Identification of a Novel Exon in Apolipoprotein E Receptor 2 Leading to Alternatively Spliced mRNAs Found in Cells of the Vascular Wall but Not in Neuronal Tissue*

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Novel members of the low density lipoprotein receptor family were identified in human endothelial and vascular smooth muscle cells utilizing a homology-cloning strategy. Four novel mRNA transcripts could be identified as isoforms of the apolipoprotein E receptor 2 (apoEr2); one form lacking three ligand binding repeats (nucleotides 497–883) but containing a novel ligand binding repeat adjacent to a unique cysteine-rich domain preceding the epidermal growth factor precursor domain of apoEr2, forms lacking the O-linked sugar domain, and forms containing a 59-amino acid deletion within the cytoplasmic tail. By fluorescence in situ hybridization for chromosome mapping, we could confirm that the novel alternative forms of apoEr2 are splice variants of transcripts from a single copy gene on chromosome 1p34. To analyze whether the different splice variants of apoEr2 mRNA are expressed in a splice variant-specific pattern, we concentrated on the central nervous system, where high expression of apoEr2 has been described originally. By means of splice variant-specific in situ hybridization, we could confirm that apoEr2 mRNA is abundantly expressed in brain tissue and, with exception of the newly identified ligand binding domain, all mRNA splice variants exhibited a similar expression pattern. The mRNA of the newly identified ligand binding domain, however, was expressed in brain only in cells of the vascular wall, confirming data from Northern blotting, where the mRNA of the newly identified ligand binding domain was found in several tissues but was absent in brain tissue.

Members of the low density lipoprotein receptor (LDLR) family (1) are multi-functional clearance receptors able to bind a large number of ligands, thus regulating lipid metabolism, extracellular proteolysis (2, 3), and growth factor/cytokine-dependent pathways (4). Within cells of the vascular wall, known members of the LDLR gene family include the LDLR (5), the LDLR-related protein (LRP) (6), and the very low density lipoprotein receptor (VLDLR) (7). Apart from overlapping ligand specificity (3), LDLR binds predominantly to plasma lipoproteins (8), whereas VLDLR and LRP also bind components of the fibrinolytic system such as tissue-type plasminogen activator and urokinase-type plasminogen activator, especially in complex with their specific inhibitor, plasminogen activator inhibitor type 1 (9–12). Based upon the observation that LRP expression is absent in vascular endothelial cells (EC) (13) and that VLDLR expression is restricted to smooth muscle cells (SMC) and ECs in specific vascular compartments (14), we speculated that additional members of the LDLR family might exist on ECs. For this purpose we designed a PCR-based homology-cloning strategy utilizing conserved structural elements within the LDLR family. Such elements are (i) the ligand binding domains containing the characteristic amino acid motif Ser-Asp-Glu (SDE) within the variable number of ligand binding repeats, (ii) the epidermal growth factor (EGF) precursor homology domains containing several Tyr-Trp-Thr-Asp (YWTD) consensus tetrapeptides, and (iii) the cytoplasmic region. Applying this cloning strategy, we have cloned novel members of the LDLR family and identified these as splice variants of apoEr2 (15). To elucidate possible functional implications of these splice variants of apoEr2, we studied the splice-specific mRNA expression pattern in the brain, where apoEr2 was reported to be expressed abundantly and splice variant-specific expression was already described (16). We found that the mRNA splice variant containing the newly identified ligand binding domain was expressed in brain only in vascular cells, whereas all other mRNA splice variants were found to be expressed in neuronal tissue in a similar pattern.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing—Poly(A+) RNA was extracted from human umbilical vein endothelial cells (HUVEC), human foreskin microvascular endothelial cells (HSMEC), and human arterial SMC, isolated, and cultured as described (17) using oligo(dT)-cellulose (Amersham Pharmacia Biotech). First-strand cDNA was synthesized from 1 µg of mRNA at 42 °C using avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals) and the degenerate oligonucleotide LDLRF31 (5'-GTGGTYYTCICRTASCRCGGRTGTCACAGGTT-3' (Y = C + T, R = A + G, S = G + C), corresponding to nucleotides 2477–2509 of human LDLR, 2604–2636 of human VLDLR, 13967–13999 of human LDLR-related protein, and 2604–2636 of human LDLR, 13967–13999 of human LDLR, and 2604–2636 of human LDLR).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Z75190. © 2001 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
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Fig. 1. Detection of apoEr2 mRNA in EC and SMC. Panel A, amplification with LDLR family specific primers as described under "Experimental Procedures." PCR products corresponding to LDLR and VLDLR and two additional bands were observed: Product 1, a 1115-bp band; Product 2, a 889-bp band with high sequence homology to VLDLR and LDLR. VLDLR-2 represents the VLDLR-specific PCR product containing the O-linked sugar domain. Panel B, identification of multiple variants of apoEr2 mRNA by Northern blotting. The blotted mRNAs were first hybridized with a probe corresponding to labeled Product 1 and then rehybridized with a VLDLR, a LDLR, and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Arrows indicate transcript sizes.

