

## 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\*

Received for publication, December 29, 2000, and in revised form, January 9, 2001  
Published, JBC Papers in Press, January 10, 2001, DOI 10.1074/jbc.M011795200

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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.

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<sup>+</sup>) 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'-GTGGTYTTCIIRTASACRGGRTTGCAAAGTT-3' (Y = C + T, R = A + G, S = G + C), corresponding to nucleotides 2477–2509<sup>2</sup> of human LDLR, 2604–2636 of human VLDLR, 13967–13999 of human LRP, and

Members of the low density lipoprotein receptor (LDLR)<sup>1</sup>

\* This work was supported in part by Austrian Science Foundation Grants F509 and P10559. An abstract of this article has been published (Korschineck, I., Ziegler, S., Breuss, J., Lang, I., Lorenz, M., Kaun, C., Ambros, P. F., and Binder, B. R. (1997) *Thromb. Haemostasis* **77**, Suppl., p. 400, Abstr. OC1630. 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Z75190.

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<sup>1</sup> The abbreviations used are: LDLR, low density lipoprotein receptor; apoEr2, apolipoprotein E receptor 2; LRP, LDLR-related protein; VLDLR, very low density lipoprotein receptor; SMC, smooth muscle cells; EGF, epidermal growth factor; EC, endothelial cells; HUVEC, human umbilical vein EC; HSMEC, human skin microvascular EC; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s).

<sup>2</sup> Nucleotide numbering corresponds to the sequence shown in Fig. 2.

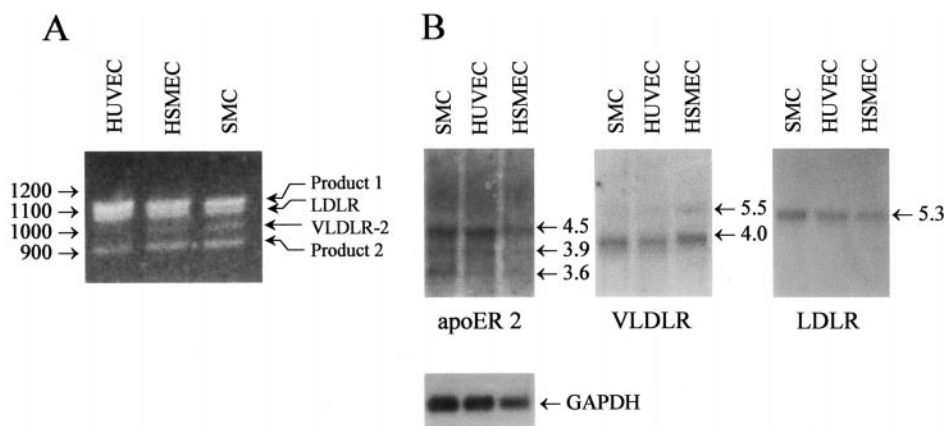


FIG. 1. **Detection of apoEr2 mRNA in EC and SMC.** Panel A, amplification with LDLR family specific primers as described under "Experimental Procedures." PCR products corresponding to LDLR and VLDLR and two additional bands were observed: Product 1, a 1115-bp band; Product 2, a 889-bp band with high 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.

13920–13952 of rat gp330 (18) cDNAs). Samples were adjusted to PCR buffer conditions in a total volume of 50  $\mu$ l with 30 pmol of primer LDLRF31 and 30 pmol of LDLRF50 (5'-CCIGMIGSIMTIGCWGTKGAYTGG-3' (M = A + C, W = A + T, K = G + T), reverse complementary to nucleotides 1439–1463 of LDLR, 1656–1680 of VLDLR, 9776–9800 of LRP, and 9942–9966 of gp330), employing 2 units of *Taq* DNA polymerase (PerkinElmer Life Sciences) and 0.1 unit of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Hot start PCR (Thermal Cycler 2400, PerkinElmer Life Sciences) was performed for 30 cycles under the following conditions: 40 s at 94  $^{\circ}$ C, 40 s at 62  $^{\circ}$ C, and 1 min at 68  $^{\circ}$ 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'-GACTCGCSIGAYGGITCIGAYGAG-3') corresponding to the amino acid motif SDE and the specific antisense primer 5 (5'-CTGAGATGGTCTTATTGCCGAG-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'-CTGGAAGCCTGCTGGGCACGT-3', nucleotides 1033–1053) was employed for 5'-adaptor amplification, and the sense primer was employed for C5 (5'-CTCAACAGTCACTGCCGTGTTATC-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  $\mu$ g 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). Blotting, prehybridization, and hybridization were performed according to standard procedures (19). Blots were hybridized utilizing  $^{32}$ P-labeled DNA probes corresponding to nucleotides 1444–2558 of apoEr<sub>2906</sub> (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  $1 \times 10^9$  cpm/ $\mu$ g using [ $^{32}$ P]dCTP (Amersham Pharmacia Biotech) and the DNA-labeling kit (Roche Molecular Biochemicals). Prehybridization was performed for 2 h at 57  $^{\circ}$ C and hybridization overnight at 57  $^{\circ}$ C. Blots were washed at a final stringency of  $1 \times$  SSC (0.15 M NaCl and 0.015 M sodium citrate), 5% SDS at 60  $^{\circ}$ 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 apoEr<sub>2906</sub> 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).

