mNotch1 leads to proteolytic cleavage of mNotch1 between Gly-1743 and Val-1744 (site 3) in the transmembrane region without interaction with its ligands, Delta or Jagged (20, 42). Fig. 1*A* shows the alignment of the amino acid sequences of mNotch1, -2, -3, and -4 around the cleavage site 3 of mNotch1. It is obvious that no amino acid residue is conserved around cleavage site 3 except the valine (Val-1744 of mNotch1, Val-1697 of mNotch2, Val-1663 of mNotch3, and Val-1463 of mNotch4). Nonetheless, all four mouse Notch family members have the RAM domain in their intracellular region, which is essential to interact with RBP-J, suggesting that a similar kind of proteolytic processing is required for all Notch members to translocate to the nucleus and transactivate the target genes.

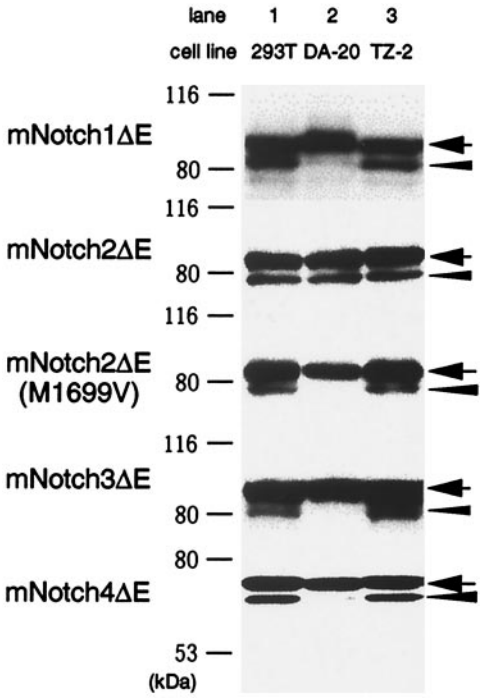

Fig. 2. Requirement of PS for processing of ΔE of mouse Notch family members. Each ΔE was transfected into 293T cells (the parental cell line), DA-20 (the cell line stably expressing D257A, dominant negative PS-1), and TZ-2 (the control cell line). Whole cell lysate of each sample was analyzed by Western blot using the 9E10 monoclonal antibody. Arrows and arrowheads indicate full-length and cleaved products of ΔE, respectively.

To examine whether this site 3 cleavage occurs in mNotch2, -3, and -4 by deletion of their extracellular domain, ΔE plasmids of mNotch2, -3, and -4 were constructed, and the signal sequence and 5xMyc tag were fused to the N and C termini of ΔE, respectively (Fig. 1*B*). These ΔE constructs were transfected into 293T cells, and Western blot analysis was performed. When probed with the anti-Myc antibody, 9E10, whole cell extracts of cells transfected with ΔE of every Notch family member revealed two fragments (Fig. 2, lane 1). The larger fragment of every Notch family member had the size equivalent to the full length of Notch ΔE and the smaller to the cleaved products. These results indicate that the spontaneous processing is triggered by the deletion of their extracellular regions in all mNotch family members.

Intramembranous Cleavage of Mouse Notch1, -2, -3, and -4 Requires Presenilin. Although it is still unclear whether PS is itself the γ-secretase or not, PS is essential for the γ-secretase activity for APP and site 3 cleavage of mNotch1 within its transmembrane region (35–38). To determine whether mNotch2, -3, and -4 require PS for their processing events, we established two kinds of stable 293T transfectant lines. One was named DA-20 that was stably expressing dominant negative PS-1 aspartic mutant (D257A) (35), which significantly reduced the γ-secretase activity in APP, and the other was named TZ-2, the control vector transfectant line. The expression of mutant PS-1 was confirmed by Western blot analysis (data not shown). ΔE constructs of mouse Notch family members were transfected into 293T, DA-20, and TZ-2 cells, and the effect of PS on each mouse Notch family member was evaluated by Western blot analysis. Cleavage of every Notch ΔE was detected in both 293T and TZ-2 cells (Fig. 2, lanes 1 and 3), whereas cleavage of all members but wild-type mNotch2 ΔE were markedly reduced in DA-20 (Fig. 2, lane 2).

