Notch Oncoproteins Depend on γ-Secretase/Presenilin Activity for Processing and Function*

Received for publication, August 20, 2003, and in revised form, May 3, 2004
Published, JBC Papers in Press, May 3, 2004, DOI 10.1074/jbc.M309252200

Indranil Das‡, Colleen Craig‡, Yasuhiro Funahashi‡, Kwang-Mook Jung‡, Tae-Wan Kim‡, Richard Byers§, Andrew P. Weng§, Jeffery L. Kutok§, Jon C. Aster§, and Jan Kitajewski‡

From the §Department of Pathology and Obstetrics/Gynecology, Columbia University, College of Physicians and Surgeons, New York, New York 10032, the ¶Dana-Farber Cancer Institute, Boston, Massachusetts 02115, and the ¶Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

During normal development Notch receptor signaling is important in regulating numerous cell fate decisions. Mutations that truncate the extracellular domain of Notch receptors can cause aberrant signaling and promote unregulated cell growth. We have examined two types of truncated Notch oncoproteins that arise from proviral insertion into the Notch4 gene (Notch4/int-3) or a chromosomal translocation involving the Notch1 gene (TAN-1). Both Notch4/int-3 and TAN-1 oncoproteins lack most or all of their ectodomain. Normal Notch signaling requires γ-secretase/presenilin-mediated proteolytic processing, but whether Notch oncoproteins are also dependent on γ-secretase/presenilin activity is not known. We demonstrate that Notch4/int-3-induced activation of the downstream transcription factor, CSL, is abrogated in cells deficient in presenilins or treated with a pharmacological inhibitor of γ-secretase/presenilins. Furthermore, we find that both Notch4/int-3 and TAN-1 accumulate at the cell surface, where presenilin-dependent cleavage occurs, when γ-secretase/presenilin activity is inhibited. γ-Secretase/presenilin inhibition effectively blocks cellular responses to Notch4/int-3, but not TAN-1, apparently because some TAN-1 polypeptides lack transmembrane domains and do not require γ-secretase/presenilin activity for nuclear access. These studies highlight potential uses and limitations of γ-secretase/presenilin inhibitors in targeted therapy of Notch-related neoplasms.

Notch proteins are a family of single-pass transmembrane receptors that regulate cell fate decisions during development, including the differentiation of arterial versus venous fates in the vascular system (1) and T cell versus B cell development in the immune system (2, 3). Appropriate levels of Notch signaling are critical for normal development because loss of Notch signaling or excessive Notch signaling leads to embryonic lethal phenotypes (4, 5). Additionally, abnormal activation of several Notch proteins is associated with tumorigenesis, including a subset of human T cell acute lymphoblastic leukemias (T-ALL).1

* This work was supported by Public Health Service Grant HL-62454 from the NHLBI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Columbia University, College of Physicians and Surgeons 16-419, 630 W. 168th St., New York, NY 10032. E-mail: jkk9@columbia.edu.

‡ The abbreviations used are: T-ALL, T cell acute lymphoblastic leukemia; CpdE, compound E; EC domain, extracellular domain; HA, hemagglutinin; HUVEC, human umbilical vein endothelial cells; IC domain, intracellular domain; MEF, mouse embryonic fibroblast; Me2SO, dimethyl sulfoxide; N1IC, Notch1 IC domain; PS, presenilin; TM domain, transmembrane domain.

In vertebrates, full-length Notch proteins are part of a family of four receptors (Notch1–4) and five ligands that include members of the Jagged and Delta families. Notch receptors have an extracellular (EC) domain consisting largely of multiple epidermal growth factor-like repeats within which are binding sites for ligands. The Notch receptors also have a cytoplasmic region containing the RAM domain, seven ankyrin repeats, and a C-terminal PEST domain. Newly synthesized Notch receptors are processed initially in the trans-Golgi by a furin-like convertase, generating a ligand binding ectodomain and a transmembrane signaling domain that remain associated during transport to the plasma membrane (6–8). Ligand binding to the ectodomain induces several successive proteolytic cleavage events in the transmembrane subunit that result in release of its intracellular domain from the cell membrane. An initial cleavage by ADAM (a disintegrin and metalloprotease)-type metalloproteases occurs just external to the transmembrane (TM) domain (9, 10) and serves to facilitate additional cleavages by γ-secretases/presenilins within the TM domain (11). Mammalian presenilins (PS-1 and PS-2), are polytopic membrane proteins that appear to function as aspartyl proteases within a high molecular mass multiprotein complex that also includes the proteins nicastrin, Aph-1, and Pen-2 (12–15). Presenilins provide the active site of the γ-secretase proteolytic activity (16).

Presenilin-mediated cleavage is involved in activation of all four mammalian Notch receptors (17, 18), and in the case of Notch1, it occurs at several sites within the TM domain (19). Notch1 cleavage is prevented by the addition of γ-secretase inhibitors or mutations within critical aspartate residues of presenilin (20, 21). Delta-1-induced signaling through Notch1 is also reduced in cells deficient in presenilins or in cells treated with γ-secretase inhibitors (21–23). Presenilins have recently been recognized as having multiple substrates in addition to Notch, including β-amyloid precursor protein, ErbB4, N- and E-cadherins, and CD44 (24–28). Presenilins are necessary, after ectodomain shedding, for the cleavage and subsequent nuclear translocation of the intracellular domains of each of these polypeptides (11, 25, 27–29). In the case of Notch, presenilin-mediated cleavage allows the Notch intracellular domain (IC) to gain access to the nucleus, where it binds the transcription factor CBF1/CSL. This binding event converts CSL from a transcriptional repressor to a transcriptional activator, leading to expression of target genes (30). Notch signaling is normally activated by the interaction of a...
Notch Oncoproteins Require Presenilins

