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A Novel Low-Density Lipoprotein Receptor-Related Protein with Type II Membrane Protein-Like Structure Is Abundant in Heart[1]

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We report herein the identification of a novel member of the low-density lipoprotein receptor (LDLR) family termed LDLR-related protein 4 (LRP4). Murine LRP4 cDNA encodes a 1113-amino-acid type II membrane-like protein with eight ligand-binding repeats in two clusters. Southern blot analysis of genomic DNA from several different organisms suggests the presence of LRP4 homologues in chicken lacking the gene encoding apolipoprotein E, which is recognized by the ligand-binding repeats of LDLR. LRP4 transcripts were detected almost exclusively in heart in mouse and humans. Despite the presence of the ligand-binding repeats, COS cells transfected with LRP4 did not show surface-binding of β-migrating very-low-density lipoprotein, suggesting that LRP4 plays a role in a pathway other than lipoprotein metabolism.

Key words: LDL receptor family, LDL receptor related protein, membrane protein, receptor.

The low-density lipoprotein receptor (LDLR) family is a growing super gene family that includes LDLR itself (*1*), apolipoprotein E (apoE) receptor 2 (apoER2) (*2, 3*), very-low-density lipoprotein receptor (VLDLR) (*4, 5*), insect vitellogenin receptors (*6, 7*), LDLR-related protein/α_2-macroglobulin receptor (LRP1) (*8*), a kidney autoantigen gp330/megalin (LRP2) (*9, 10*), and a recently identified member termed LDLR relative with 11 binding repeats (LR11/sorLA1) (*11, 12*). All members of this gene family contain the following five structural motifs: (i) complement-type cysteine-rich repeats, termed LDLR ligand-binding repeats or LDLR class A repeats; (ii) cysteine-rich epidermal growth factor (EGF) precursor-type repeats, termed growth factor repeats or LDLR class B repeats; (iii) cysteine-poor spacer regions, with five copies of the sequence YWTD, separating the growth-factor repeats; (iv) a single membrane-spanning region; and (v), a cytoplasmic region with at least one copy of the "NPXY" internalization signal. LDLR is the best characterized protein in this superfamily and the relationship between structure and function for each module of LDLR has been elucidated by analysis of mutations in patients with familial hypercholesterolemia (*13, 14*).

Among members of the LDLR family, VLDLR and apoER2 most closely resemble LDLR in structure and, like LDLR, bind apoE-rich β-VLDL with high affinity (*2-4*). In the chicken, VLDLR is expressed almost exclusively in oocytes and mediates uptake of yolk precursors, VLDL and vitellogenin (*15*). This receptor-mediated process is critical in non-mammalian vertebrate oogenesis: female chicken mutants lacking VLDLR are sterile (*16*). In contrast to the chicken, mammalian VLDLR mRNA is abundant in heart, skeletal muscle, brain, and adipose tissues (*4*). Frykman *et al.* have shown that mice lacking VLDLR exhibit modest decreases in body weight, body mass index, and adipose tissue mass, while their plasma cholesterol levels, triacylglycerol levels, and lipoprotein profiles are not altered (*17*). Furthermore, knockout mice lacking both VLDLR and LDLR exhibit a modest hypercholesterolemia (*17*), whereas apoE knockout mice exhibit a profound hypercholesterolemia (*18*). These data suggest the presence of other apoE receptors.

To extend our studies on receptors that may play a role in the clearance of apoE-containing lipoproteins from the circulation, we have been characterizing cDNAs belonging to the LDLR superfamily. In the previous study, we have characterized a new LDLR-related protein termed LRP3 (*19*). Human and rat LRP3 consist of a 770-amino-acid type I membrane protein with the following regions: a putative signal sequence; two isoleucine/leucine/valine-rich regions with an RGD sequence; two ligand-binding repeat regions; a putative transmembrane region; and a proline-rich cytoplasmic region with a tyrosine-based internalization signal. Despite the presence of the ligand-binding repeats, CHO cells transfected with LRP3 failed to bind β-VLDL.

In this study, we have isolated a near full-length cDNA encoding a new member of the LDLR family, termed

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Abbreviations: apoE, apolipoprotein E; apoER2, apolipoprotein E receptor 2; LDLR, low-density lipoprotein receptor; LRP, low-density lipoprotein receptor-related protein; VLDLR, very-low-density lipoprotein receptor; β-VLDL, β-migrating very-low-density lipoprotein.

