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Molecular Cloning of Delta-4, a New Mouse and Human Notch Ligand

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Complementary DNAs encoding a previously unidentified mouse Notch ligand and its human ortholog were isolated. The new Notch ligand contains a signal sequence, a DSL domain, eight epidermal growth factor–like repeats, a transmembrane domain, and an intracellular region, all of which are characteristics of members of the Delta protein family. The new protein was therefore designated Delta-4. Several previously unidentified sequences in both the extracellular and intracellular regions were shown to be conserved among vertebrate Delta proteins. The tissue distribution of *Delta-4* mRNA resembles that previously described for *Notch-4* (*Int-3*) transcripts. However, *in situ* hybridization with mouse lung revealed that *Delta-4* mRNA is abundant in squamous alveolar cells that neighbor endothelial cells; *Notch-4* expression is largely restricted to the latter cell type. Soluble forms of the extracellular portion of Delta-4 inhibit the apparent proliferation of human aortic endothelial cells, but not human pulmonary arterial endothelial cells.

Key words: consensus sequence, Delta, endothelial cells, Notch.

The Notch signaling molecule is essential for determining the fate of various cell types during development (*1*, *2*). Notch signaling as a result of cell-to-cell interactions thus determines whether certain cells proliferate or differentiate. One characteristic consequence of Notch signaling is lateral inhibition (*1*, *2*), in which the random expression of the Notch ligand Delta by a single cell among a group of equivalent cells expressing Notch results in inhibition of the differentiation of the neighboring cells through stimulation of Notch signaling. Such lateral inhibition occurs during determination of the fate of neural and epidermal precursor cells in the ventral ectoderm of *Drosophila* embryos. Inductive Notch signaling also occurs between nonequivalent cells; thus, the Delta-expressing R8 cells stimulate the adjacent Notch-expressing R7 cells and induce their differentiation during eye development in *Drosophila*.

Members of the Notch family of proteins are transmembrane receptors that contain characteristic multiple epidermal growth factor (EGF)–like repeats as well as conserved domains such as RAM, ankyrin-like repeat, and PEST sequences. Ligands for Notch proteins include Delta and Serrate in *Drosophila melanogaster*, LAG-2 (*3*) and APX-1 (*4*) in *Caenorhabditis elegans*, and Delta and Serrate (or Jagged) in vertebrates. These ligands are also transmembrane proteins and contain a highly conserved DSL (Delta-Serrate-LAG-2) motif (*5*) upstream of a variable number of EGF-like repeats. The DSL domain is a characteristic feature of Notch ligands and is important for protein function; thus, point mutation of the DSL domain in LAG-2 results in a loss of activity (*3*). Although the Delta and Jagged (Serrate) proteins of vertebrates exhibit similar structures, each group of proteins also possesses several distinct features (*6–10*). Thus, whereas vertebrate Delta proteins contain eight EGF-like repeats, Jagged proteins contain 16 such repeats. Furthermore, the EGF domains are followed by a cysteine-rich domain in Jagged proteins but not in Delta proteins. However, the consequences of these structural differences remain unclear.

Mouse Jagged-1 interacts with Notch-1, -2, and -3, and it has been thought that Notch ligands exert redundant actions (*11*). However, *Jagged-2* knockout mice show specific defects in limb, craniofacial, and thymic development (*12*). The specific interactions between Notch proteins and their ligands are thought to be determined by the corresponding patterns of expression, given that both the receptors and ligands are membrane-bound proteins that require cell-cell contact for their interaction. In an attempt to identify new Notch ligands with distinct physiological roles, we have now cloned a new member of the vertebrate Delta family, which we have termed *Delta-4*, from both mouse and human. The tissue distribution of *Delta-4* was shown to resemble that of *Notch-4* (*Int-3*). We also prepared soluble forms of Delta-4 and showed that they inhibit the growth of human aortic endothelial cells (HAECs) but not that of human pulmonary arterial endothelial cells (HPAECs).

MATERIALS AND METHODS

Isolation of Mouse Delta-4 cDNA—Total RNA was isolated from fetal livers dissected from five pregnant mice (C57BL6) at 14.5 days postcoitum. First-strand cDNA was synthesized from 50 μg of total RNA with an oligo(dT) primer and SuperscriptII (GIBCO-BRL). A portion of the synthesized cDNA was then subjected to PCR with degen-

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Abbreviations: EGF, epidermal growth factor; HAEC, human aortic endothelial cell; HPAEC, humal pulmonary arterial endothelial cell; RT-PCR, reverse transcription and polymerase chain reaction; IgG, immunoglobulin G; PAGE, polyacrylamide gel electrophoresis.

erate forward (Kur-1, 5′-ATGAATTCTGYMRNAAYGGNG-GNACNTGYMAR) and reverse (Del-2, 5′-ATGAATTCRT-ANCCNDKNTYRCANWTRCANTKRTA) primers corresponding to the amino acid sequences C(K/R)NGGTC(Q/K) and Y(H/N/K)C(M/I/N)C(N/E/D/K)(M/K/N/P/Q)GY, respectively.