13929–13952 of rat gp330 (18) cDNAs. Samples were adjusted to PCR buffer conditions in a total volume of 50 μl with 30 pmol of primer LDLRF31 and 30 pmol of LDLRF50 (5′-CCGCGCSTTGGCAGTG-3′, nucleotides 1033–1053) was employed. GAAGCCTGCTGGGCACGT-3′, nucleotides 1048–1068 of LDLR (5′-CCGCGCSTTGGCAGTG-3′, nucleotides 1033–1053) was employed. For that purpose, the antisense primer 15 (5′-CCTGAGATGGTCTATTATTG-3′, nucleotides 1494–1516). Amplification was performed (2400, PerkinElmer Life Sciences) was performed for 30 cycles under the conditions: 40 s at 94 °C, 40 s at 62 °C, and 1 min at 68 °C. Amplified products were separated on 1% agarose gels and stained with ethidium bromide. Products were purified using the QIAEX DNA gel extraction kit (Qiagen, Hilden, Germany) and analyzed by DNA sequencing with a model 310 DNA sequencer (Applied Biosystems Inc., Foster City, CA). In the course of the subsequent cloning of the 5′ sequence we used the degenerate sense primer LDLRF51 (5′-CCTGAGATGGTCTATTATTG-3′, nucleotides 1494–1516). Amplification was performed as above. The 5′- and 3′-ends were identified from HUVEC and HSMEC mRNAs by a rapid amplification of cDNA ends protocol using the Marathon kit (CLONTECH, Palo Alto, CA) according to the manufacturer's protocol. For that purpose, the antisense primer 15 (5′-CGTGAAGCGCTCAGGCACGT-3′, nucleotides 1033–1053) was employed for 5′-adaptor amplification, and the sense primer was employed for C5 (5′-CTCAACATCATTTGGCAG-3′, nucleotides 2409–2433) for 3′-adaptor amplification. Both strands of PCR products, in each case from three independent amplifications, were completely sequenced.

Northern Blot Analysis—Six μg of mRNAs from cultured HUVEC, HSMEC, or SMC were electrophoresed on a denaturing formaldehyde-agarose gel (1%) and transferred to a nylon membrane (Duralon UV, Stratagene, La Jolla, CA). Blots were hybridized utilizing 32P-labeled DNA probes corresponding to nucleotides 1444–2558 of apoEr22iso (Fig. 2), nucleotides 1439–2509 of LDLR, nucleotides 1656–2604 of VLDLR, and rat glyceraldehyde-3-phosphate dehydrogenase. The probes were labeled by random priming to a specific activity of 1 × 106 cpm/μg using [32P]dCTP (Amersham Pharmacia Biotech) and the DNA-labeling kit (Roche Molecular Biochemicals). Prehybridization was performed for 2 h at 57 °C and hybridization overnight at 57 °C. Blots were washed at a final stringency of 1× SSC (0.15 M NaCl and 0.015 M sodium citrate), 5% SDS at 60 °C. Blots were exposed to autoradiography film (Amersham Pharmacia Biotech).

Chromosomal Localization—The chromosomal localization of the transcripts was determined by fluorescence in situ hybridization (20) using nucleotides 1444–2558 of apoEr22iso as a probe. The probe was labeled with biotinylated-dUTP using a standard nick translation protocol and hybridized to chromosomes isolated from peripheral blood lymphocytes. Probe detection was performed using tetramethylrhodamine isothiocyanate-labeled antibodies. To further define the regional localization, chromosome banding was performed (21).