Notch receptor with ligand expressed on an adjacent cell. Truncated forms of Notch containing deletions spanning most, or all, of the EC domain bypass the need for ligand binding and signal constitutively. Two such Notch deletions, involving mouse Notch4 and human Notch1 (initially named TAN-1 for translocation-associated Notch homolog-1), were originally identified as oncogenes. The int-3 oncogene, which we refer to as Notch4/int-3, arises from retrotiral integration of the mouse mammary tumor virus into the Notch4 locus and causes murine mammary tumors (31–33). In all cases, viral integration results in the production of a truncated transcript that encodes both the TM and the IC domain of Notch4. In a similar fashion, the TAN-1 oncogene associated with T-ALL is generated from a truncation in human Notch1 caused by a recurrent (7, 9) chromosomal translocation involving the Notch1 gene and the T cell receptor β gene (34). All breakpoints identified to date occur within the intron just prior to the exon encoding the 34th epidermal growth factor repeat and result in identified oncogenes. The int-3 oncogene, which we refer to as Notch4/int-3, arises from retroviral integration of the mouse oncogenes. The int-3 oncogene, which we refer to as Notch4/int-3, arises from retroviral integration of the mouse oncogenes.

In this study, we asked whether Notch4/int-3 and TAN-1, oncogenic forms of Notch proteins, require presenilins for proper processing, signaling function, and activity. Based on the known sites of truncation within the Notch1 and Notch4 genes during their oncogenic conversion, both TAN-1 and Notch4/int-3 are predicted to encode proteins including TM domains, but lacking signal peptides. It was thus unclear whether these oncoproteins would insert into membranes and require presenilin activity for activation.

Our results show that both Notch4/int-3 and TAN-1 are targeted to the membrane and are subject to presenilin-mediated proteolytic processing. Notch4/int-3 requires presenilin activity for CSL-mediated signaling and biological activity in endothelial cells. TAN-1, however, demonstrates only partial dependence on presenilin activity. Although blockade of presenilin function alters TAN-1 processing, it does not affect the growth of T-ALL cells, apparently because some TAN-1 polypeptides bypass the requirement for presenilin processing for nuclear access. The finding that some oncogenic forms of Notch depend on presenilin activity for function suggests potential avenues for therapeutic intervention.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Compound E (CpdE) was a gift from T. Golde. The antibodies PS-1-loop and PS-2-loop were provided by G. Thimakan. The immortalized fibroblasts lacking PS-1 and PS-2 were provided by B. De Strooper.

**Luciferase Reporter Assays**—Transient transfections were performed by calcium phosphate precipitation. For assessment of activated Notch signaling in HeLa cells, cells were plated 24 h prior to transfection in 6-well plates at 1.0 × 10⁵ cells/well. Cells were transfected in triplicate with 250 ng of pQNCN1IC-HA or 250 ng of pLNCNotech4/int-3-HA in combination with 500 ng of CSL luciferase reporter (pGA981-8) and 350 ng of pLNClacZ (control for transfection efficiency). Activated Notch signaling in MEFs (wild type or PS-1.2(–/–)) was assessed by plating cells 24 h prior to transfection in 6-well plates at 3 × 10⁶ cells/well. MEFs were transfected in triplicate with 1 μg of pQNCN1IC-HA or 1 μg of pLNCNotech4/int-3-HA in combination with 670 ng of pGA981-6 and 333 ng of pLNClacZ.

**To determine ligand-induced Notch signaling, coculture assays were performed using HeLa and 293-derived Bosc cells.** HeLa cells plated 1 day earlier in 10-cm plates at 1.5 × 10⁵ were transfected with 7.5 μg of pBOS Notch1 or pBOS Notch2, 4 μg of pGA981-6, and 1 μg pLNC lacZ. Bosc cells plated 1 day prior in 10-cm plates at 4 × 10⁵ were transfected with either 25 μg pHyTc-Jagged1, pHyTc-Delta-1, pHyTc-Delta-4, or pHyTc-EP-Δ (empty vector). One day after transfection, cells were cocultured in triplicate (HeLa:Bosc, 1:2) on 12-well plates for 24 h. In all cases, cultures were treated with CpdE 16 h prior to harvest of cells. Cells were harvested, and luciferase activity was determined by performing a dual injection luminescence assay. Reporter activation was normalized for transfection efficiency. Fold reduction by CpdE was calculated as the ratio of the normalized reporter activity from untreated cells to CpdE-treated cells.

**Biotinylation Assays**—The membrane expression of TAN-1, Notch4/int-3, and N1IC-HA was determined by performing surface biotinylation experiments. Notch4/int-3 or N1IC was transiently transfected into HeLa cells by Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). SUPT1 cells (expressing TAN-1) and transfected HeLa cells were treated with CpdE 16 h prior to harvest. SUPT1 cells and transfected HeLa cells were washed with phosphate-buffered saline and then labeled with EZ-Link Sulfo-NHS-Biotin (Pierce) for 30 min at 4°C. Biotin was quenched with phosphate-buffered saline and glycine, and cells were washed with phosphate-buffered saline. Cells cultured in 6-cm plates were lysed in TENT buffer, and after clearing lysates, a small aliquot was saved to load 50 ng of whole cell lysate. The remainder of the lysate was used to purify surface proteins by incubating the membrane-immobilized streptavidin-agarose overnight at 4°C. Streptavidin–precipitated proteins and whole cell lysates were treated with λ-phosphatase (New England Biolabs), separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibodies: TC to detect TAN-1 (41), CoRb2–2 to detect Notch4/int-3 (42), 12CA5 to detect HA-tagged N1IC, anti-glycogen synthase kinase–β (Transduction Laboratories), anti-α-tubulin (Sigma), anti-β integrin (43) provided by Eugene Marcantonio, Columbia University), and the PS-1-loop or PS-2-loop antibodies to detect presenilins-1 and 2, respectively (44). Proteins were visualized with ECL (Amersham Biosciences).