The PCR products were digested with *Eco*RI and cloned into pBluescriptII KS+ (Stratagene). One of the resulting clones encoded a previously uncharacterized EGF-like repeat sequence that was subsequently shown to correspond to the region of mouse Delta-4 spanning amino acids 341 to 473 (Fig. 1). To obtain a full-length *Delta-4* cDNA clone, we constructed a murine fetal brain (14.5 days postcoitum) cDNA library with the use of a ZapExpress kit (Stratagene). Screening of the library with the initial cDNA fragment yielded one positive clone. This clone lacked the DNA

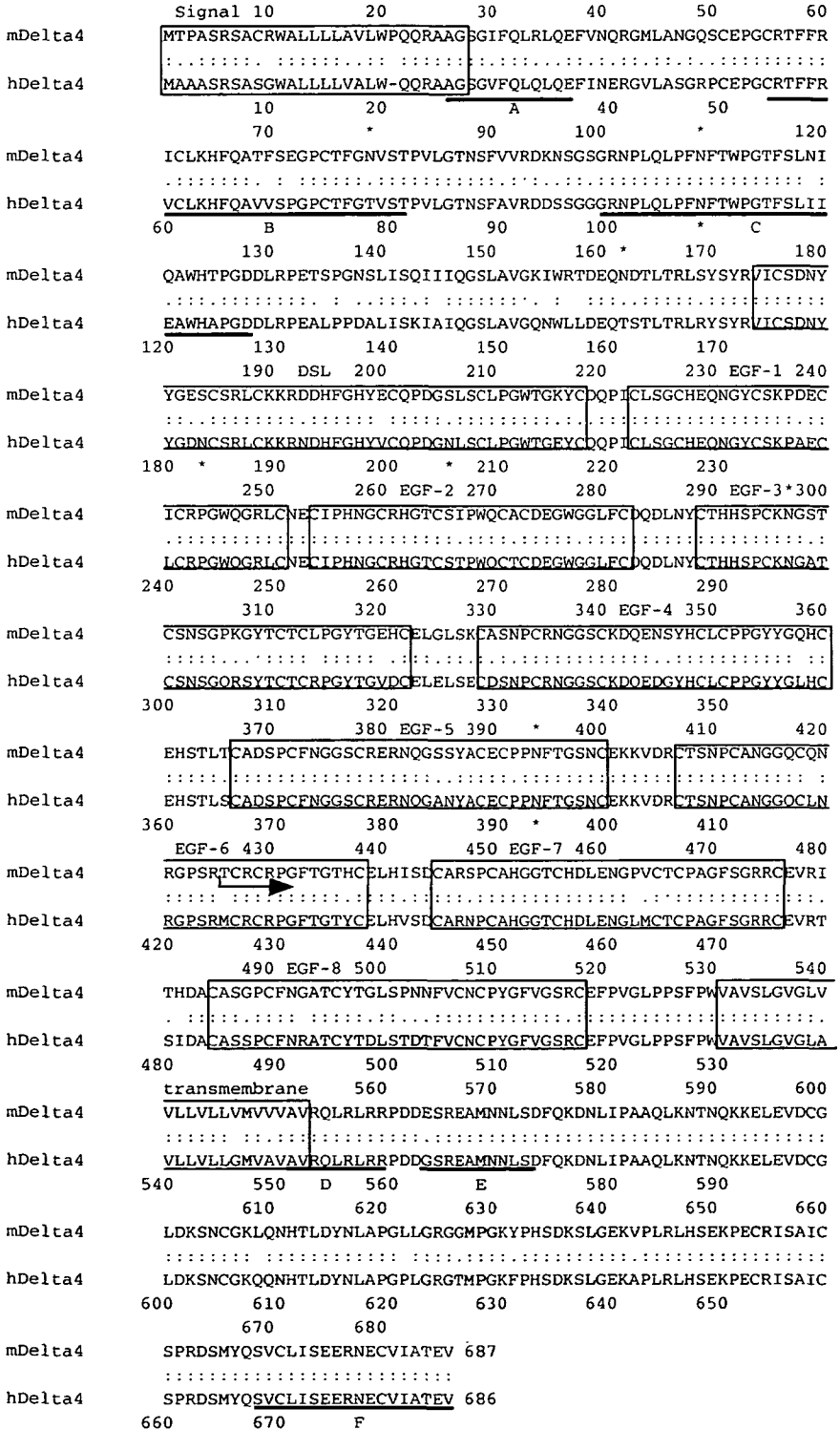

Fig. 1. **Predicted amino acid sequences of murine and human Delta-4.** The complete sequences of mouse (m) and human (h) Delta-4 proteins were aligned by DNASIS software (Hitachi, Tokyo). The signal sequence, DSL, EGF-like repeat, and transmembrane domains are boxed. The NH_2-terminal residues of the truncated mouse proteins used for functional assays are indicated by arrows. Potential *N*-glycosylation sites are represented by asterisks. Regions corresponding to those shown in Fig. 2 are underlined. The nucleotide sequences of mouse and human *Delta-4* cDNAs have been deposited in DDBJ under the accession codes AB043893 and AB043894, respectively.

sequence corresponding to the NH_2-terminal 28 residues of Delta-4 (Fig. 1). The cDNA fragment encoding the NH_2-terminal region was therefore obtained by 5′ rapid amplification of cDNA ends with murine brain Marathon-ready cDNA (Clontech).

