**RESEARCH COMMUNICATION**

**Dll4, a novel Notch ligand expressed in arterial endothelium**

John R. Shutter,1 Sheila Scully,2 Wei Fan,3 William G. Richards,3 Jan Kitajewski,4 Gisele A. Deblandre,5 Chris R. Kintner,5 and Kevin L. Stark1,6

1Departments of Molecular Genetics, 2Pathology, and 3Neurobiology, Amgen, Inc., Thousand Oaks, California 91320 USA; 4Department of Pathology and OB/GYN, Columbia University, College of Physicians and Surgeons, New York, New York 10032 USA; 5Molecular Neurobiology Laboratory, The SALK Institute for Biological Studies, La Jolla, California 92037 USA

We report the cloning and characterization of a new member of the Delta family of Notch ligands, which we have named Dll4. Like other Delta genes, Dll4 is predicted to encode a membrane-bound ligand, characterized by an extracellular region containing several EGF-like domains and a DSL domain required for receptor binding. In situ analysis reveals a highly selective expression pattern of Dll4 within the vascular endothelium. The activity and expression of Dll4 and the known actions of other members of this family suggest a role for Dll4 in the control of endothelial cell biology.

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Cell-to-cell communication is required for many biological processes such as differentiation, proliferation, and homeostasis. One system utilized by a wide range of eukaryotes is the Notch-signaling pathway. The Notch gene family consists of structurally conserved cell surface receptors that are activated by one or more membrane-bound ligands of the emerging Delta/Serrate/Lag-2 (DSL) gene family (Fleming 1998). Previous studies with invertebrates have shown that the Notch-signaling pathway allows a subset of cells within an equipotent precursor population to acquire a specific cell fate in a spatially and temporally restricted manner. In this process, known as lateral inhibition or specification, cells destined for one fate inhibit neighboring cells from adopting the same fate (Artavanis-Tsakonas et al. 1999). In *Drosophila*, for example, equipotent cells in the embryonic ectoderm give rise to both neural and epidermal cells. As cells assume a neural fate, expression of neurogenic genes results in increased expression of Delta on the cell surface, leading to an inhibition of these same genes in neighboring cells via activation of their Notch receptors. Notch-receptor activation results in the trans-activation of the Enhancer of Split complex [E(spl)-C] which, in turn, suppresses the expression of downstream genes, including Delta, necessary for neurogenesis (Artavanis-Tsakonas et al. 1999). Accordingly, mutant flies lacking Notch have excessive numbers of neuronal cells, whereas activating mutations of Notch lead to an inhibition of neuron production (Struhl et al. 1993). Although Delta–Notch interactions in vertebrates are less well understood, the identification of several family members suggests that these genes play an important role in mammalian development.

Four mammalian Notch receptors have been identified, Notch1/TAN-1 (Ellisen et al. 1991; del Amo et al. 1993), Notch2 (Weinmaster et al. 1992), Notch3 (Lardelli et al. 1994), and Notch4/int-3 (Uyttendaele et al. 1996). Dysregulated Notch signaling has been shown to result in several developmental abnormalities and diseases. Chromosomal translocations resulting in the truncation of the human Notch1 homolog, TAN1, have been found in certain T-cell acute lymphoblastic leukemias (T-ALL) (Ellisen et al. 1991). A frequent site for insertion activation by the mouse mammary tumor virus [MMTV] in malignant mammary carcinomas is the Notch4 locus. The insertion of MMTV results in an aberrantly expressed intracellular form of Notch4 originally identified as Int-3 (Gallahan and Callahan 1987). Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a disorder that leads to ischemic strokes and dementia in adults, and has been traced recently to missense mutations of the Notch3 receptor (Joutel et al. 1997).

Notch ligands are divided into two subclasses, the Delta family and the Serrate family. All Notch ligands share some structural features (Fleming 1998) including epidermal growth factor [EGF]-like repeats, a characteristic DSL domain necessary for Notch binding (Muskhatch 1994) and a transmembrane region. However, an extracellular cysteine-rich domain and insertions that interrupt some EGF-like repeats are common only to the Serrate family. It is these structural differences that categorize a Notch ligand as a Delta or Serrate family member. Mammalian ligands reported include two members of the Serrate family, Jagged1/JAG1 (Lindsell et al. 1995; Oda et al. 1997a) and Jagged2 (Shawber et al. 1996), and two members of the Delta family, murine Dll1 (Bettenhausen et al. 1995) and murine Dll3 (Dunwoodie et al. 1997).