**Analysis of Notch4/int-3 Expression in 293 Cells**—293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Stable cell lines (293 cells) were transfected in 100-mm dishes with 10 μg of pLNCint-3-HA or Notch1ΔE plasmids using Superfect transfection reagent according to the manufacturer’s instructions (Qiagen). Cultures were treated with CpdE, L-685,458 (Calbiochem), AEB5F (Roche Applied Science), pepstatin A (Roche Applied Science), or Me2SO for 16 h prior to harvest of cells. At 48 h post-transfection, cells were lysed using buffer IP (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM EDTA) supplemented with protease inhibitor cocktail mixture tablet (Roche Applied Science). Protein quantitation, SDS-PAGE, and Western blot analyses were carried out as described previously (27). The primary anti-α-tubulin antibody (HA 11, Covance) was used at a dilution of 1:1,000, the anti-cleaved Notch1 (Val1799) antibody (Cell Signaling) was used at a dilution of 1:1,000, and anti-α-tubulin (Sigma) was used at 1:15,000.

**Culture of Human Umbilical Vein Endothelial Cells (HUVEC)**—HUVEC were isolated from human umbilical vein by the method described previously (45). HUVEC were grown in EGM2 kit (BioWhittaker, San Diego), containing vascular endothelial growth factor, basic fibroblast growth factor, epidermal growth factor, insulin, and 2% fetal bovine serum, and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Stable cell lines (293 cells) were transfected in 100-mm dishes with 10 μg of pLNCint-3-HA or Notch1ΔE plasmids using Superfect transfection reagent according to the manufacturer’s instructions (Qiagen). Cultures were treated with CpdE, L-685,458 (Calbiochem), AEB5F (Roche Applied Science), pepstatin A (Roche Applied Science), or Me2SO for 16 h prior to harvest of cells. At 48 h post-transfection, cells were lysed using buffer IP (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM EDTA) supplemented with protease inhibitor cocktail mixture tablet (Roche Applied Science). Protein quantitation, SDS-PAGE, and Western blot analyses were carried out as described previously (27). The primary anti-α-tubulin antibody (HA 11, Covance) was used at a dilution of 1:1,000, the anti-cleaved Notch1 (Val1799) antibody (Cell Signaling) was used at a dilution of 1:1,000, and anti-α-tubulin (Sigma) was used at 1:15,000.

**Culture of Human Umbilical Vein Endothelial Cells (HUVEC)**—HUVEC were isolated from human umbilical vein by the method described previously (45). HUVEC were grown in EGM2 kit (BioWhittaker, San Diego), containing vascular endothelial growth factor, basic fibroblast growth factor, epidermal growth factor, insulin, and 2% fetal bovine serum. The supernatant was removed from type 1 collagen-coated 10-cm dishes and the cells were washed with phosphate-buffered saline. Adenoviral Gene Transfer—7.5 × 10⁵ cells of HUVEC at passage 3 were seeded into type 1 collagen-coated 6-well plates on the day before adenoviral infection. Adenoviral infection with Ad-lacZ or Ad-Notch4/
int-3 virus (42) was performed at a multiplicity of infection of 60 and incubated at 37 °C for 1 h with occasional swirling of plates. Infected cells were harvested the day after infection for further experiments.

**Sprouting Assay**—For making collagen gels, an ice-cold solution of porcine type I collagen (Nitta gelatin, Tokyo, Japan) was mixed with 10 × RPMI 1640 medium and neutralization buffer at the ratio of 8:1. Aliquots of collagen gel (400 μl) were then added to 24-well plates and allowed to gel for at least 1 h at 37 °C. After adenosviral infection (above), HUVEC were harvested and plated at 1.3 × 10^5 cells/well onto the medium of the gel in 24-well plates in 0.8 ml of EGM2 medium. HUVEC became nearly confluent 48 h after plating. After seeding, the medium was changed every 2 days for the next 3 weeks. Treatment with CpdE was started at day 2 and continued for 3 weeks. Sprouting was observed in photographs taken after 3 weeks with an Olympus (Japan) camera mounted to a microscope. For quantification of the number of sprouts, five fields each well were selected randomly, and sprouting was counted under microscopy in a blind manner by two investigators.

**Cell Cycle Analysis**—SUPT1 cells were seeded at 1 × 10^6/ml in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and the indicated concentrations of CpdE or carrier alone (MeSO). Cells were split every 2 days into fresh medium and drug to maintain cell density between 0.5 and 1.5 × 10^5/ml. At day 4, cells were harvested, fixed in 70% ETOH, stained with propidium iodide, and analyzed by flow cytometry for DNA content. Cell cycle fractions were determined using MultiCycle software.