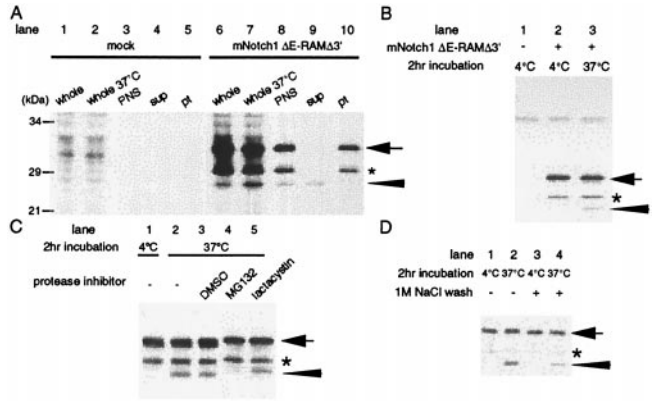

Fig. 3. (*A*) Analysis of the cells transfected with mNotch1 ΔE-RAMΔ3′. Whole cell extract was homogenized in the isotonic buffer (lanes 1 and 6) and incubated at 37°C for 2 h (lanes 2 and 7) or centrifuged at 700 × *g* for 10 min to obtain PNS (lanes 3 and 8). PNS were separated into the supernatant (sup, lanes 4 and 9) and the pellet (pt, lanes 5 and 10) by centrifugation at 100,000 × *g* for 1 h. (*B*) Cell-free Notch cleavage assay for mNotch1 ΔE-RAMΔ3′. The membrane of transfected cells was incubated at either 4°C or 37°C for 2 h. (*C*) Effects of 20 μM MG132 and 50 μM β-*clasto*-lactacystin in cell-free Notch cleavage assay. (*D*) Effect of washing the membrane with 1 M NaCl before 2-h incubation. Notch fragments were detected by Western blot using 9E10. Arrows, full length of mNotch1 ΔERAMΔ3′; arrowheads, cleaved product; asterisks, alternatively translated product.

Because the methionine (Met-1699, circled in Fig. 1*A*) near the putative cleavage site is the second methionine in mNotch2 ΔE, Met-1699 might be used as an alternative initiation codon. In fact *in vitro* translated mNotch2 ΔE contained two products corresponding to the peptides initiated at the first and second methionines (data not shown). Therefore, we introduced a mutation (M1699V) into mNotch2 ΔE to eliminate the product alternatively initiated from the second methionine. As expected, cleavage of mNotch2 ΔE (M1699V) was markedly reduced in DA-20 cells as compared with in 293T and TZ-2 cells. These facts indicated that PS is required for site 3 cleavage of mNotch2, -3, and -4 as well as that of mNotch1 *in vivo*.

Establishment of Cell-Free Notch Cleavage Assay. To gain further insight into biochemical mechanisms of mouse Notch signaling, we established a cell-free system for site 3 cleavage of Notch. Cells (293T) were transiently transfected either with an empty vector or with mNotch1 ΔE-RAMΔ3′ plasmid, which encodes the signal peptide, the C-terminal portion (20 residues) of the extracellular region, the total transmembrane region (24 residues), and the N-terminal 60 residues of the intracellular region (RAM domain) of mNotch1 (Fig. 1*B*). The intracellular region was truncated and fused to 5xMyc tag.