Isolation of Human Delta-4 cDNA—The GenBank database was searched with the mouse *Delta-4* sequence. Only one highly homologous sequence (accession no. Z63545) was detected. A pair of PCR primers (hLDE-1F, 5′-TCTTCCGCATCTGCCTTAAGCAT; hLDE-1R, 5′-AGTCTCTGGCCGCAGGTCGTCTCC) was designed on the basis of this human sequence and synthesized. A human *Delta-4* cDNA fragment was obtained by PCR with these two primers and human brain cDNA as template. A human lung cDNA library (Clontech) was then screened by plaque hybridization with this cDNA fragment, and four positive clones were isolated. The clone containing the longest cDNA lacked the sequence encoding the NH_2-terminal 35 residues of human Delta-4 (Fig. 1). We therefore obtained a cDNA encoding this NH_2-terminal region of the human protein by 5′ rapid amplification of cDNA ends with human fetal kidney Marathon-ready cDNA (Clontech).

Northern Hybridization—A ^{32}P-labeled probe corresponding to the 3′ region of mouse *Delta-4* cDNA (encoding amino acids 83 to 687) was prepared with the Megaprime labeling system (Amersham). A mouse MTN membrane (Clontech) was subjected to hybridization with this probe at 42°C in the presence of 50% formamide. The membrane was washed with a final stringency of 0.1× standard saline citrate containing 0.1% SDS at 65°C, followed by exposure to an imaging plate (Fujifilm) overnight and analysis with a BAS2000 bioimage analyzer (Fujifilm).

In Situ Hybridization—Digoxigenin-labeled antisense and sense riboprobes were transcribed with the use of T7 and T3 RNA polymerases from linearized pBluescriptII SK+ containing the 3′ untranslated region of mouse *Delta-4* cDNA (450-bp *Pst*I–*Xho*I fragment) (*13*). Lungs were isolated from adult C57BL6 mice, fixed overnight with 4% paraformaldehyde in phosphate-buffered saline, and washed three times with ice-cold phosphate-buffered saline. The fixed tissue was then equilibrated with 30% sucrose, embedded in OCT compound (Miles), and frozen for cryosectioning. In situ hybridization of the cryosections was performed as described (*14*).

Multiple Alignment—Multiple alignment was performed with ClustalW (*15*) and DNASIS software (Hitachi), and was modified manually.

Expression and Purification of sDl4V5H—A cDNA fragment encoding the extracellular domain of mouse Delta-4 (amino acids 1 to 527) followed by the V5 epitope and six-histidine tag was inserted into the pTracer-CMV vector (Invitrogen). CHO ras clone1 cells were transfected with the resulting plasmid, and stable transfectants were established. The cells were cultured, and 50 liters of conditioned medium was recovered and filtered. The medium was then concentrated with a Biomax-10 device (Millipore), and sDl4V5H was purified by sequential chromatography with Hi-trap chelating resin (5 ml) (Pharmacia), DEAE-Toyopearl (TOSOH), and SP-Toyopearl (TOSOH).

Expression and Purification of sDl4Fc—A cDNA fragment encoding the extracellular domain of mouse Delta-4 (amino acids 1 to 527) followed by the Fc region of human IgG was inserted into the pTracer-CMV vector. CHO ras clone1 cells were transfected with the resulting plasmid, stable transfectants were established and cultured, and 50 liters of conditioned medium was recovered and filtered. The sDl4Fc protein was purified by sequential chromatography through Hi-trap protein A (5 ml) (Pharmacia), DEAE-Toyopearl, and SP-Toyopearl.

Amino Acid Sequence Analysis—Purified soluble Delta-4 proteins were separated by SDS-PAGE under reducing conditions, transferred to a polyvinylidene difluoride membrane, and stained with Coomassie Brilliant Blue. The protein bands were excised from the membrane and subjected to NH_2-terminal amino acid sequencing with an PPSQ-10 protein sequencer (Shimadzu).

Analysis of Cell Proliferation—HAECs and HPAECs were obtained from KURABO (Osaka) and maintained under an atmosphere of 5% CO_2 and 95% air at 37°C in 25-cm^2 flasks containing HuMedia-EG medium (KURABO) supplemented with 5% fetal bovine serum, EGF, basic fibroblast growth factor, heparin, hydrocortisone, and antibiotics. Cell proliferation was measured by an MTS colorimetric assay (Promega) based on the dehydrogenase activity in metabolically active cells. One of sDl4V5H, sDl4Fc, or human IgG at 4 or 40 μg/ml in 100 μl of culture medium was added to the wells of a 96-well tissue culture plate. HAECs or HPAECs were suspended in culture medium at a density of 1×10^5 cells/ml, and 100 μl of the cell suspension was added to the wells of the 96-well plate. After cell culture for 4 days, 20 μl of MTS/PMS reagent was added to each well and the plate was incubated for an additional 2 h at 37°C. The absorbance at 492 nm was then measured with a plate reader (Well reader SK601; Seikagaku Kogyo).