Isoform Analysis by PCR—A series of primers distinguishing the apoEr2 isoforms by size were utilized to analyze cell type-specific expression. Primer positions are indicated in Fig. 4. Each experiment was carried out with outer primers for the first amplification and inner primers for nested PCR. Outer primer pairs were primer A (5′-AAAGACTGGAGGGTGAG-3′, nucleotides 454–474) and primer B (5′-CCGAAGACAAATCTTTTGG-3′, nucleotides 3093–3114). Primer L5 (5′-ACATGGTCTTCGAAATCAAGGC-3′, nucleotides 541–585) and primer L3 (5′-GGTGGGACAGCTTCTGAC-3′, nucleotides 938–959), primer O5 (5′-ATCCGGCTAAGGTTGCTGAG-3′, nucleotides 1653–1671) and primer O3 (5′-GGCTATACCACTATTGCGAGC-3′, nucleotides 2441–2463), primer C5 and primer C3 (5′-GATCCACCTCGTGGTTGAGC-3′, nucleotides 2829–2851) were utilized as inner primers. cDNAs were reverse-transcribed from cultured HUVEC, HSMEC, and SMC, and systemic artery mRNAs, respectively. oligo(dT) primers served as templates for PCR amplification (40 s at 94 °C, 40 s at 58 °C, and 1 min at 68 °C, 25 cycles). Negative controls in the absence of reverse transcriptase were included in the analysis. All products were sequenced.

To confirm alternative splicing of the novel sequences at the level of genomic DNA, we used primer pair L5 and 155 (5′-GGGGTCTCAGGACAAAGGGTCTG-3′, nucleotides 833–854 of apoEr22iso) containing 250 μg of human chromosomal DNA purified from peripheral blood lymphocytes (QIAamp blood kit, Qiagen) as PCR template.

In Situ Hybridization—Nonradioactive in situ hybridization was performed on cryosections (7 μm) of neuronal tissues obtained from a rhesus monkey tissue bank (22). Three riboprobes covering specific regions of the new splice variants of the human apoEr2 mRNA found by us (Fig. 4) were transcribed from the respective vectors and designated as 1) "ligand binding repeat region" (nucleotides 616–956; 340 base pairs long), 2) "O-linked sugar domain region" (nucleotides 2085–2310; 225 base pairs long), and 3) "cytoplasmatic tail region" (nucleotides 2629–2809; 180 base pairs long) using the digoxigenin-RNA labeling kit (Roche Molecular Biochemicals).