The transfected cells were harvested and homogenized in an isotonic buffer. When these homogenates were incubated at 37°C for 2 h, Western blot analysis using the 9E10 monoclonal antibody revealed that the intensity of the lowest band increased (Fig. 3*A*, lanes 6 and 7, arrowhead). To characterize this band, the homogenate was centrifuged at 700 × *g* for 10 min, and PNS was isolated. Western blot analysis showed that PNS contained three distinct bands, two of which migrated into the pellet after the centrifugation at 100,000 × *g* for 1 h (Fig. 3*A*, lanes 8 and 10). The larger fragment in the pellet is presumably the full length of mNotch1 ΔE-RAMΔ3′, whereas the smaller one is the alternatively translated product initiating from the second methionine of mNotch1 ΔE-RAMΔ3′, as this product was extinguished when a mutation was introduced to replace the intramembranous methionine with valine (M1727V; data not shown; ref. 42). Although these two bands were membrane-bound, the smallest

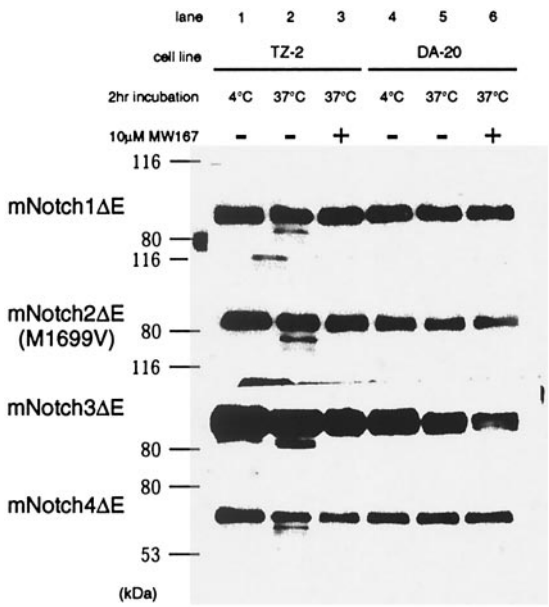

Fig. 4. Site 3 cleavage of mouse Notch family members in cell-free Notch cleavage assay. Each ΔE was transfected into DA-20 and TZ-2 cells. The membrane of the transfected cells was incubated at 37°C or 4°C for 2 h with or without 10 μM MW167 and analyzed by Western blot using 9E10.

band was recovered exclusively in the supernatant (Fig. 3*A*, lane 9), suggesting that this band may be the fragment generated by site-3 cleavage.

To directly confirm this possibility, the plasma membrane fraction of the transfected cells was isolated by the sucrose layer centrifugation of PNS and incubated at 37°C or 4°C for 2 h. Western blot analysis showed that, when incubated at 4°C, the isolated plasma membrane contained only two Myc-tagged fragments corresponding to the upper two bands of PNS (Fig. 3*B*, lane 2). Incubation of this membrane fraction at 37°C for 2 h resulted in formation of the putative cleavage product of ΔE-RAMΔ3′ (Fig. 3*B*, lane 3). The cleavage was blocked by 20 μM of MG132, which has been known to block the γ-secretase activity (Fig. 3*C*, lane 4) (42). Because MG132 is also known to inhibit the proteasome activity, the most specific proteasome inhibitor, β-*clasto*-lactacystin, was tested and shown to be unable to inhibit the cleavage (Fig. 3*C*, lane 5). In addition to MG132, the cleavage was also blocked by MW167, the γ-secretase-specific inhibitor developed by Wolfe *et al.* (35) (data not shown). These results demonstrate that the site 3 cleavage is carried out by γ-secretase-like activity. When the membrane of the transfected cells was washed with 1 M NaCl for 15 min before being incubated at 37°C, site 3 cleavage was still observed (Fig. 3*D*), suggesting that the protease for site 3 cleavage may be an integral membrane protein.

Site 3 Cleavages of All Notch Family Members Depend on PS and γ-Secretase-Like Activity. We next tested whether ΔE of mNotch1, 2, -3, and -4 could be cleaved similarly by cell-free Notch cleavage assay after transfection into TZ-2 and DA-20 cells. mNotch2 ΔE (M1699V) was used instead of wild-type mNotch2 ΔE. ΔE of mNotch1, -2, -3, and -4 were cleaved in the membrane of TZ-2 but not in the membrane of DA-20 (Fig. 4, lanes 2 and 5). These facts are consistent with the intact cell experiments described above. The γ-secretase-specific inhibitor, MW167 (10 μM) (35), almost completely inhibited the proteolytic processing of ΔE of all Notch family members in TZ-2 (Fig. 4, lanes 2 and 3). These results indicate that the proteolytic cleavage of all mouse Notch family members are carried out by γ-secretase-like activity and are dependent on PS.