RESULTS

Molecular Cloning of Mouse and Human Delta-4 cDNAs—Using degenerate oligonucleotide primers corresponding to the conserved pattern of EGF-like repeats in the Delta family of proteins, we screened several mouse tissues for the expression of new members of this family by reverse transcription and the polymerase chain reaction (RT-PCR). The presence of similar EGF-like domains in many other proteins, including receptors (such as Notch, Tie, and the low density lipoprotein receptor) and cell adhesion molecules (such as laminin, integrin, and tenascin), resulted in the isolation of many clones that encode EGF-like repeats from both known (Delta-1, Jagged-1, Notch-1 to -3, Nel, Fat, and fibrillin) and previously uncharacterized proteins. One of these clones, initially termed *Lde1* (fetal liver-derived EGF-like motif gene 1), encoded a DSL motif and was shown to correspond to a member of the family of Notch ligands after extensive sequence analysis of an almost full-length cDNA subsequently obtained by plaque hybridization. The nucleotide sequence of the mouse *Lde1* cDNA was compared with sequences in the GenBank database, and, with the exception of a short human genomic sequence (Z63545), no identical or highly homologous sequences were detected.

We succeeded in isolating full-length cDNAs for both mouse and human *Lde1* genes. The deduced amino acid sequences of murine and human LDE1 comprise 687 residues (74,987 Da) and 686 residues (74,601 Da), respectively, with the human protein containing a one-amino acid deletion in the signal sequence (Fig. 1). The mouse and

human proteins share 86% amino acid sequence identity. Both proteins contain a signal sequence, a DSL domain, eight EGF-like repeats, a transmembrane domain, and an intracellular domain. The mouse and human proteins possess seven and six *N*-glycosylation motifs, respectively; two of the putative glycosylation sites are conserved between mouse and human LDE1, but none is conserved in other members of the Delta family.

Multiple Alignment of Vertebrate Delta Proteins—The two groups of Notch ligands in vertebrates (Delta and Jagged) and Drosophila (Delta and Serrate) are distinguished on the basis of the number of EGF-like repeats, with members of the Delta and Jagged (Serrate) families containing 8 and 16 such repeats, respectively. In addition, members of the Jagged (Serrate) family possess a characteristic cysteine-rich domain downstream of the EGF-like