**Immunostaining**—SUPT1 cells and Jurkat cells grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum were washed twice in phosphate-buffered saline, fixed for 15 min in 10% buffered formalin, and centrifuged at 1,000 × g for 5 min. Cell pellets were then dehydrated, embedded in paraffin, and sectioned at 5 μm. After dewaxing in graded ethanols, the sections were subjected to "antigen retrieval" by heating in 1× x EDTA, pH 8.0 (Zymed Laboratories Inc., South San Francisco, CA), in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, CA) according to the manufacturer's instructions. After washing in distilled water, slides were pretreated with Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 min, followed by goat serum (1:5 in 50 mM Tris-Cl, pH 7.4) for 20 min. Sections were then incubated with rabbit anti-Notch1IC (TC) (1:5,000) (41) in 50 mM Tris-Cl, pH 7.4, containing 3% goat serum, followed by goat anti-rabbit horseradish peroxidase-conjugated antibody (Envision detection kit, DAKO). After further washing, immunoperoxidase staining was developed using a diaminobenzidine chromogen (DAKO), followed by an enhancing solution (Zymed Laboratories Inc., South San Francisco, CA).

**Spectral Imaging**—Sections were stained with anti-Notch1IC (TC) antibody as above and subjected to analysis using a CRI Nuance spectral imaging system (CRI, Cambridge, MA). Briefly, conventional bright-field images (×400 magnification) were captured sequentially at 10-nm wavelength intervals from 420 nm (blue) to 800 nm (red), and the resultant image cube (the concatenated stack of images at each wavelength interval per pixel) was analyzed using CRI Nuance software. The diaminobenzidine spectral profile was applied across all image cubes to determine the intensity of diaminobenzidine staining per pixel after which the staining intensities were converted to composite false color images.

**RESULTS**

**Presenilin Activity Is Required for Ligand-induced Activation of Notch1 and Notch2**—It is established through genetic analysis that presenilins function in ligand-dependent cleavage of cell surface Notch proteins and that processing of NotchΔE proteins is mediated by presenilin-dependent γ-secretase activity. Use of specific γ-secretase inhibitors or dominant-negative forms of presenilin has established a requirement for γ-secretase/presenilin in processing of NotchΔE proteins and also in processing of the Notch1 receptor upon Delta-1 activation.

We wanted to establish that γ-secretase/presenilin activity is a general requirement for ligand-induced Notch signaling; that is, we wanted to determine whether other Notch family proteins in addition to Notch1 depend on γ-secretase/presenilin.

To address this issue, we used a pharmacological inhibitor of γ-secretase, CpdE (46), in conjunction with an *in vitro* co-cultivation assay in which ligand and receptor were expressed independently on two different cell types and then cocultured. CpdE has been shown to bind to both PS-1 and PS-2 at the cell membrane (46). Additionally, CpdE blocks the cleavage event that releases NotchΔE from the cell surface (27) and also blocks the growth of T-ALL cells, which were transformed by membrane-tethered Notch1ΔE (40). Ligand-mediated signaling through Notch1 and Notch2 was assessed via a reporter construct encoding six CSL binding sites which was transfected into the Notch-expressing cells to assess Notch activation. CSL-mediated signaling was observed through both Notch1 and Notch2 by Jagged1 (Fig. 1A) and also by Delta-1 and Delta-4 (data not shown). Jagged1 stimulated Notch1 activity >5-fold over control and stimulated Notch2 activity >10-fold over control. When CpdE was added to the *in vitro* coculture assays 16 h prior to harvest of cells, CSL reporter activity was reduced through both Notch1 and Notch2 via the different ligands tested (Fig. 1B). CpdE caused a 3-fold inhibition in Jagged1-induced signaling and a 5-fold inhibition in Delta-4-induced signaling through both Notch1 and Notch2. Additionally, Delta-1-induced signaling was reduced 5-fold and <4-fold through Notch1 and Notch2, respectively. Thus, attenuation of signaling by a γ-secretase/presenilin inhibitor demonstrates the requirement of γ-secretase/presenilin activity in both Notch1 and Notch2 signaling whether it is via the ligand Jagged1, Delta-1, or Delta-4.

**Presenilin Activity Is Necessary for Notch4/int-3 Signaling**—To test the dependence of Notch4/int-3 on presenilin activity, we relied on an expression construct that drives expression of the same polypeptides observed in mouse mammary tumors caused by proviral insertion. Notch4/int-3 translation is initiated from this construct from an internal Notch4 start codon, resulting in synthesis of a polypeptide that includes 30 amino acids of the EC domain, the TM domain, and the IC domain; the Notch4 signal peptide is absent from this polypeptide. A second construct encoding just the IC domain of Notch (N1IC), which signals in a presenilin-independent fashion, was used as a negative control.

Transient expression of either Notch4/int-3 or N1IC led to robust induction of CSL-mediated reporter activity in HeLa cells (Ref. 47 and data not shown). We determined whether blocking γ-secretase/presenilin activity would affect reporter activity induced by these constitutively active Notch proteins. Notch4/int-3 and N1IC were each expressed independently in HeLa cells or MEFs in the presence or absence of CpdE. In both cell lines, CpdE attenuated the activity of Notch4/int-3; 1.7-fold in HeLa cells and 3.8-fold in MEFs (Fig. 2, A and B). In contrast, CpdE did not inhibit N1IC-induced activity (Fig. 2, A and B). Furthermore, Notch4/int-3 activity, in a MEF line deficient in both PS-1 and PS-2, was almost completely abrogated compared with the activity in wild type MEFs (Fig. 2C). The level of N1IC signaling was similar in wild type or PS-1/-/- MEFs. These results indicate that presenilins are necessary for Notch4/int-3-induced CSL signaling and are most consistent with an intramembrane cleavage by presenilin in the TM domain of Notch4/int-3. N1IC does not encode for the TM domain and therefore is not dependent on presenilin for cleavage and subsequent nuclear translocation.