In brief, slides were fixed in buffered, freshly prepared 4% paraformaldehyde solution for 10 min and then incubated in PBS containing 100 mM Tris-HCl, 50 mM EDTA, pH 8.0 containing 1 mg/ml RNase-free proteinase K. Sections were then postfixed in 4% paraformaldehyde, washed in PBS, and treated in triethanolamine/acetate buffer solution (0.1 M triethanolamine, pH 8.0, containing 0.25% acetic anhydride) on a rocking platform. After another PBS washing step, the slides were wiped dry around the tissue and laid out flat in airtight boxes on top of filter paper soaked in box buffer (4× SSC, 50% formamide). Each section was covered with 20 μl of hybridization buffer (50% formamide, 0.6 x SSC, 10 x Tris-HCL, pH 7.5, 1 x EDTA, 50 mM magnesium heparin, 10 μM dithiothreitol, 10% polyethylene glycol 8000, and Denhardt's solution). 1 μl of riboprobe in 40 μl of hybridization buffer was added to each section and incubated for 16 h at 52 °C. Hybridization was followed by two washes (10 min each) at room temperature in wash buffer (2× SSC, 10 x 2-mercaptoethanol, 1 x EDTA). Slides were then immersed in RNase A solution (20 mg/ml) for 30 min at 37 °C and washed again twice in wash buffer at room temperature then for 2 h in wash buffer containing 0.1 x SSC at 55 °C.
Identification of New Splice Variants of apoEr2 mRNA—The degenerate upstream primer LDLRF31, corresponding to the coated pit signal Asn-Pro-Val-Tyr (NPVY) within the cytoplasmic tail (23), and the degenerate downstream primer LDLRF50, corresponding to the tetrapeptide sequence Ala-Val-Asp-Trp (AVDW) within the EGF precursor domain, were used for initial reverse transcriptase-PCR experiments. Inosine was utilized in each position having more than two bases degeneracy. Using this strategy, a LDLR fragment of 1070 bp and a VLDLR fragment of 889 bp were readily identified in cultured HUVEC, HSMEC, and SMC. In all three cell types, two new PCR fragments (1115 and 889 bp) were obtained (Product 1 and Product 2 in Fig. 1A). The fragment corresponding to LRP (4200 bp) could only be detected in SMC utilizing a semi-nested PCR strategy (data not shown). All four PCR products shown in Fig. 1A were sequenced and confirmed as LDLR and VLDLR, respectively, containing the 84-bp O-linked sugar domain (VLDLR-2). The two novel fragments with partial sequence identity carried an open reading frame corresponding to an EGF precursor region with YWTD motifs and a transmembrane domain. The two clones differed by 225 bp upstream of the transmembrane domain that were similar to the O-linked sugar domain of LDLR and VLDLR. Northern blot analysis (Fig. 1B) utilizing the 1115-bp fragment as a probe yielded multiple transcripts corresponding to the 4.5, 3.9, and 3.6-kb mRNA species, respectively, in HUVEC, HSMEC, and SMC. In comparison, the VLDLR probe hybridized to a 4.0- and a 5.5-kb transcript and the LDLR probe to a 5.3-kb transcript, which were all distinct from the transcripts detected with the 1115-bp probe.

**RESULTS**

**PCr-Cloning and Sequence Analysis—**Amplification of the 5'-end was achieved by combining the degenerate downstream primer LDLRF51 corresponding to the SDE sequence within the ligand binding region and a specific upstream primer (primer 5) overlapping the novel sequence by 80 bp. Using the 5'- and 3'-rapid amplification of cDNA ends technique with specific primers 90 bp downstream and 150 bp upstream of the sequence, the missing 5'- and 3'-ends of the cDNA were identified. One large open reading frame was identified coding for a protein with 906 amino acids and corresponding to a calculated molecular mass of 100 kDa (Fig. 2). The sequence was partially identical to sequences of the originally described apoEr2. The apoEr2 gene on chromosome 1p34, suggesting that the apoEr2 gene is localized as a single copy on human chromosome 1444–2558 of apoEr2 common to all forms (Fig. 3). This sequence corresponds to the splice site generating the apoEr2 710 in Fig. 3.

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Detection of the labeled and hybridized probe was performed using the DIG nucleic acid detection kit (Roche Molecular Biochemicals) by incubation for 30 min with alkaline phosphatase-labeled anti-digoxigenin Fab fragment (Roche Molecular Biochemicals, 1:700 in 0.1% goat serum, Tris-buffered saline at room temperature); the signal was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate. Consecutive sections were analyzed the three different antisense probes as well as the respective sense probe as control for nonspecific hybridization.

**Chromosomal Localization and Genomic Organization—**To clarify the nature of the various forms with partial identity to apoEr2, genomic analysis was performed using nucleotides 1444–2558 of apoEr2 corresponding to all fragments (Fig. 3). This sequence could be localized as a single copy on human chromosome 1p32 by fluorescence in situ hybridization (Fig. 3). Kim et al. (24) identify the apoEr2 gene on chromosome 1p34, suggesting that the different forms found by us are splice variants of apoEr2. Exon 6 of apoEr2 was found to be separated from the novel exon 6a by a ~0.75-kb intron. Both the ligand binding

**FIG. 2.** Nucleotide sequences and deduced amino acid sequence of human apoEr2mRNA. The signal sequence and the transmembrane sequence are boxed. The internalization motif is given in bold. The signal sequence is different from apoEr2932 and 2952. Arrowheads indicate the start and end of alternatively spliced regions and mark alternative splicing sites. The first arrowhead marks the site of ligand binding repeats 4–6 of the published apoEr2 sequence (15). The nucleotide sequence could encode transcripts of novel exon 6a as indicated in gray in Fig. 3. Arrowhead 3 points to another alternative splicing site that has been identified but is not further discussed. Arrowhead 4 corresponds to the splice site generating the apoEr2,isoform shown in Fig. 3. Underlining highlights the four binding sites of the degenerate primers.
repeat 8 and the novel cysteine-rich region of apoEr2906 are encoded by this exon located within the 3.7-kb intron region identified by Kim et al. (24). The exon-intron junctions conform to the GT/AG rule (25) (Fig. 2).