We next investigated the effect of PS on the transcriptional activities of ΔE of all mouse Notch family members. Notch ΔE was cotransfected with pGa981-6, the luciferase reporter plasmid that harbors the hexamerized RBP-J binding site (41, 46), and luciferase activities were compared between DA-20 and TZ-2 cells. Whereas Notch ΔE of all family members showed increasing transcriptional activities in TZ-2 by the increment of ΔE amounts, little augmentation was seen in DA-20 (Fig. 5*A*). Expression levels of full-length ΔE were similar between DA-20 and TZ-2 cells, and the cleaved products were hardly detectable in DA-20 cells (Fig. 5*C*). Small amounts of cleavage products detectable after longer exposure may explain residual activities in DA-20 cells (data not shown). To exclude the possibility that the dominant negative PS-1 (D257A) expressed in DA-20 cells affects the transcriptional activities of ΔE by other ways than the inhibition of proteolytic processing of ΔE, the Notch intracellular region, designated RAMIC, was used instead of ΔE for this reporter assay. Because RAMIC does not require the processing event, the luciferase transcription activity should not be inhibited in DA-20 cells. In fact, RAMIC of all Notch family members exhibited similar levels of the transcriptional activities in both DA-20 and TZ-2 cells (Fig. 5*B*). Furthermore, ΔE of Notch2, -3,

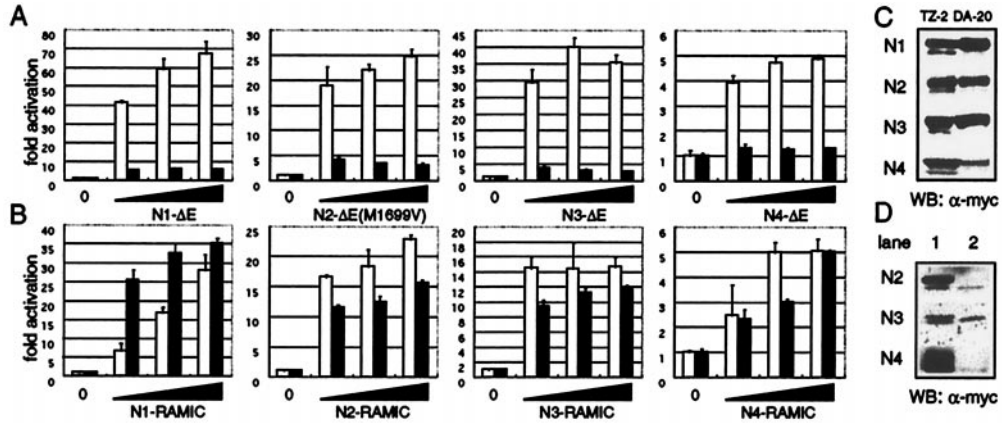

Fig. 5. Relative transcriptional activities of TP-1 promoter by ΔE (*A*) or RAMIC (*B*). White and black bars indicate transcriptional activities in TZ-2 and DA-20 cells, respectively. The results are presented as the mean ± SD for triplicate determination. (*C*) Expression levels of ΔE in DA-20 and TZ-2 cells at the maximal amount of DNA (2 μg DNA per well) were confirmed by Western blot analysis. (*D*) Nuclear fraction contains only cleaved products of ΔE. Lane 1, whole cell extract; lane 2, nuclear fraction.

and -4 migrated to nuclei after cleavage (Fig. 5*D*) as shown previously for Notch1 ΔE (36, 42). These data indicate that PS is essential for the transcriptional activities of ΔE of all Notch family members at the step of their processing and releasing the intracellular region of ΔE, which migrates to the nucleus.