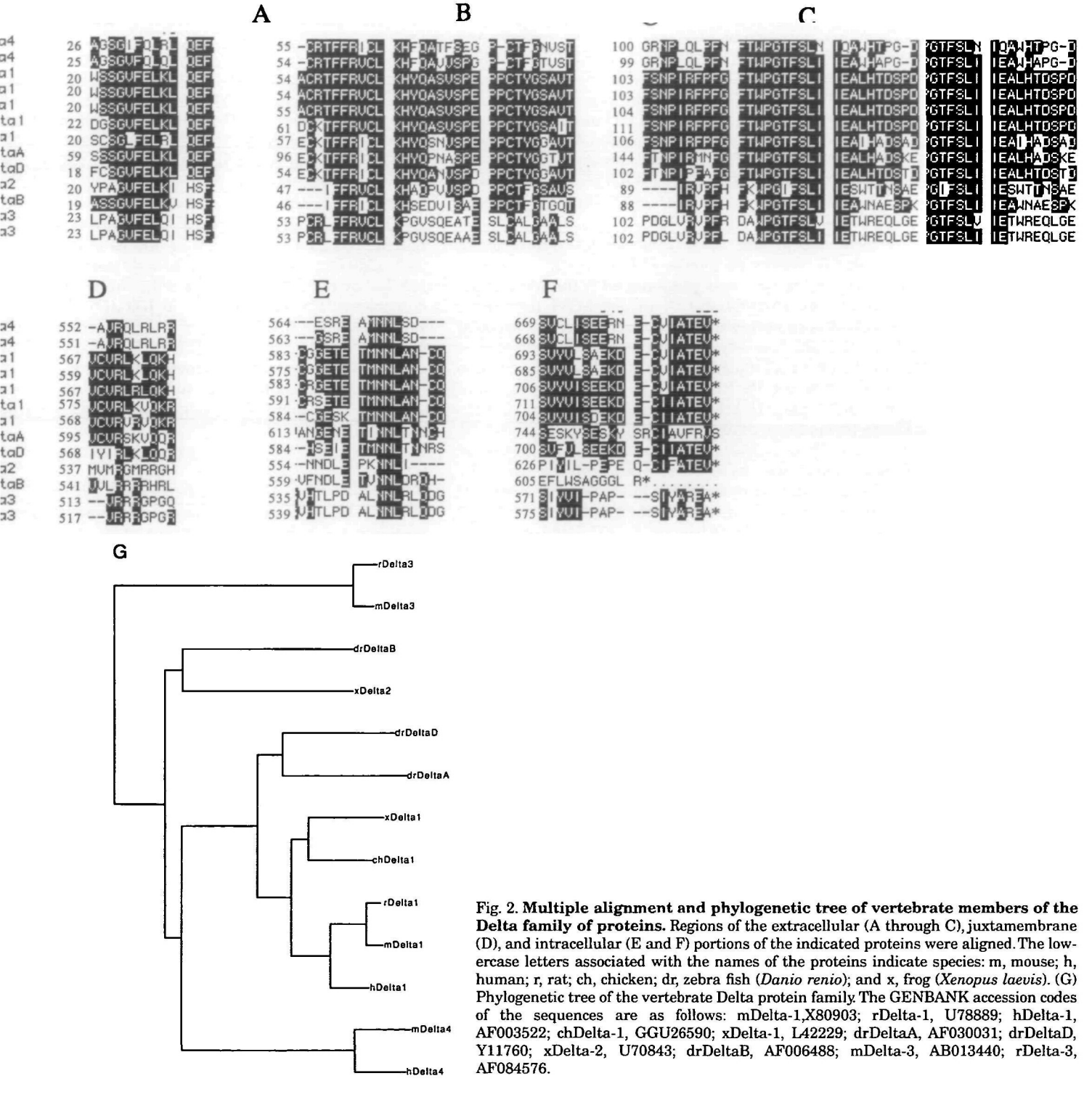

Fig. 2. **Multiple alignment and phylogenetic tree of vertebrate members of the Delta family of proteins.** Regions of the extracellular (A through C), juxtamembrane (D), and intracellular (E and F) portions of the indicated proteins were aligned. The lowercase letters associated with the names of the proteins indicate species: m, mouse; h, human; r, rat; ch, chicken; dr, zebra fish (*Danio renio*); and x, frog (*Xenopus laevis*). (G) Phylogenetic tree of the vertebrate Delta protein family. The GENBANK accession codes of the sequences are as follows: mDelta-1,X80903; rDelta-1, U78889; hDelta-1, AF003522; chDelta-1, GGU26590; xDelta-1, L42229; drDeltaA, AF030031; drDeltaD, Y11760; xDelta-2, U70843; drDeltaB, AF006488; mDelta-3, AB013440; rDelta-3, AF084576.

repeats. Both mouse and human LDE1 proteins contain eight EGF-like repeats that are not followed by a cysteine-rich domain. We therefore propose that LDE1 is a previously unidentified member of the Delta family, which we now term Delta-4. Multiple alignment of vertebrate members of the Delta family revealed that Delta-4 has stretches showing high sequence similarity to other proteins of this family (Fig. 2, A through F), suggesting that Delta-4 also plays important roles in development. Phylogenetic analysis revealed that mouse and human Delta-4 appear to constitute a distinct subfamily of Delta proteins (Fig. 2G).

The extracellular regions of mouse and human Delta-4 share conserved sequences in addition to the DSL domain and the EGF-like repeats with other vertebrate Delta proteins. Three such conserved sequences are apparent between the NH_2-termini and DSL domains of these proteins (Fig. 2, A through C), and are not detected in members of the Jagged (Serrate) family or in LAG-2. The intracelluar regions of mouse and human Delta-4 appear more highly conserved than those of other Delta proteins. Thus, whereas the extracellular, transmembrane, and intracellular regions of mouse and human Delta-4 share 84, 84, and 95% sequence identity, respectively, the corresponding values for human and mouse Delta-1 are 91, 92, and 82%, respectively. Although substantial sequence homology among the intracellular regions of Delta proteins has not previously been described, we detected two consensus sequences, (E/D)X(E/D)(A/T)XNNL and EXX(E/Q)C(V/I)(I/F)ATEV, in these regions (Fig. 2, E and F). Furthermore, the juxtamembrane regions of these proteins share a cluster of positively charged amino acids (PC domain) that resembles the RAM domain of the Notch family (Fig. 2D). These newly identified sequence similarities suggest potential roles of the intracellular domains of members of the Delta family in the regulation of Notch signaling.

Analysis of Delta-4 Expression—Northern hybridization using mouse MTN blot (Clontech) revealed that the murine *Delta-4* mRNA is ~3.5 kb. Transcripts were detected in various tissues, but most abundantly in lung and in smaller amounts in heart, liver, skeletal muscle, and kidney (Fig. 3). The expression of *Delta-4* was also apparent in all tissues examined (brain, heart, liver, smooth muscle, bone marrow, spleen, and thymus) by RT-PCR (data not shown). The expression profile of *Delta-4* is thus highly similar to that of *Notch-4* (*Int-3*) (*16*).