Ligand mutations can also result in the disruption of the Notch-signaling pathway, leading to developmental abnormalities. Mutations of human JAG1 have been attributed to the development of Alagille syndrome (AGS), an autosomal dominant disorder characterized by developmental abnormalities of the heart, skeleton, muscle, liver, and eyes (Li et al. 1997; Oda et al. 1997b). Mice homozygous for the pudgy (pu) mutation exhibit severe deformities of the ribs and vertebrae, and this mutation
The pleiotropic effects of dysregulated Notch signaling validate this pathway as an important regulator of development, and the existence of multiple ligands and receptors suggests a more specialized role for these genes in mammals than in lower eukaryotes. Here we report the molecular cloning and characterization of Dll4, the third mammalian member of the Delta family of Notch ligands. The ability to activate Notch receptors and the specific expression pattern of Dll4 implicate a role for this gene in the regulation of vascular biology.

Results and Discussion

cDNA cloning

An EST database from a murine, white adipose cDNA library was found to contain a clone with homology to known members of the DSL family of Notch ligands. This EST consisted of 450 bp of sequence encoding a DSL domain (Tax et al. 1994) followed by an EGF-like repeat. RACE of double-stranded DNA synthesized from white adipose tissue yielded a 3.4-kb cDNA. Analysis of the insert sequences from multiple clones revealed a 2058-bp ORF which was predicted to encode a 686 amino acid protein (Fig. 1). These clones also contained ~300 bp of 5' untranslated region (UTR) and 1000 bp of 3' UTR, including a polyadenylation signal sequence, and a poly[A] tail [data not shown]. The human DLL4 gene was also isolated and the ORF is ~86% identical at the nucleotide level and 87% identical at the amino acid level to murine Dll4.

Dll4 is a novel Delta family member

The predicted protein sequences for both human DLL4 and murine Dll4 exhibit all of the hallmarks of the Delta family of Notch ligands. The extracellular region contains 8 EGF-like repeats, as well as a DSL domain that is conserved among all Notch ligands and is necessary for receptor binding. The predicted protein also contains a transmembrane region, and a cytoplasmic tail lacking any catalytic motifs. The absence of an extracellular cysteine-rich domain and the lack of insertions between the EGF-like repeats clearly exclude Dll4 from the Jagged family of ligands. Sequence comparisons between Dll4

Figure 1. Murine Dll4 nucleotide sequence with murine and human-deduced amino acid sequence shown. The DSL domain is framed, the eight EGF-like repeats are shaded, and the predicted transmembrane region is underlined. For the human amino acid sequence, only the residues differing from mouse are shown. Human and mouse amino acid sequences are ~86% identical. The GenBank accession nos. for human and mouse are AF253468 and AF253469, respectively.
and the mu-rine Dll1 and Dll3, and Xenopus X-Delta1 (Chitnis et al. 1995) and X-Delta2 (Jen et al. 1997) proteins are shown in Figure 2. The Dll1 and X-Delta1 homologs are >70% identical at the amino acid level, whereas each of these genes is ~50% identical to Dll4. Similarly, Dll4 is ~50% identical to X-Delta2, suggesting Dll4 is not a mammalian homolog of the X-Delta2 gene, but rather a novel fourth member of the Delta gene family.

Dll4 is expressed in arterial endothelium

Northern blots containing mRNA from a variety of murine tissues were hybridized with a Dll4 probe. Expression of the 4.3-kb Dll4 message was highest in lung, followed by heart, kidney, skeletal muscle, and brain (Fig. 3). Transcripts were barely detectable in spleen and testis, but hybridization with GAPDH showed little detectable mRNA in these samples. Northern analysis of whole mouse embryos demonstrated increased expression of Dll4 from E7 through E17 of development. Analysis by RT–PCR revealed a low level of Dll4 expression in all tissues examined with higher levels in lung, brown and white adipose, and adrenal (data not shown).