Discussion

At least three steps of proteolytic processing events are involved in the signal transduction of mNotch1 (15–18). Site 3 cleavage is the most critical event for the signal transduction of mNotch1 (35, 42). Here, we developed a cell-free system for the evaluation of site 3 cleavage of mouse Notch family members. Taking the advantage of this cell-free Notch cleavage assay, we demonstrated here that site 3 cleavage occurs in mNotch2, mNotch3, and mNotch4 as well as in mNotch1 and is carried out by γ-secretase-like activity, which depends on PS. PS is also required in the cleavage of all four notch family members in experiments using intact cells.

MW167 was designed on the basis of the primary amino acid sequence of the cleavage site in APP by γ-secretase (35). MW167 also inhibited site 3 cleavage of all mouse Notch family members, although their amino acid sequences of putative cleavage sites are not conserved except for valine and quite different from that of APP. These facts are consistent with the fact that the sequence specificity of γ-secretase is low. Although it is unclear whether site 3 protease(s) for mNotch1, mNotch2, mNotch3, and mNotch4 are identical or not, our data clearly indicate the conservation of the processing system of the Notch family members with respect to the requirement of PS and the remarkable inhibitory effect of MW167.

After the cytoplasmic portion of mouse Notch is released from the plasma membrane by the proteolytic mechanism, we show that all members of mouse Notch family are capable of activating transcription from a promoter carrying the RBP-J binding motif. The present and previous studies (36, 41, 42) indicate that this is done by translocation of the cytoplasmic portion of Notch into the nucleus and by association with RBP-J. We also demonstrate that ΔE of all mouse Notch family members require PS at the step of the cleavage to gain the transcriptional activity via RBP-J.

Mutations of PS-1 and PS-2 found in familial Alzheimer's disease have the ability to enhance γ-secretase activity on APP (30, 32–34). We investigated the effect of one familial Alzheimer's disease mutation of PS-1, A246E, on the cleavage of mouse Notch family members. A246E does not have any effect on the processing of mouse Notch family members (data not shown). This result suggests that the biological function of PS in γ-secretase cleavage on APP and site 3 cleavage of Notch family members can be distinct.

Disruption of the mouse RBP-J gene results in early embryonic death before 10.5 days of gestation (47). RBP-J null mutant mice showed severe growth retardation as early as 8.5 days of gestation and developmental abnormalities, including incomplete turning of the body axis, microencephaly, abnormal placental development, anterior neuropore opening, and defective somitogenesis at 9.5 days of gestation (47). Mice lacking both PS genes also showed early embryonic lethality at 9.5 days of gestation, a little bit earlier than RBP-J knockout mice (48). They exhibited multiple early patterning defects, including lack of somite segmentation, disorganization of the trunk ventral neural tube, midbrain mesenchyme cell loss, anterior neuropore closure delays, and abnormal heart and second branchial development (48, 49). Comparing these two kinds of mice, the phenotype of PS-1 and PS-2 double knockout mice is severer than that of RBP-J knockout mice. Considering our results that PS is essential for the cleavage and transcriptional activity from RBP-J of all mouse Notch family members, the difference between mice lacking both PS genes and RBP-J gene implicates (*i*) γ-secretase may have other substrates, which play important role in development, other than Notch family members, (*ii*) mouse Notch family members may have other ways of transduction systems that do not require the proteolytic cleavage, or (*iii*) mouse Notch family members may have other effectors than RBP-J.

γ-Secretase has been considered to be an important target for the development of therapeutics for AD, for which no effective treatment has been found. Because γ-secretase inhibitors have their effects on not only APP but also mNotch1 (35, 36), they would cause side effects by inhibition of mNotch1 signaling, for example in hematopoiesis (35). The present study implicated that they would have additional problems by blocking the signaling of mNotch2, mNotch3, and mNotch4. Although functions of mNotch2, mNotch3, and mNotch4 in the adult mammals are less investigated than that of mNotch1, it would be more difficult to use γ-secretase inhibitors as medication for Alzheimer's disease than expected. Consequently, it is required to develop a γ-secretase inhibitor that blocks γ-secretase cleavage of APP without any effects on the cleavage of Notch family members or to target other steps involved in production, accumulation, and degradation of Aβ for treatment of AD.

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