The expression of *Delta-4* in mouse lung was investi-

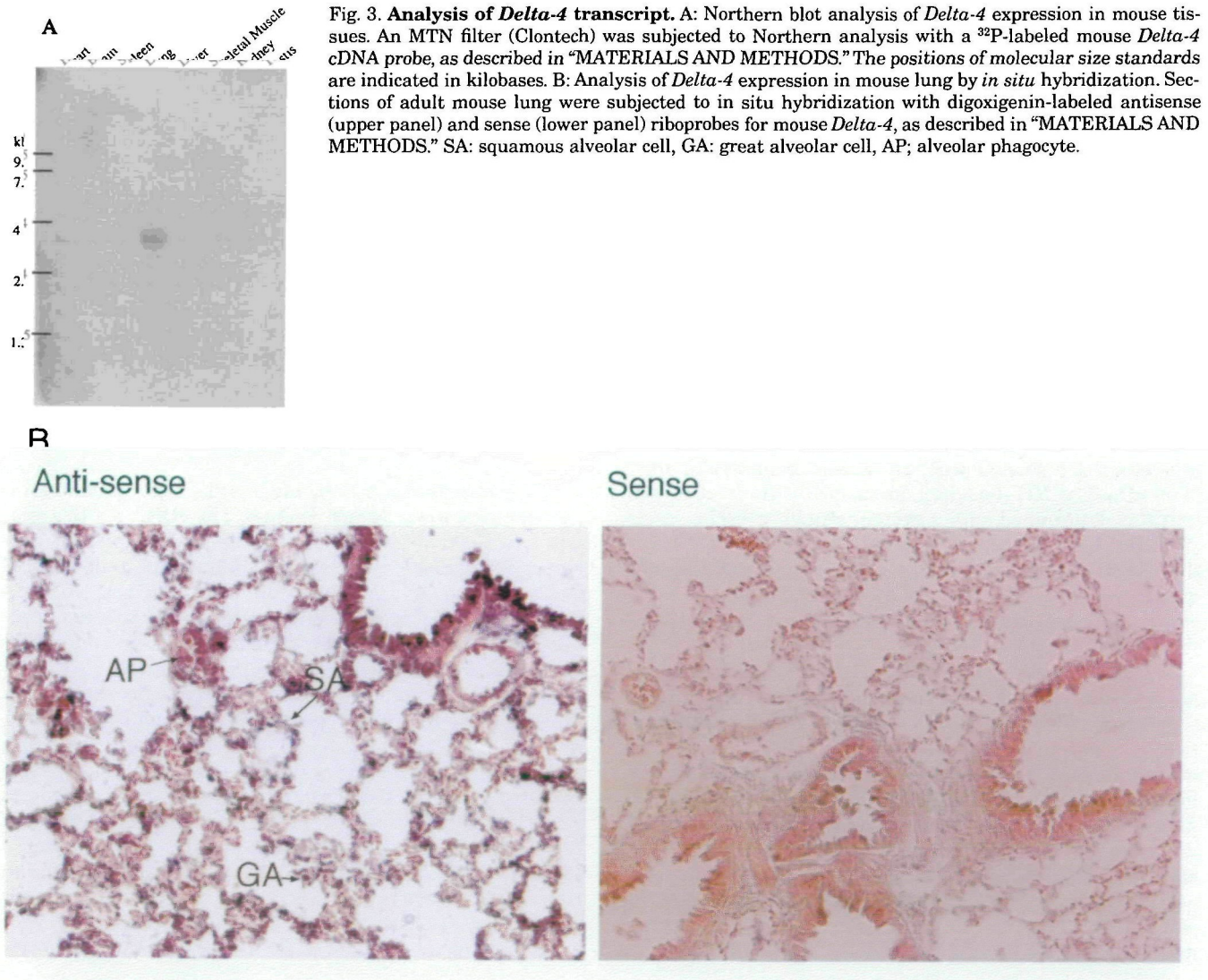

Fig. 3. **Analysis of *Delta-4* transcript.** A: Northern blot analysis of *Delta-4* expression in mouse tissues. An MTN filter (Clontech) was subjected to Northern analysis with a ^{32}P-labeled mouse *Delta-4* cDNA probe, as described in "MATERIALS AND METHODS." The positions of molecular size standards are indicated in kilobases. B: Analysis of *Delta-4* expression in mouse lung by *in situ* hybridization. Sections of adult mouse lung were subjected to in situ hybridization with digoxigenin-labeled antisense (upper panel) and sense (lower panel) riboprobes for mouse *Delta-4*, as described in "MATERIALS AND METHODS." SA: squamous alveolar cell, GA: great alveolar cell, AP; alveolar phagocyte.