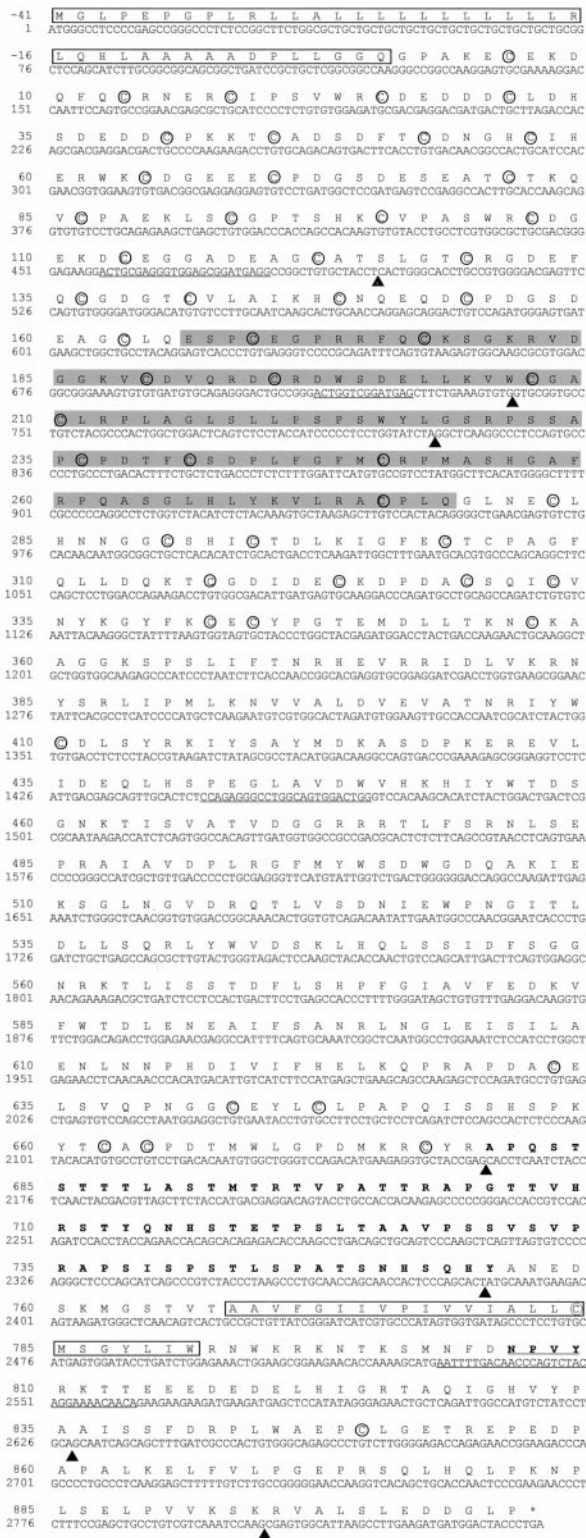
**Isoform Analysis by PCR**—A series of primers distinguishing the apoEr2 isoforms by size were utilized to analyze cell type-specific ex-