In situ hybridization was performed on a variety of mouse tissues with an antisense Dll4 riboprobe, and a strikingly specific expression pattern was observed. In both embryonic and adult tissues, the predominant site of Dll4 expression was the vasculature (Fig. 4). Although it is difficult to rule out expression in the underlying smooth muscle, the expression of Dll4 in the choroid plexus (Fig. 4a), which lacks smooth muscle cells, suggests that the endothelium is the predominant site of Dll4 expression. In brain, Dll4 expression was observed in blood vessels (Fig. 4b), although some non-vascular expression was also seen in neural epithelium (not shown). Non-vascular expression was also observed in adrenal cortex, spleen, and lymph node (not shown). The precise cell types that express Dll4 in these tissues requires further investigation. Expression of Dll4 was observed in the glomeruli of the kidney (Fig. 4c) and the vasculature of the lung (Fig. 4d). Within the vasculature, Dll4 is relatively restricted to arteries, arterioles, and capillaries. This is illustrated strikingly in the umbilical cord (Fig. 4e), in which high expression is observed within the umbilical artery but not in the adjacent vein. The arterial expression of Dll4 is further illustrated by whole-mount in situ analysis of developing embryos (see the accompanying paper by Krebs et al.). In situ analysis of human DLL4 was also performed, and a similar expression pattern was seen (data not shown).

Chromosomal localization

To determine whether the human or mouse gene mapped near any human disease syndromes or pre-exist-
Dlg4 activates Notch1 and Notch4

In developing Xenopus embryos, activation of the Notch-signaling pathway results in the inhibition of neurogenesis (Coffman et al. 1993), which can be visualized by staining for the presence of N-tubulin. The ability of Dll4 to activate the Xenopus Notch receptor (X-Notch) was examined by injecting Dll4 RNA into a single blastomere of two-celled albino embryos, which were subsequently labeled for N-tubulin expression at stage 14. Consistent with X-Notch activation, Dll4 suppresses N-tubulin formation in approximately one-half of the embryos tested when compared with uninjected controls [Fig. 5a,b]. To further verify Notch activation by Dll4, expression levels of ESR-1 (Wettstein et al. 1997) and ESR-7 (Deblandre et al. 1999) in neural ectoderm were quantified in animal cap experiments in the presence or absence of Dll4 [Fig. 5c, columns 1–6]. The Xenopus ESR genes are related to Drosophila E(spl)-C, and are transcriptionally activated by the Notch-signaling pathway. Dll4 activated endogenous X-Notch, leading to increases in ESR-1 and ESR-7 levels. The coexpression of murine Notch1 with Dll4 resulted in higher ESR levels, similar to those resulting from activation of X-Notch by X-Delta1. This effect is most likely due to a higher affinity between the murine genes, as coexpression of murine Notch1 with X-Delta1 did not significantly increase ESR gene expression. The Notch4 receptor is highly expressed in the vascular endothelium of both embryos and adults (Uyttendaele et al. 1996). To investigate the potential of Dll4 as a Notch4 ligand, we performed animal cap experiments with murine Notch4 [Fig. 5c, columns 7–10]. Similar to the Notch1 signaling seen previously, the activation of ESR gene expression upon Dll4/Notch4 coinjection was >3.5-fold higher than injection of Dll4 alone. Although the specificity of Dll4 for each of the Notch receptors has yet to be elucidated, Dll4 clearly functions as a ligand for Notch1 and Notch4.

The isolation and characterization of Dll4 further expands the DSL family of mammalian Notch ligands. The structural characteristics of the protein clearly establish Dll4 as a novel Delta gene, and the expression pattern implicates Notch signaling in the regulation of blood vessel biology. The specific expression of Dll4 in arterial endothelium is reminiscent of ephrin-B2; another membrane-bound ligand (Wang et al. 1998). During vascular development, ephrin-B2 is expressed in arterial endothelial cells, whereas its cognate receptor, Eph-B2, is expressed only in venous endothelium. Targeted disruption of the murine ephrin-B2 gene affects angiogenesis, but not vasculogenesis, in developing embryos. Because the Notch-signaling pathway is an important regulator of neurogenesis and myogenesis (Nye and Kopan 1995) a similar involvement in the regulation of vascularization could be envisioned. In fact, the study of receptor mutants and downstream genes is providing evidence that Notch signaling is pivotal in vascular development. The E(spl)-C genes are regulated by Notch activation, and a new E(spl)-C family member in zebrafish, gridlock, is
required for development of the aorta (Zhong et al. 2000). CADASIL patients have missense mutations within the extracellular EGF domains of their Notch3 receptors. These mutations result in the addition or deletion of cysteine residues, possibly altering the formation of disulfide bonds necessary for normal Notch3 signaling. In the accompanying manuscript by Krebs et al. [2000], mice lacking the Notch1 and Notch4 receptors develop vascular defects, and expression analysis of known Notch ligands suggests that Dll4 is the only ligand that exhibits spatial and temporal expression consistent with the activation of Notch1 and Notch4 during vascular development. The identification of Dll4 reveals a candidate ligand for Notch receptors involved in blood vessel biology, although no vascular disorders map near the Dll4 locus in mice or humans. A more detailed examination of receptor-ligand specificities and the cell types involved should provide new insight into the role of the Notch-signaling pathway in the development and regulation of the vasculature.