**Blockade of Presenilin Activity Alters the Protein Expression Pattern of Notch4/int-3 and TAN-1**—We noted previously that membrane-tethered forms of truncated Notch1 (NotchΔE) accumulate in the presence of presenilin inhibitors (40), raising the possibility that presenilin blockade would also stabilize Notch4/int-3 and TAN-1 polypeptides. Transient expression of Notch4/int-3 in transfected 293 cells led to the appearance of multiple closely spaced Notch4 isoforms that are phosphorylated differentially (47). Treatment with two different presenilin inhibitors, CpdE or L-685,458, shifted the protein band pattern toward a higher molecular mass (Fig. 3, A and B). After
drug treatment, there was a decrease in levels of the lowest molecular mass band and an increase in levels of the two higher molecular mass bands that became more pronounced with increasing drug concentrations. This up-regulation of the higher molecular mass Notch4/int-3 proteins can likely be attributed to the accumulation of the unprocessed larger forms of Notch4/int-3 that would arise if the protein were not cleaved in the TM domain by presenilin. Consistent with this result, a similar shift in the Notch4/int-3 proteins was observed in 293 cells stably coexpressing dominant-negative forms of presenilin (D385A-PS1 and D366A-PS2) (48) relative to 293 cells stably coexpressing the wild type forms of the presenilins (Fig. 3C). To demonstrate that the inhibitory effects of CpdE and L-685,458 were specific to \( / \)-secretase activity, experiments were performed with two unrelated protease inhibitors, AEBSF and pepstatin A, which should not affect \( / \)-secretase/presenilin cleavage at the indicated concentration. Use of these nonspecific inhibitors at relatively high concentrations (1 \( \mu \)M) did not alter the processing of Notch4/int-3 (Fig. 3E) or Notch1AE (Fig. 3F), as was observed with CpdE (Fig. 3, E and F).

The effects of \( \gamma \)-secretase/presenilin inhibitors on processing of TAN-1 polypeptides were examined in the human T lymphoblastic cell-line SUPT1, which carries two copies of the \( t \) (7;9) and no normal Notch1 genes (49). Multiple truncated Notch1 mRNA species are observed in SUPT1 cells that initiate from cryptic promoters lying near the chromosomal breakpoint (34). These transcripts give rise to a series of polypeptides ranging in size from 100 to 125 kDa (Fig. 3D); these same polypeptides were detected with two different anti-Notch1IC antibodies (35). These polypeptides likely arise, in part, from cryptic translational start sites (35). Treatment with CpdE altered the expression pattern of TAN-1 polypeptides, increasing the level of a prominent broad band of \( \sim 110 \) kDa and decreasing the level of a band of slightly lower molecular mass (Fig. 3D), suggesting a precursor/product relationship. Normally, this cleavage might occur spontaneously, but in the presence of the \( \gamma \)-secretase/presenilin inhibitor this conversion is prevented. Thus, TAN-1 and Notch4/int-3 polypeptides accumulate when \( / \)-secretase/presenilin cleavage is inhibited.

**Notch4/int-3 and TAN-1 Are Targeted to the Membrane**—Presenilin-mediated cleavage within the TM domain of Notch is thought to occur predominantly at the cell surface within the plasma membrane (18, 19, 50). If Notch4/int-3– and TAN-1–derived polypeptides are substrates for presenilin, blockage of presenilin activity would thus be expected to increase levels of Notch4/int-3 and TAN-1 polypeptides at the cell surface.

To determine the localization of Notch4/int-3, TAN-1, and presenilins, we used cell surface biotinylation followed by streptavidin affinity purification and immunoblotting. In transiently expressing HeLa cells, Notch4/int-3 proteins were ex-
pressed at both the cell surface and in whole cell lysate (Fig. 4 A), whereas N1IC, which localizes at the nucleus, was not biotinylated. To examine TAN-1 polypeptides, SUPT1 cells were biotinylated. Western analysis performed using an antibody that specifically recognizes the intracellular domain of Notch1 revealed a minor TAN-1 polypeptide that was precipitated with streptavidin-agarose (Fig. 4 B). As a further control for nonspecific labeling of intracellular proteins, samples were probed with an antibody to the intracellular protein, glycogen synthetase kinase-3β/H9252. As expected, glycogen synthetase kinase-3β/H9252 was not detected in biotin-streptavidin-precipitated samples, indicating that cytoplasmic proteins were not biotinylated (Fig. 4, A and B).

Normal Notch polypeptides associate with presenilin early in the secretory pathway within the Golgi/endoplasmic reticulum and then move to the cell surface (51). We found that HeLa cells express both PS-1 and PS-2, whereas SUPT1 cells express only PS-1 (Fig. 4, A and B, and data not shown). In both cell lines, presenilins were present at the cell surface, based on their susceptibility to biotinylation (Fig. 4, A and B).

If cleavage of Notch oncproteins by presenilins occurs at the cell surface, then one may expect that inhibition of γ-secretase/presenilin activity would alter the membrane-bound forms of these proteins by preventing their cleavage. Analysis was performed by treating cells with CpdE to examine the effects on membrane expression of the truncated Notch proteins. The cell surface expression level of Notch4/int-3 proteins in transfected HeLa cells was increased noticeably after CpdE treatment (Fig. 4A). Similarly, CpdE treatment of SUPT1 cells led to a dramatic increase in the levels of a specific TAN-1 polypeptide at the cell surface (Fig. 4B). CpdE did not nonspecifically alter the expression of cell surface proteins because integrin levels at the membrane were similar before and after CpdE treatment. Thus, presenilin inhibition causes the cell surface accumulation of Notch4/int-3 and TAN-1 polypeptides that presumably represent presenilin substrates.