pression. 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'-AAGCAGTGCAGGGTGGAGCG-3', nucleotides 454–474) and primer B (5'-CCAGCAACCAACATCTTCTG-3', nucleotides 3093–3114). Primer L5 (5'-ACATGTGTCCTTGAATCAAGCAC-3', nucleotides 541–565) and primer L3 (5'-GGATGGACAAGCTCTTAGCAC-3', nucleotides 938–959), primer O5 (5'-ATCTGGGCTCAACGGTGTGGAC-3', nucleotides 1653–1671) and primer O3 (5'-GGTATCACCCTATGGGCACG-3', nucleotides 2441–2463), primer C5 and primer C3 (5'-GATCCATCCTCAGGGTAGTCC-3', nucleotides 2829–2851) were utilized as inner primers. cDNAs were reverse-transcribed from cultured HUVEC, HSMEC, SMC, and systemic artery mRNAs, respectively. Oligo(dT) primers served as templates for PCR amplification (40 s at 94  $^{\circ}$ C, 40 s at 58  $^{\circ}$ C, and 1 min at 68  $^{\circ}$ 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'-GAGGGTCAGAGCAGAAAGTGTC-3', nucleotides 833–854 of apoEr<sub>2906</sub>) employing 250 ng 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  $\mu$ m) 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) "cytoplasmic 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 glycine and PBS containing 0.3% Triton X-100. After a short washing step with PBS, sections were digested for 30 min at 37  $^{\circ}$ C with TE buffer (100 mM Tris-HCL, 50 mM EDTA, pH 8.0) containing 1 mg/ml RNase-free proteinase K. Sections were again postfixed in 4% paraformaldehyde, washed in PBS, and treated in triethanolamine/acetic anhydride 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 $\times$  SSC, 50% formamide). Each section was covered with 20  $\mu$ l of prehybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris-HCL, pH 7.5, 1 mM EDTA, 50 mg/ml heparin, 10 mM dithiothreitol, 10% polyethylene glycol 8000, and Denhardt's solution). 7  $\mu$ l of riboprobe in 40  $\mu$ l of hybridization buffer were added to each section and incubated for 16 h at 52  $^{\circ}$ C. Hybridization was followed by two washes (10 min each) at room temperature in wash buffer (2 $\times$  SSC, 10 mM 2-mercaptoethanol, 1 mM EDTA). Slides were then immersed in RNase A solution (20 mg/ml) for 30 min at 37  $^{\circ}$ C and washed again twice in wash buffer at room temperature then for 2 h in wash buffer containing 0.1 $\times$  SSC at 55  $^{\circ}$ C.



**FIG. 2. Nucleotide sequences and deduced amino acid sequence of human apoEr2<sub>906</sub>.** The signal sequence and the transmembrane sequence are boxed. The internalization motif is given in bold underline. The O-linked sugar domain (between nucleotides 2162 and 2386) is given in bold. Cysteine residues are circled. Arrowheads indicating the start and end of alternatively spliced regions mark alternative splicing sites. The first arrowhead marks the site of ligand binding repeats 4–6 of the published apoEr2 sequence (15). The nucleotide stretch between arrowheads 2–5 represents 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<sub>710</sub> isoform shown in Fig. 3. Underlining highlights the four binding sites of the degenerate primers.

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.

**RESULTS**

**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 980 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.

**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 new sequence found was different from apoEr2<sub>922</sub> by lacking repeats 4–6 of the ligand binding domain and containing additional sequence homologous to ligand binding repeat 8 of VLDLR and an additional cysteine-rich region with no homology to any sequence of a member of the low density lipoprotein receptor family.

**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 common to all forms (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