Materials and methods

**Marine Dll4 cDNA cloning**

A marine adipose Marathon library (Clontech) was constructed and RACE was performed. For the 5’ RACE reaction, PCR was performed with a gene-specific primer (5’-TGCTTGAGTTAGATTTTGCGCAAA-C3’) and the API primer (5’-CCATCTCTATACGTCTACTATAGGCGC-3’) using the Advantage PCR kit components (Clontech). A 5’ RACE was performed using a gene-specific primer (5’-GTTGAATCCGGC-CACAGGTCAAATGC3’) and the API primer. To generate the full-length *Dll4* cDNA ORF, PCR was performed on marine adipose cDNA library containing the following oligos: [5’-AGCCACCATGACGCCTGTCGCT-C] and [5’-TCTTATTACCTCCTGTCGCAATCAC-3’].

**Human Dll4 cDNA cloning**

The murine *Dll4* sequence was searched against the Genbank database (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI) and a 409-bp sequence (accession no. T33811) from a human brain cDNA library was found that had 81.37% identity to the 3’ end of *Dll4*. Oligos were designed from this sequence (5’-AGAAAGGACCTG-GAAATGACTCTG-3’) and [5’-ATCAAAACACACAGCTGTACA-C3’] and used to amplify a 245-bp product from a Marathon human brain cDNA library (Clontech). This fragment was used to screen a human fat cell 5’ Stretch Plus cDNA [gt10] library (Clontech) as per instructions. One phage clone was isolated using the Wizard Lambda Prep DNA Purification System (Promega). This clone contained 215 bp of 5’ UTR and 1980 bp of the *Dll4* coding region, lacking the last 85 bp of the coding region. Oligos from the *Dll4* phage clone sequence and from T33811 sequence (5’-ACCTGATCCTGAGCGCAGCAG-3’) and [5’-GATGTTCAGTACGGTCTGCG-3’], respectively, were used to amplify the full-length human *Dll4* gene from a Marathon human lung cDNA library (Clontech).

**Northern blot analysis**

Nylon filters containing immobilized poly(A) RNA [2 ug/lane] from a variety of murine tissues (Clontech) were probed with a 275-bp fragment of the mouse *Dll4* gene as per the manufacturer’s instructions. Hybridization signals were visualized with a PhosphorImager (Molecular Dynamics).

**In situ hybridization**

A panel of normal embryonic [E10.5–E18.5] and adult mouse tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Prior to in situ hybridization, tissues were permeabilized with 0.2 μM HCL, followed by digestion with Protease K, and acetylation with triethanolamine and acetic anhydride. Sections were hybridized overnight at 55°C with a 32P-labeled riboprobe corresponding to murine *Dll4* ORF. Following RNase digestion, the slides were subjected to a high-stringency wash in 0.1× SSC at 55°C. Slides were dipped in Kodak NTB2 emulsion, exposed at 4°C for 2–3 weeks, developed, and counterstained. Sections were examined with darkfield and standard illumination to allow simultaneous evaluation of tissue morphology and hybridization signal.