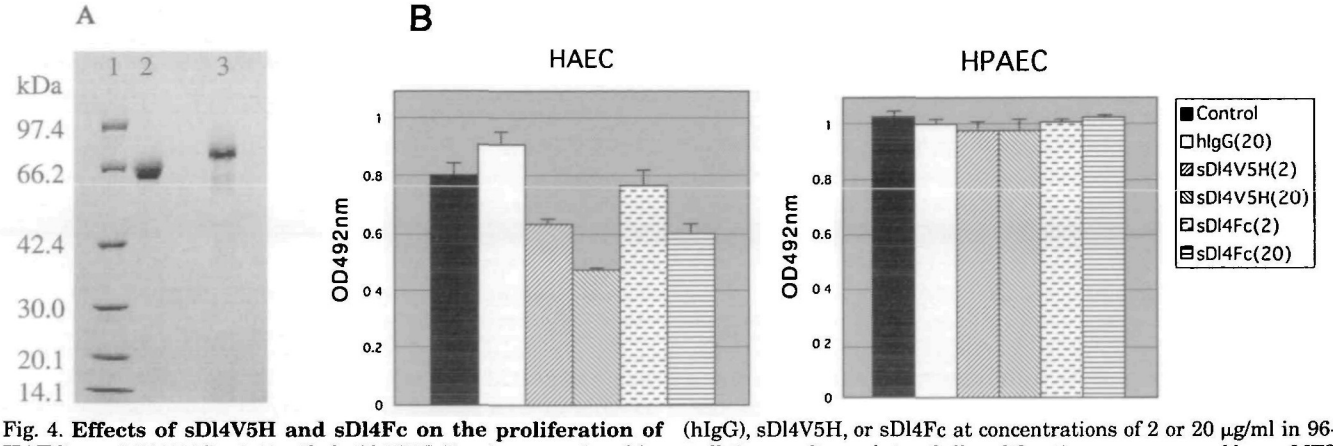

Fig. 4. **Effects of sDl4V5H and sDl4Fc on the proliferation of HAECs and HPAECs.** A: Purified sDl4V5H (2 μg) was analyzed by SDS-PAGE under reducing (lane 2) or nonreducing (lane 3) conditions. Lane 1, molecular size standards (in kilodaltons). B: Cells were cultured for 4 days in the absence (control) or presence of human IgG (hIgG), sDl4V5H, or sDl4Fc at concentrations of 2 or 20 μg/ml in 96-well tissue culture plates. Cell proliferation was measured by an MTS colorimetric assay based on dehydrogenase activity in metabolically active cells; absorbance at 492 nm (A_{492}) was determined with a plate reader.

gated further by in situ hybridization (Fig. 3). Intense staining was detected in alveolar walls where capillaries were associated with squamous alveolar cells, and punctate staining was apparent predominantly in squamous alveolar cells. Great alveolar cells also appeared to express *Delta-4*, but alveolar phagocytes did not.

Expression and Purification of Soluble Mouse Delta-4—For functional studies, we prepared soluble forms of the mouse Delta-4 protein with either a V5 epitope and a six-histidine tag, or the Fc portion of human immunoglobulin G (IgG) fused to the extracellular region; these constructs were termed sDl4V5H and sDl4Fc, respectively. Both soluble proteins were expressed in CHO ras clone1 cells (*17*), which yielded concentrations of each of several micrograms per milliliter in the conditioned medium. The sDl4V5H protein was purified by chelating chromatography, anion exchange chromatography, and cation exchange chromatography. SDS–polyacrylamide gel electrophoresis (PAGE) and staining with either Coomassie Brilliant Blue (Fig. 4) or silver (data not shown) indicated that the purified protein was >95% homogeneous. The sDl4V5H protein was expressed as a monomer with an estimated molecular mass of 66 kDa. The NH_2-terminal amino acid of the mature protein was determined with a protein sequencer to be serine-28. The apparent size of sDl4V5H is thus slightly larger than that (57,785 Da) calculated from the amino acid sequence, probably as a result of the addition of sugar chains, which also likely explains the broad nature of the band in the SDS-PAGE gel.

The sDl4Fc protein was purified by affinity chromatography, anion exchange chromatography, and cation exchange chromatography. SDS-PAGE analysis of the purified preparation revealed 90 and 40 kDa proteins under reducing conditions, and 200 and 130 kDa proteins under nonreducing conditions (data not shown). It is likely that the 200-kDa protein is a homodimer of the 90-kDa protein, and that the 130-kDa protein is a heterodimer of the 90- and 40-kDa proteins. Determination of the NH_2-terminal sequences of the 90- and 40-kDa proteins revealed that the NH_2-terminal residue of the former (serine-28) is identical to that of sDl4V5H, whereas the 40-kDa protein initiates at threonine-426 in the sixth EGF-like motif, and therefore lacks most of the extracellular region. The apparent sizes of 90 and 40 kDa determined for sDl4Fc are also slightly larger than those calculated from the amino acid sequences (80,215 and 36,518 Da, respectively), suggesting that sDl4Fc is also glycosylated.

Effects of sDl4V5H and sDl4Fc on the Proliferation of HAECs and HPAECs—To investigate the possible role of Delta-4 in endothelial cell proliferation, we incubated HAECs and HPAECs with sDl4V5H or sDl4Fc. Both sDl4V5H and sDl4Fc inhibited the apparent proliferation of HAECs but not HPAECs (Fig. 4). No marked difference was apparent between the activities of sDl4V5H and sDl4Fc. Given that Delta-Notch signaling is important in the regulation of hematopoietic cell differentiation (*18*, *19*), we also examined whether the soluble Delta-4 proteins affect the proliferation or differentiation of mouse hematopoietic stem cells; however, neither sDl4V5H nor sDl4Fc exhibited a marked effect (data not shown).