### Differential Biological Effects of Notch Oncoproteins Are Observed after γ-Secretase Inhibition

—We next asked whether inhibition of γ-secretase/presenilin activity would abrogate the biological activity of Notch4/int-3 proteins in an *in vitro* angiogenesis assay and through TAN-1 proteins in a growth inhibition assay of SUPT1 cells. Recent studies indicate that Notch signaling is involved in endothelial cell development and angiogenesis (42, 52–54). Several *in vitro* assays have been developed that demonstrate a role for Notch signaling during angiogenesis. In a three-dimensional collagen gel bioassay, expression of Notch4/int-3 in HUVEC induces endothelial cell sprouts (Fig. 5A). These results help define the potential roles for Notch4 signaling in angiogenesis. Treatment with the presenilin inhibitor, CpdE, caused a dramatic decrease in HUVEC sprouting (Fig. 5B), indicating that this Notch4/int-3 activity is

---

**FIG. 2.** CSL-mediated signaling through Notch4/int-3 is dependent on presenilin activity. Notch4/int-3-HA or N1IC-HA was transfected into HeLa cells (A) or MEFs (B) together with a CSL reporter. Cells were treated in the presence or absence of 100 nM CpdE 16 h prior to harvest. The graphs depict the fold change in Notch4/int-3 or N1IC signaling by CpdE treatment. C, Notch4/int-3-HA or N1IC-HA was transfected into native MEFs or MEFs deficient in both PS-1 and PS-2 (PS1,2+/−). Cells were treated with CpdE and assayed as above. Normalized values for reporter activity are relative to cells transfected with a control plasmid. WT, wild type. * indicates p < 0.005.

---

*Y. Funahashi, manuscript in preparation.*
Notch Oncoproteins Require Presenilins

Constitutive Notch1 signaling induces the appearance of T cell lymphoblastic neoplasms (T-ALL) (37, 55) that require sustained Notch1 signaling for growth and survival (40). In murine T-ALL cell lines transformed by Notch1, which require presenilin cleavage for nuclear access, treatment with CpdE led to growth arrest and apoptosis of these cells (40). Similarly, dominant-negative inhibitors of the CSL/N1IC nuclear complex cause G1 cell cycle arrest and apoptosis of SUPT1 cells (40), indicating that nuclear Notch1 is also needed for growth and survival of human T-ALL cells. Despite this requirement, treatment of SUPT1 cells for up to 7 days with 5 μM CpdE had no effect on growth, as assessed by cell number (not shown) or cell cycle analysis (Fig. 6).

These observations, together with the Western blot data shown in Fig. 3C, suggest only a partial requirement for γ-secretase/presenilin activity for TAN-1 polypeptides to enter the nucleus. To study the extent to which nuclear access of TAN-1 polypeptides is γ-secretase/presenilin-dependent, SUPT1 cells treated with CpdE or carrier were immunostained with an antibody specific for a C-terminal portion of the intracellular domain of Notch1 (anti-TC) (Fig. 7). Nuclear and cytoplasmic staining was observed in the absence of drug (Fig. 7A), but nuclear staining was decreased preferentially by treatment with CpdE (Fig. 7B). This was documented further by analysis of anti-Notch1IC (TC) staining in control and treated SUPT1 cells with a spectral imager, which confirmed a substantial redistribution of TAN-1 polypeptides from the nucleus to the cytoplasm of cells treated with CpdE (Fig. 7, C and D). In contrast, the distribution and intensity of anti-Notch1IC (TC) staining was unchanged in CpdE-treated Jurkat cells, a control T cell leukemia cell line that expresses normal Notch1 polypeptides at modest levels (Fig. 7, E and F). These data are consistent with the existence of two pools of TAN-1 polypeptides, a major pool dependent on γ-secretase/presenilin for nuclear access, and a minor pool that can access the nucleus in a γ-secretase/presenilin-independent fashion (see Fig. 8).

Support for the existence of the latter type of TAN-1 polypeptide is seen in Western blots (Fig. 3C), in which one TAN-1 polypeptide of slightly smaller size than the membrane-associated presenilin-sensitive polypeptides is unaffected by drug treatment. This polypeptide also fails to appear at the cell surface (Fig. 4B), presumably because it lacks a TM domain, and thus would not be expected to require γ-secretase/presenilin activity for processing and nuclear access. This same TAN-1 polypeptide may generate sufficient constitutive Notch activity to maintain the growth of SUPT1 cells in the presence of γ-secretase/presenilin blockade.
FIG. 4. Notch4/int-3 and TAN-1 are expressed at the cell membrane and accumulate after γ-secretase inhibition. A, HeLa cells were transfected with either Notch4/int-3-HA or N11C-HA. Transfected HeLa cells (A) or SUPT1 cells (B) incubated with or without CpdE for 16 h were treated with membrane-impermeable biotin to label cell surface proteins. Detergent lysates were made and biotinylated proteins captured with streptavidin-agarose beads. Proteins bound to beads (Mem) and whole cell lysates (CL) were analyzed by Western blot for the indicated proteins. An antibody to β1-integrin was used as a control, demonstrating that CpdE did not nonspecifically affect membrane protein levels. An antibody to glycogen synthetase kinase-3β (GSK-3β) was used as a cytosolic protein control.