Structural Comparison of the Splice Variants of ApoEr2—Protein domains in alignment with the exon organization are shown in Fig. 4. In theory, 12 apoEr2 isoforms could result from the combinations of the various alternative regions. The shortest apoEr2 mRNA isoform apoEr2659 encodes four ligand binding repeats, an EGF precursor domain, a transmembrane domain, and a short cytoplasmic region (isoform not shown in Fig. 4). The apoEr2906 variant consists of five ligand binding repeats, the novel cysteine-rich domain, an EGF precursor domain, an O-linked sugar domain, a transmembrane domain, and a cytoplasmic region. Additional alternative forms of apoEr2, for example isoforms containing the O-linked sugar domain and the 59-amino acid deletion within the cytoplasmic region, are shown in Fig. 4. The isoforms apoEr2716 and apoEr2apoEr2734, both lacking the ligand binding repeat 4–6, were also described recently by Clatworthy et al. (16).

Tissue-specific Expression of Splice Variants of ApoEr2906—To analyze which splice variants are actually expressed in different tissues, a reverse transcriptase-PCR strategy with several primer combinations as presented in Fig. 5A was used. Splicing-mediated insertion or deletion of sequence(s) is revealed by generation of multiple PCR fragments of different sizes (Fig. 5B). As expected, mRNA of the novel ligand binding repeat and the novel cysteine-rich domain were present in HUVEC, HSMEC, and also in SMC and placenta, whereas none of the other tissues contained these alternative regions. HUVEC and SMC were found to express an apoEr2 mRNA isoform containing the novel cysteine-rich region but not the novel ligand binding repeat (Fig. 5B, 1, apoEr2710 as shown in Fig. 4). In brain and placenta, the predominant products contained the O-linked sugar domain, whereas in heart, an exclusive PCR product lacking the O-linked sugar domain was detected (Fig. 5B, 2). Both alternate cytoplasmic forms were identified in placenta. In heart, the predominant mRNA product contained the 59-amino acid insertion, whereas in brain, the shorter product was detected (Fig. 5B, 3). ApoEr2 mRNA containing the cytoplasmic region without the 59-amino acid insertion and the isoform

![Fig. 3. Fluorescent in situ hybridization of labeled human apoEr2 cDNA (nucleotides 1444–2558) to R-banded human metaphase chromosomes. A schematic representation of human chromosome 1 is shown on the right.](http://www.jbc.org/)

![Fig. 4. Protein domains of ApoEr2 isoforms in alignment with the exon organization. The ligand binding repeats are numbered, and the growth factor repeats are indicated by capital letters. Arrows mark the position of the introns. The bold line separating ligand binding repeats five and six in apoEr2922 indicates the linker region. Transmembrane stands for transmembrane domain, cytoplasmic represents the cytoplasmic region, Cysteine-rich indicates the cysteine-rich region, ligand binding indicates the ligand binding regions, and EGF precursor homology stands for the EGF precursor homology-region.](http://www.jbc.org/)
with and the isoform lacking the O-linked sugar domain was identified in coronary arteries from healthy individuals (data not shown).