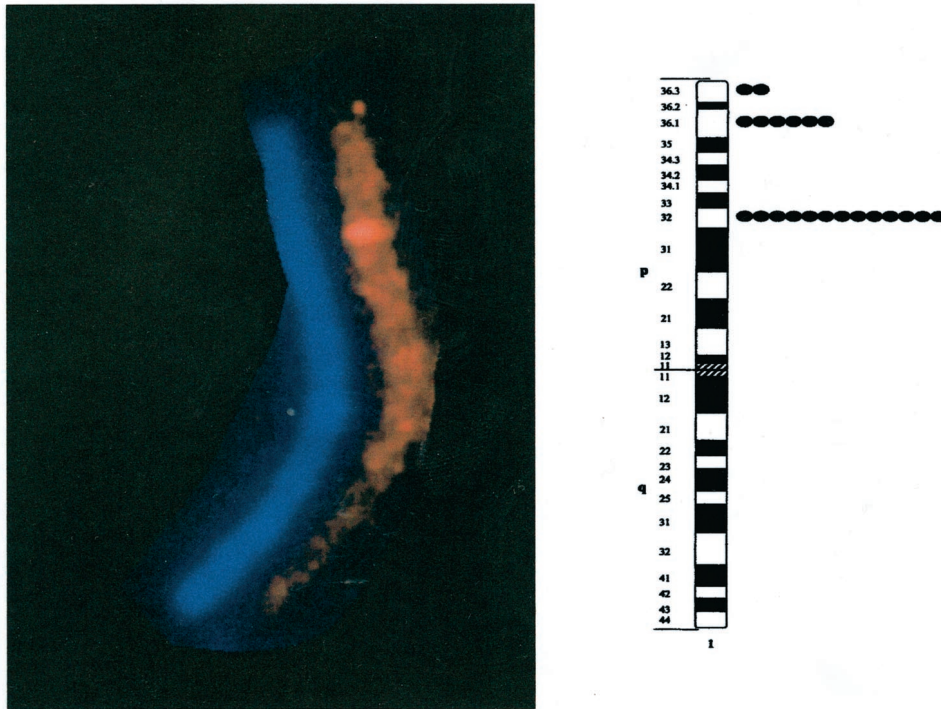
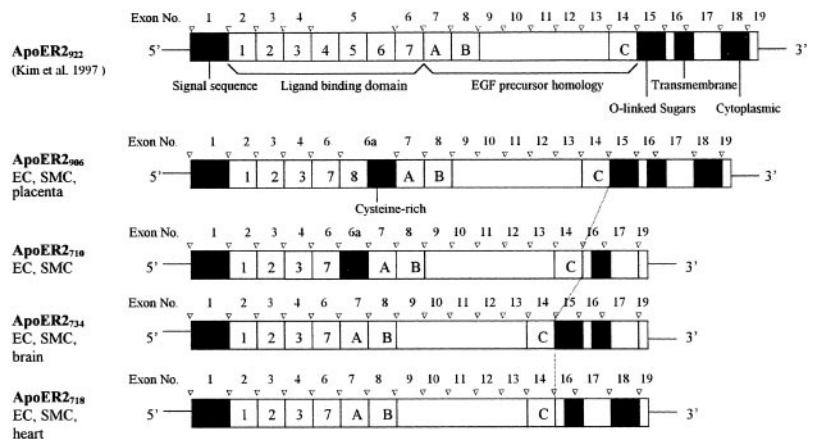


FIG. 3. **Fluorescent *in situ* hybridization of labeled human apoEr2 cDNA (nucleotides 1444–2558) to R-banded human metaphase chromosomes.** A schematic representation of human chromosome 1 is shown on the right.

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*. *Arrowheads* mark the position of the introns. The *bold line* separating ligand binding repeats five and six in apoEr2<sub>922</sub> 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.