FIG. 5. γ-Secretase inhibition prevents Ad-Notch4/int3-induced sprouting of HUVEC cultured on collagen gel. A and B, morphology of adenovirus-infected HUVEC. A, Ad-LacZ-infected HUVEC; B, Ad-Notch4/int3-infected HUVEC. Ad-LacZ-infected HUVEC made a confluent monolayer, whereas Ad-Notch4/int3-infected HUVEC made sprouts beneath the monolayer. Arrows show branched sprouting of Ad-Notch4/int3 infected HUVEC. Photographs were taken at ×130. C, effect of CpdE treatment on the number of Ad-Notch4/int3-induced sprouts of HUVEC. The number of sprouts was determined as described under “Experimental Procedures.” Experiments were performed in triplicate.
Our studies demonstrate that both Notch4/int-3 and TAN-1 composite false color spectral images. B and CpdE (embedded SUPT1 cells treated for 24 h with Me2SO) were stained with propidium iodide and analyzed for cell cycle fractions as described under "Experimental Procedures."

**FIG. 6. Growth of SUP-T1 cells is unaffected by ts-secretase inhibition.** SUPT1 cells cultured for 4 days with the indicated doses of CpdE or drug carrier (Me2SO) were stained with propidium iodide and analyzed for cell cycle fractions as described under "Experimental Procedures."

**FIG. 7. Effect of ts-secretase inhibition on nuclear localization of TAN-1 polypeptides in SUPT1 cells.** 5-μm sections of paraffin-embedded SUPT1 cells treated for 24 h with Me2SO (A and C) or 1 μM CpdE (B and D) were stained with the antibody specific for the intracellular domain of Notch1 (anti-TC). A and B show representative conventional light microscopic images of cells stained using an immunoperoxidase method that produces a brown stain; C and D show composite false color spectral images. Arrows in B denote the redistribution of Notch staining to the cytoplasm (E and F). Jurkat cells were sectioned and immunostained for Notch1, as above, after culturing in the absence (E) or presence of 1 μM CpdE (F).

**DISCUSSION**

Proteolytic cleavage by ts-secretase/presenilin is a critical event in the ligand-induced proteolytic processing of Notch1. We demonstrate that ts-secretase/presenilin cleavage is a general requirement for the activation of the Notch signaling pathway via both Notch1 and Notch2. This conclusion is based on the finding that ts-secretase/presenilin inhibition greatly reduced the ability of Notch1 and Notch2 receptors to activate CSL upon exposure to the ligand Jagged1, Delta-1, or Delta-4. Our studies are consistent with the observation that Notch1–4AE polypeptides all require ts-secretase/presenilin to activate CSL (17, 18). The Notch4/int-3 and TAN-1 oncoproteins are also truncated and lack most of the EC domain. However, unlike the engineered NotchΔE proteins, Notch4/int-3 and TAN-1 do not possess signal peptides, and it was thus unclear whether these polypeptides would be inserted into membranes and require ts-secretase/presenilin for downstream signaling. Our studies demonstrate that both Notch4/int-3 and TAN-1 polypeptides are inserted in membranes and that treatment with a ts-secretase/presenilin inhibitor results in the accumulation of Notch4/int-3 and TAN-1 proteins at the cell surface.

**Membrane Targeting of Notch4/int-3 and TAN-1—** Presenilins and Notch interact early in the secretory pathway within the Golgi/endoplasmic reticulum (51), even with Notch constructs devoid of most of the EC domain (50, 51). Perhaps this association promotes membrane insertion of truncated Notch polypeptides lacking signal peptides but retaining TM domains. After insertion, truncated Notch would be competent for transport to the cell surface as part of a complex containing presenilin. This is consistent with coinmunoprecipitation experiments showing that most Notch proteins targeted to the membrane are associated with presenilin (50). Presenilins are expressed at the membrane in a biologically active complex with nicastrin (15, 56), and membrane-tethered Notch has also been observed in a complex with nicastrin (14). Thus, it is possible that the affinity between Notch and presenilin and/or nicastrin mediates the localization of Notch oncoproteins to the cell surface.

The idea that presenilins assist in the cell surface targeting of Notch is supported by studies on the role of presenilins in the trafficking of β-amyloid precursor protein (βAPP) (56, 57). Aberrant processing of β-amyloid precursor protein by presenilin is associated with Alzheimer's disease. In addition to this role, presenilins are also necessary in the trafficking of β-amyloid precursor protein from the trans-Golgi and endoplasmic reticulum (57) and from the cell surface to endosomes (56). Additionally, presenilins are required for the proper maturation and trafficking of nicastrin to the cell membrane (58–60). Presenilins may serve a similar function in the trafficking of Notch. Such a function would be independent of proteolytic activity because mutations of critical aspartate residues abrogate the proteolytic activity of presenilin without affecting trafficking of Notch to the cell surface (21).

**Differential Effects of Presenilin Inhibitor on Biological Activity Mediated through TAN-1 and Notch4/int-3—** Although Notch4/int-3 and TAN-1 polypeptides both exhibit altered proteolytic processing and accumulate at the cell surface when presenilin activity is blocked, ts-secretase/presenilin inhibition yields different effects on the biological activity of these oncoproteins. The biological activity of Notch4/int-3 was examined in a bioassay established to detect endothelial sprouting from HUVEC. Notch4/int-3 induced a strong sprouting response in this assay, demonstrating a role for Notch4 signaling in the initial processes of angiogenesis, an effect that was almost completely inhibited by a ts-secretase/presenilin inhibitor. These studies suggest that blockade of presenilin activity may be a means of inhibiting Notch-mediated angiogenesis, which could be a useful tool in regulating tumor-associated angiogenesis.