DISCUSSION

We have identified a new member of the Delta protein family, which we have termed Delta-4. The cDNA for this protein was isolated by RT-PCR with degenerate primers corresponding to the conserved EGF-like repeat domain of Delta proteins. In addition to the *Delta-4* cDNA, we identifed cDNAs for several previously uncharacterized proteins that possess EGF-like repeats, the analysis of which will be described elsewhere.

The Delta-4 protein contains a signal sequence, a DSL domain, eight EGF-like repeats, a transmembrane domain, and an intracellular region, all of which are characteristics of members of the Delta family. Phylogenetic analysis revealed, however, that Delta-4 is only distantly related to other vertebrate members of the Delta family of proteins.

Sequence analysis allowed us to identify three extracellular domains located upstream of the DSL domain that are conserved in vertebrate members of the Delta family but not in Jagged (Serrate) proteins. Delta and Jagged (Serrate) proteins are thought to perform distinct functions; for

example, whereas activation of Notch signaling by Serrate is inhibited by Fringe, the activation of Notch by Delta is enhanced by Fringe (*20*). The newly identified conserved domains in the extracellular region of Delta may contribute to the differences in function between Delta and Jagged (Serrate).

The intracellular domains in Notch ligands appear to exert specific functions in Notch signaling. Thus, ectopic expression of a *Xenopus* Delta-1 (xDelta-1) protein that lacks the intracellular domain results in an increase in the number of primary neurons (*21*). This effect is opposite to that of either full-length xDelta-1 or activated Notch-1, and therefore is likely a consequence of a dominant negative action of the truncated protein. Carboxyl-terminal truncation mutants of *Drosophila* Delta or Serrate are able to bind to Notch, but they fail to trigger signaling (*22*). These observations suggest that truncated Notch ligands interfere with the interaction between Notch and full-length ligands, and that the intracellular domains of Notch ligands are important for the ability of these proteins to regulate Notch signaling. The intracellular region of Delta proteins is functionally replaceable by β-galactosidase (*3*), which forms homotetramers; the resultant Delta-β-galactosidase fusion protein also forms oligomers and is able to mediate signaling. In spite of the evidence in support of a functional role for the intracellular regions of Notch ligands, no conserved domains have been previously described in these regions. We have now identified two conserved domains, (E/D)X(E/D)(A/T)XNNL and EXX(E/Q)C(V/I)(I/F)ATEV, as well as a cluster of positively charged amino acids (PC domain) in the intracellular regions of vertebrate Delta proteins. The two conserved sequences are not present in Delta proteins of invertebrates or in Jagged or Serrate proteins. The PC domain is present in members of both the Delta and the Jagged (Serrrate) families. These observations support the notion that the intracellular domains of Notch ligands are important in Notch signaling.

Northern hybridization and RT-PCR analysis revealed *Delta-4* to be expressed in all tissues examined. This expression profile is similar to that of *Notch-4* (*Int-3*), which was originally identified as an oncogene in mammary carcinoma and is expressed predominantly in endothelial cells (*16*). *In situ* hybridization analysis revealed that *Delta-4* is expressed predominantly in squamous alveolar cells that are associated with neighboring endothelial cells. Thus, *Notch-4* (*Int-3*) and *Delta-4* appear to show complementary expression profiles at the cellular level, suggesting the possibility that Delta-4 is the physiological ligand for Notch-4 (Int-3).

Soluble forms of Delta-4 inhibit the apparent proliferation of HAECs but not of HPAECs, demonstrating that the action of these proteins is target cell specific. Soluble forms of the *C. elegans* Notch ligands APX-1 and LAG-2 (*23*), and those of mammalian Notch ligands, including peptides corresponding to the DSL domain, exhibit signaling activity with hematopoietic cells (*19*, *24*). The soluble forms of Delta-4 also appear able to bind to receptors on HAECs and thereby to affect the proliferation of these cells. But it is not clear whether this observation is caused by the activation of Notch signaling pathway or by a dominant-negative effect, or whether it results from an enhancement of cell death or a reduction of metabolic activity. Overexpression of Int-3 in mouse mammary epithelial cells promotes aberrant cell growth (*25*), whereas activated Notch-1 induces aberrant growth of T lymphocytes (*26*). We propose that the soluble forms of Delta-4 function as Notch ligands with respect to growth regulation. It remains to be determined whether Delta-4 acts as the physiological ligand for Notch-4 (Int-3) and thereby regulates the growth or differentiation of vascular endothelial cells.

We have identified a new mouse Notch ligand and its human ortholog, which we have designated Delta-4. Several conserved sequences were identified in both the extracellular and intracellular regions of vertebrate Delta proteins, suggesting that these sequences may be important in protein function. The tissue distribution of *Delta-4* mRNA is similar to that of *Notch-4* (*Int-3*) mRNA, and the two genes show complementary expression profiles at the cellular level. The proliferation of HAECs was inhibited by soluble forms of Delta-4. Our results suggest that Delta-4 may be the physiological ligand for Notch-4 (Int-3).

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