In Situ Hybridization of Different Splice Variants of ApoEr2 mRNA—To support the tissue PCR data by an independent method, we used in situ hybridization specific for different splice variants of apoEr2 mRNA. We chose brain tissue because of the high apoEr2 expression in human brain observed by our own Northern analysis and because of the existing in situ hybridization data for the rat brain (15), human brain (16), and mouse brain (25). Cerebral tissue sections of rhesus monkey were employed together with digoxigenin-labeled probes specific for the different splice variants of apoEr2 mRNA. We could detect the same differences in the expression pattern between the different splice forms of the apoEr2 mRNAs as revealed from PCR experiments. Fig. 6 shows the expression pattern for the riboprobes specific for the newly identified ligand binding domain, the O-linked sugar domain, and the cytoplasmic tail in the central nervous system (Fig. 6, A–C). Using the riboprobe specific for the new ligand-binding site (Fig. 6A), we could not detect any signal in neuronal tissue but only strong expression in vascular cells (inset). We observed a similar mRNA expression pattern using the other two riboprobes specific for the O-linked sugar domain (Fig. 6B) and the cytoplasmic tail (Fig. 6C), for which large and small neurons were found to be positive. In vessels, the endothelium expressed all three splice variants of apoEr2 mRNA, whereas the basket cells and the granule cells show low levels of these two splice variants of the apoEr2 mRNA. Furthermore the oligodendrocytes in the white matter expressed low levels of these two splice variants of the apoEr2 mRNA.

**DISCUSSION**

In search for novel members of the LDLR family in vascular tissue, we found several hitherto unknown mRNA transcripts using a homology-cloning strategy. Analyzing the origin of these novel transcripts, they were found to be splice variants of the apoER2 (15). Theoretically, 12 apoER2 isoforms could result from the combinations of the various alternative regions. apoEr2906 lacks ligand binding repeats 4–6 (nucleotides 497–883 of apoE2) and, therefore, the short linker region between ligand binding repeats 5 and 6, thought to be required for low density lipoprotein binding by LDLR (8) is absent. The fifth ligand binding repeat of apoEr2906 exhibits the highest homology (55%) to ligand binding repeat 8 of VLDLR. A comparable ligand binding repeat of apoEr2906 exhibits the highest homology (55%) to ligand binding repeat 8 of VLDLR. A comparable ligand binding repeat has recently been described within the chicken lipoprotein receptor LR8B (27), which has high sequence homology to apoEr2. The cysteine-rich domain of apoEr2906 is a novel sequence with no homology to any known protein. It is important to note that these two regions each contain an odd number of cysteines (n = 5), suggesting tertiary structural disulfide bridge formation. Another AG nucleotide sequence within exon 6a gives rise to a possible additional alternative spliced variant with a truncated cysteine-rich region encoding for four cysteines (not shown). Whether new motifs with new ligand binding properties result from formation of disulfide bonds between cysteines of adjacent domains in addition to the possible variation in ligand binding properties caused by alternative splicing remains to be determined.
Data from fluorescence in situ hybridization localizing apoEr2 on chromosome 1p32 indicate that the novel alternative transcripts and the original apoEr2 mRNA are alternatively spliced variants of the same gene locus, since Kim et al. (24) identify the apoEr2 gene on chromosome 1p34.

Previously published data have already indicated alternative splicing of the O-linked sugar domain of VLDLR (28) and chicken lipoprotein receptor 8 (LR8) (27) as well as of the apoEr2 itself (16). The intracellular domain of apoEr2 contains motifs that have previously been described in human gp330 (29). The alternatively spliced cytoplasmic region of human apoEr2 contains the amino acid sequence motifs LPGEPRs and LPKNPLs, with two potential Src homology 3 binding regions (30, 31), indicating a possible intracellular signaling through these motifs. In contrast, these motifs are not present in LDLR, VLDLR, or LRP.

Our data revealed by in situ hybridization confirm and expand with single cell resolution earlier results by Kim et al. (15) obtained with radioactive in situ hybridization. The data are also consistent with results on normal mouse embryo brain, published by Trommsdorff et al. (26) and with the detection of apoEr2 in human brain (16). These authors also could not detect splice variants containing the ligand binding domain 4–6 of apoEr2 within the brain, consistent with our data. To assure that comparable parts of tissue were analyzed, consecutive sections were used for the three different riboprobes and the respective sense controls.

In conclusion, we have identified a family of new isoforms of apoEr2 mRNAs originating from alternative splicing. The restriction of the expression of a mRNA isoform coding for a new ligand binding domain in brain tissue to vascular cells could indicate possible binding of a specific ligand only to vascular cells but not to neuronal tissue. This could indicate a specific function of this splice variant in vascular cells or, alternatively, disruption of the vascular cell-specific expression and expression also in neuronal tissue and, in turn, uptake of a novel ligand into neuronal cells normally not internalized could contribute to brain pathologies (15).

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