**Chromosomal localization**

The human *Dll4* gene was localized using the Stanford G3 RH panel (Research Genetics, Baltimore, MD). Oligonucleotides that amplified human, but not hamster DNA [5’-GGCTTACTGCTGCTGGC-3’] and [5’-GGCTTTCCGGCGGACGACCC-3’], were used to amplify RH panel DNA under the following PCR conditions: an initial denaturation at 94°C for 3 min, 35 cycles of 94°C at 30 sec, 68°C at 45 sec, and 72°C for 10 min. A score of 00000100000000000000000000000000-000001000000001010000010000000000000020 was obtained with the Stanford G3 RH panel and was submitted to the appropriate server (http://www.shgc.stanford.edu/RH/serverform.html). These data place *Dll4* within 15R201000 of SHGC-5930 and SHGC-57568 (LOD 7.74 for each). The Genome Database (http://www.gdb.org) was used to determine the cytogenetic location of these markers and indicated that *Dll4* maps to 15q21.1. For mouse chromosomal localization, DNA from a BAC clone (Genome Systems, St. Louis, MO) containing the murine *Dll4* gene was labeled with digoxigenin-dUTP by nick translation. Labeled probe was combined with sheared mouse genomic DNA and hybridized to metaphase chromosomes derived from a mouse fibroblast cell line in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI, resulting in preliminary localization to chromosome 2. A second hybridization was then performed with a probe specific for the centromeric region of chromosome 2 as well as the *Dll4* probe. Measurements of 10 specifically labeled chromosomes demonstrated that *Dll4* was located at a position that is 58% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome 2, an area that corresponds to band 2E3. A total of 80 metaphase cells were examined with 71 exhibiting specific labeling.

**Embryos and injections**

Embryos were obtained from *Xenopus laevis* adult frogs by hormone-induced egg-laying and in vitro fertilization by standard methods. *Xenopus* embryos were staged according to Nieuwkoop and Faber (1967). Constructs cloned in pCS2+ vectors [Turner and Weintraub 1994] were transcribed into capped RNA using SP6 RNA polymerase. Templates for generating RNA encoding *Dll4* were obtained by linearization of the CS2 construct by Asp718. The templates for generating the *Notch1* and *Notch4* RNAs (Kopan et al. 1996) were obtained by linearization by HpaII in situ hybridization analysis. Synthetic n-loc2 RNA (20 pg), encoding a nuclear-localized form of β-galactosidase, along with 1–2-ng test RNAs (stated if otherwise) were injected into single blastomeres of albino embryos at the two-cell stage as described previously [Coffman et al. 1993]. For animal caps, two-cell stage *Xenopus* embryos were injected in the animal region of each blastomere with 0.5–2 ng of the indicated capped synthetic RNAs. To generate neutralized ectoderm, 0.1–0.5 ng of RNA encoding Noggin [Lamb et al. 1993] was co-injected in each blastomere.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization of *Xenopus* embryos was performed according to Harland [1991] with modifications described by Knecht et al. [1995] using digoxigenin-labeled anti-sense RNA probe for *Xenopus Notch1* and *Notch4* RNAs (Kopan et al. 1996) obtained by linearization by HpaII in situ hybridization analysis. Synthetic n-loc2 RNA (20 pg), encoding a nuclear-localized form of β-galactosidase, along with 1–2-ng test RNAs (stated if otherwise) were injected into single blastomeres of albino embryos at the two-cell stage as described previously [Coffman et al. 1993]. For animal caps, two-cell stage *Xenopus* embryos were injected in the animal region of each blastomere with 0.5–2 ng of the indicated capped synthetic RNAs. To generate neutralized ectoderm, 0.1–0.5 ng of RNA encoding Noggin [Lamb et al. 1993] was co-injected in each blastomere.

**Animal caps experiments and RNA protection assays**

Animal caps were dissected at stage 9 and cultured on agarose-coated Petri dishes in 0.5× MMR containing gentamycin until sibling controls reached stage 13. RNA was isolated and analyzed by RNA protection assay using 32P-labeled antisense RNA probes as described previously [Kintner and Melton 1987; Kintner and Dood 1991]. The template used for the ESR-7 probe corresponds to the Prull–HindIII fragment containing most of the ORF and the first half of the 3’ UTR, whereas that for ESR-1 has been described previously [Wetstein et al. 1997]. RNA samples isolated from eight animal caps were analyzed simultaneously along with
the EF1-α probe to control for sample recovery during the assay. Quan-
tification was carried out on a PhosphorImager (Molecular Dynamics),
and for each lane, specific band intensities were normalized to the amount of EF1-α RNA.

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