The biological activity of TAN-1 was studied in a growth assay of SUPT1 cells, which have two truncated and no normal Notch1 genes. SUPT1 cells contain increased levels of nuclear Notch1 and required persistent Notch1 signaling for growth, based on the effect of dominant-negative inhibitors of N1IC/CSL function (40). However, SUPT1 cell growth is unaffected by CpdE, despite evidence of altered TAN-1 processing and accumulation of TAN-1 polypeptides at the cell surface. The unresponsiveness of SUPT1 cells may be the result of the expression of TAN-1 polypeptides that are not dependent on presenilin-mediated proteolysis for nuclear access. Specifically, the smallest TAN-1 polypeptide detected in SUPT1 whole cell lysates was unaltered by CpdE (Fig. 3D). This polypeptide may arise from a cryptic translational start site downstream of the region encoding the extracellular domain. Such aberrant initiation codons have been demonstrated within the TM domains of murine Notch1 (61) and feline Notch2 (62). Whether such
sites are purely pathophysiologic or utilized under some normal circumstances is unknown. We hypothesize that this TAN-1 polypeptide accesses the nucleus in a presenilin-independent fashion, accounting for the refractoriness of SUPT1 cells to presenilin blockade.

Model for Notch4/int-3 and TAN-1 Cleavage by Presenilins—Our studies are consistent with current models for Notch processing and activation (9, 63) illustrated in Fig. 8. These models propose that the Notch receptor is normally inhibited by very low levels of nuclear Notch, are not readily available. Means of measuring Notch activation, which can be mediated by presenilin cleavage and the release of NotchIC from the membrane. Notch4/int-3 lacks most of the ectodomain but is inserted into membranes and tra cies to the cell surface where it is cleaved readily by presenilins. Blockade of presenilin-mediated activity can inhibit signal induction through both Notch4/int-3 and FL-Notch. TAN-1 polypeptides demonstrate partial dependence on presenilins. Like Notch4/int-3, some TAN-1 polypeptides are inserted into membranes and depend on proteolytic cleavage by presenilins (mTAN-1); some TAN-1 polypeptides, however, may not be localized to the cell surface and are targeted to the nucleus in a presenilin-independent manner (cTAN-1).

It is still controversial whether presenilins recognize a specific consensus sequence within Notch that is required for its cleavage. All four Notch receptors are cleaved by presenilin at a conserved valine within the TM domain, and mutation of this valine residue within Notch1 and Notch4 greatly reduces the efficiency of cleavage (18). However, Notch cleavage is also observed at another site within the TM domain N-terminal to the conserved valine (19). Additionally, studies in Drosophila show that it is the size of the Notch EC domain that is critical to whether presenilin can cleave, such that the shorter the EC domain the more efficient the cleavage (63). This lack of cleavage site specificity was also observed for other substrates and suggests that presenilins may serve a general role in facilitating the cleavage of particular single-pass TM proteins with short EC domains.

Notch Oncoproteins and Cancer—N-terminal truncations within the Notch proteins can lead to oncogenic gain of function phenotypes. Such truncations are observed in human T-ALLs with chromosomal translocations of Notch1, or in T-ALLs in mice and cats with proviral insertions into Notch1 (64), or Notch2 (65), respectively, and in murine mammary tumors with proviral insertions into Notch4 (33, 66). Interestingly, various human tumor cell lines exhibit up-regulation of a RNA species encoding for a truncated form of Notch4 that also has transforming potential (67). From our studies, one might predict that regulation of presenilin-mediated proteolysis and subsequent activation of these Notch oncoproteins is dependent upon whether the protein encodes for the TM domain. In tumors driven by Notch oncoproteins containing the TM domain, γ-secretase/presenilin inhibitors may be an effective therapy. The Notch4/int-3 oncoprotein is a potent mammary oncogene that is dependent on presenilin for signal activation and function. In addition, ligand-dependent signaling through full-length Notch receptors may also be important in some neoplasms. For example, ligand-dependent activation of full-length Notch receptors contributes to the growth of Hodgkin and anaplastic large cell lymphoma cell lines (68). Many neoplasms express one or more Notch receptors, but reliable means of measuring Notch activation, which can be mediated by very low levels of nuclear Notch, are not readily available. Presenilin inhibitors may be generally useful tools in evaluating the role of Notch signaling in various neoplasms.

Acknowledgments—We thank Gina Finan and Kimara Glaser-Kirschenbaum for technical assistance.

REFERENCES
1. Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A., and Weinstein, B. M. (2001) Development 128, 3675–3683
2. Pui, J. C., Allman, D., Xu, L., DeRocco, S., Karsell, F. G., Balkezur, S., Lee, J. Y., Kadesch, T., Hardy, R. R., Aster, J. C., and Pear, W. S. (1999) Immunity 11, 299–308
3. Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meervijk, J., MacDonald, H. R., and Aegert, M. (1999) Immunity 10, 547–558
4. Krebs, L. T., Xue, Y., Norton, C. R., Shutter, J. R., Maguire, M., Sundberg, J. P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., Smith, G. H., Stark, K. L., and Grudle, T. (2000) Genes Dev. 14, 1343–1352
5. Uyttendaele, H., Ho, J., Rossant, J., and Kitajewski, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5643–5648
6. Blaumeller, C. M., Qi, H., Zagours, P., and Artavanis-Tsakonas, S. (1997) Cell 89, 281–291
7. Logeat, F., Bessia, C., Brow, C., LeBail, O., Jarriault, S., Seidah, N. G., and Israel, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8108–8112