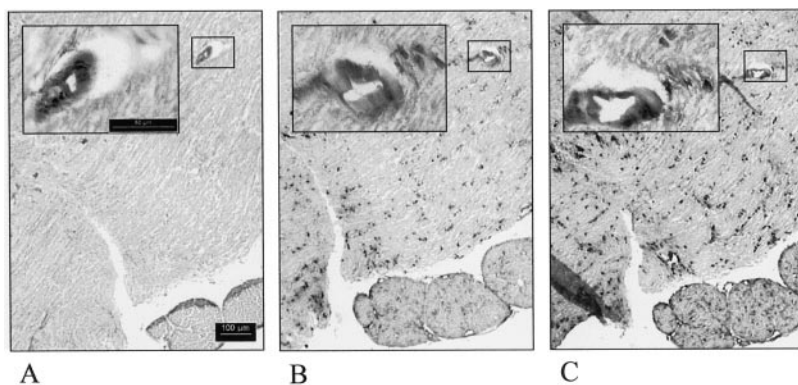
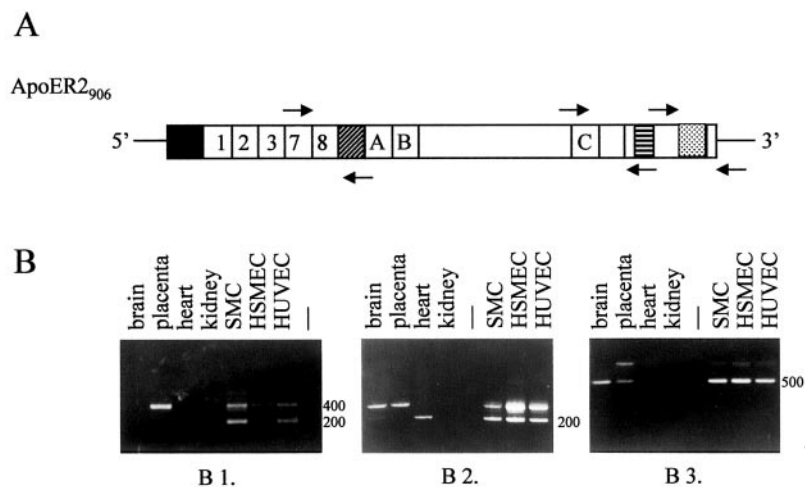
repeat 8 and the novel cysteine-rich region of apoEr2<sub>906</sub> are encoded by this exon located within the 3.7-kb intron region identified by Kim *et al.* (24). The exon-intron junctions conform to the GT/AG rule (25) (Fig. 2).

**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 apoEr2<sub>659</sub> encodes four ligand binding repeats, an EGF precursor domain, a transmembrane domain, and a short cytoplasmic region (isoform not shown in Fig. 4). The apoEr2<sub>906</sub> 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 apoEr2<sub>718</sub> and apoEr2<sub>734</sub>, both lacking the ligand binding repeat 4–6, were also described recently by Clatworthy *et al.* (16).

*Tissue-specific Expression of Splice Variants of ApoEr2<sub>906</sub>*

**mRNA and Expression in Cultured Cells**—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, apoEr2<sub>710</sub> 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. 5. Structural characterization of apoEr2<sub>906</sub> mRNA isoforms.** Panel A, schematic drawing of apoEr2<sub>906</sub> mRNA, illustrating alternatively expressed regions, with the positions of the specific primers used for PCR analysis. Panel B, 1, PCR products obtained with primers L5 and L3, resulting in the amplification of ligand binding repeats 4 and 5 and the novel cysteine-rich region of apoEr2<sub>906</sub>. Panel B, 2, PCR products obtained with primers O5 and O3 flanking the alternatively spliced O-linked sugar domain. Panel B, 3, PCR products obtained from amplification with primers flanking the alternatively spliced cytoplasmic region.



**FIG. 6. In situ hybridization in brain tissue (brain stem).** A–C, nonradioactive *in situ* hybridization of the different apoEr2 mRNAs using digoxigenin-labeled RNA antisense strand probes visualized by phosphatase-labeled anti-digoxigenin antibodies. A, the riboprobe, specific for the ligand binding domain, did not reveal any positive staining in neuronal cells but showed positive signals within the endothelium of a small vessel (*inset*). B and C, hybridization of the riboprobes specific for the O-linked sugar domain (B) and the cytoplasmic tail (C) in several small and large neurons; in endothelium, the same positive signal for all three different riboprobes is seen (*inset* in B and C).

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 analyzed in a similar pattern (*insets* in Fig. 6, A–C). The negative control using a sense strand probe was always found negative (not shown). In other parts of the brain (not shown), the O-linked sugar domain and the cytoplasmic tail but not the new ligand binding domain were found positive in several types of cells. In the cerebellum Purkinje cells, the large neurons located at the border of the granular and the molecular

layer expressed high levels, whereas the basket cells and the granule cells show low levels of these two apoER2 mRNA variants. 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. ApoEr2<sub>906</sub> 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 apoEr2<sub>906</sub> 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 apoEr2<sub>906</sub> 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 sites. 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).

*Acknowledgment*—We appreciate the expert technical help of Thomas Nardelli in the preparation of the artwork.

#### REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1986) *Science* **232**, 34–47
2. Strickland, D. K., Koungas, M. Z., Williams, S. E., and Argraves, W. S. (1994) *Fibrinolysis* **8**, 208–215

3. Krieger, M., and Herz, J. (1994) *Annu. Rev. Biochem.* **63**, 601–637
4. Hajjar, D. P., and Pomerantz, K. B. (1992) *FASEB J.* **6**, 2933–2941
5. Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., and Russell, D. W. (1984) *Cell* **39**, 27–38
6. Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H., and Stanley, K. K. (1998) *EMBO J.* **7**, 4119–4127
7. Takahashi, S., Kawarabayasi, Y., Nakai, T., Sakai, J., and Yamamoto, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9252–9256
8. Russell, D. W., Brown, M. S., and Goldstein, J. L. (1989) *J. Biol. Chem.* **264**, 21682–21688
9. Bu, G., Williams, S., Strickland, D. K., and Schwartz, A. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7427–7431
10. Nykjaer, A., Petersen, C. M., Moller, B., Jensen, P. H., Moestrup, S. K., Holtet, T. L., Etzerodt, M., Thogersen, H. C., Munch, M., and Andreasen, P. A. (1992) *J. Biol. Chem.* **267**, 14543–14546
11. Orth, K., Madison, E. L., Gething, M. J., Sambrook, J. F., and Herz, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7422–7426
12. Heegaard, C. W., Madsen, E., Ellgaard, L., Chan, L., and Andreasen, P. A. (1995) *J. Biol. Chem.* **270**, 20855–20861
13. Moestrup, S. K., Gliemann, J., and Pallesen, G. (1992) *Cell Tissue Res.* **269**, 375–382
14. Wyne, K. L., Pathak, K., Seabra, M. C., and Hobbs, H. H. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 407–415
15. Kim, D. H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H., Suzuki, H., Kondo, H., Saeki, S., and Yamamoto, T. (1996) *J. Biol. Chem.* **271**, 8373–8380
16. Clatworthy, A. E., Stockinger, W., Christie, R. H., Schneider, W. J., Nimopf, J., Hyman, B. T., and Rebeck, G. W. (1999) *Neuroscience* **90**, 903–911
17. Gimbrone, M. A., Cotran, R., and Folkman, J. J. (1974) *J. Cell Biol.* **60**, 673–684
18. Saito, A., Pietromonaco, S., Loo, A. K., and Farquhar, M. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9725–9729
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 7.37–7.52, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Lichter, P., Tang, C. J. C. T., Call, K., Hermanson, G., Evans, G. A., Hausman, D., and Ward, D. C. (1990) *Science* **247**, 64–69
21. Schweizer, D., and Ambros, P. (1994) *Methods Mol. Biol.* **29**, 97–112
22. Breuss, J. M., Gillett, N., Lu, L., Sheppard, D., and Pytela, R. (1993) *J. Histochem. Cytochem.* **41**, 1521–1527
23. Chen, W. J., Goldstein, J. L., and Brown, M. S. (1990) *J. Biol. Chem.* **265**, 3116–3123
24. Kim, D. H., Magoori, K., Inoue, T. R., Mao, C. C., Kim, H. J., Suzuki, H., Fujita, T., Endo, Y., Saeki, S., and Yamamoto, T. T. (1997) *J. Biol. Chem.* **272**, 8498–8504
25. Marchuk, D., Drumm, M., Saulino, A., and Collins, F. S. (1991) *Nucleic Acids Res.* **19**, 1154
26. Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimopf, J., Hammer, R. E., Richardson, J. A., and Herz, J. (1999) *Cell* **97**, 689–701
27. Novak, S., Hiesberger, T., Schneider, W. J., and Nimopf, J. (1996) *J. Biol. Chem.* **271**, 11732–11736
28. Bujo, H., Lindstedt, K. A., Hermann, M., Dalmau, L. M., Nimopf, J., and Schneider, W. J. (1995) *J. Biol. Chem.* **270**, 23546–23551
29. Hjalm, G., Murray, E., Crumley, G., Harazim, W., Lundgren, S., Onyango, I., Ek, B., Larsson, M., Juhlin, C., Hellman, P., Davis, H., Akerstrom, G., Rask, L., and Morse, B. (1996) *Eur. J. Biochem.* **239**, 132–137
30. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Paeson, T. (1991) *Science* **252**, 668–674
31. Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994) *Cell* **76**, 933–945