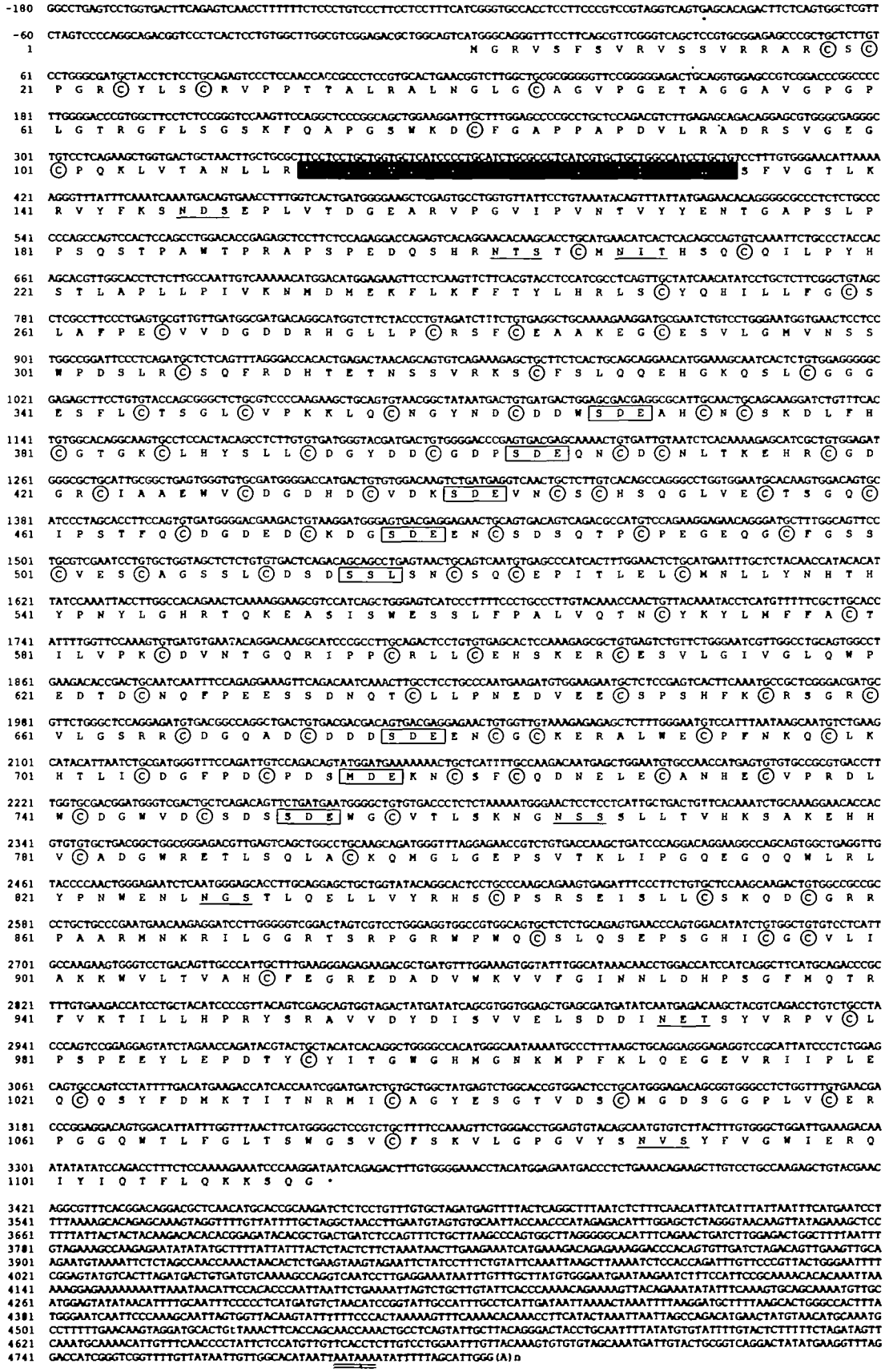

Fig. 1

Fig. 1. **Nucleotide and deduced amino acid sequence of murine LRP4 cDNA.** Nucleotide and amino acid residues are numbered on the left. Nucleotide 1 is the A of the initiator AUG codon. Negative numbers refer to the 5′-untranslated region. Two in-frame translation termination codons at −87 and 3342 are indicated by asterisks. The putative transmembrane region is boxed in black. Cysteine residues are circled and the ligand-binding motif SDE and similar sequences are boxed. Potential N-linked glycosylation sites are underlined and a potential polyadenylation signal is doubly underlined.

LDLR-related protein 4 (LRP4) and describe here the molecular characterization of this new receptor-like protein.

MATERIALS AND METHODS

Standard Procedures—Standard molecular biology techniques were carried out essentially as described by Sambrook *et al.* (*20*). Nucleotide sequencing was performed by the dideoxy-chain termination method (*21*) using M13 primers, T3 and T7, or specific internal primers. Sequence reactions were carried out using Taq DNA polymerase with fluorescently labeled nucleotides on an Applied Biosystems Model 373A DNA sequencer. To analyze RNA in murine and human tissues, commercially available Northern blots (Clontech) were used for Northern blot analysis.

cDNA Cloning—A murine heart cDNA library was constructed in pBluescript vector using poly(A) RNA and the cDNA synthesis kit from Pharmacia. The library was screened with a mixture of degenerative oligonucleotides corresponding to a highly conserved amino acid sequence, WRCDGD, among the ligand-binding domains of LDLR, VLDLR, and apoER2: 5′-TGG(A/C)G(A/C/G/T)TG(C/T)-GA(C/T)GG(A/C/G/T)GA-3′. Positive clones hybridizing with the oligonucleotide probe were the reprobed with LDLR and VLDLR probes to eliminate cDNAs for these receptors. By screening 5×10^5 clones, we obtained one positive clone that hybridized with the oligonucleotide probe alone.

"Zoo" Southern Blot Analysis—Genomic DNAs (10 μg) prepared from a normal man, a male BALB/c mouse, a white Leghorn hen, and a female *Xenopus laevis* were digested with a large excess of *Eco*RI for electrophoresis in a 0.8% agarose gel, then transferred onto a nylon membrane. The membrane was hybridized with the entire region of murine LRP4 cDNA. Hybridization was at 42°C in 5×SSC, 5×Denhardt's solution, 200 μg/ml denatured salmon sperm DNA, 50% (v/v) formamide, and 1% (w/v) SDS. The blot was then washed twice with 0.3×SSC and 1% (w/v) SDS at 60°C, followed by autoradiography.

Expression of LRP4 cDNA in COS-7 Cells—To construct an LRP4 expression plasmid (pLRP4-SRα), the entire coding region of murine LRP4 cDNA was inserted into an expression vector (pcDL-SRα296) (*22*) by multiple ligations of restriction fragments. The expression plasmid was transfected into COS-7 cells according to the transfection protocol described by Chen and Okayama (*23*).

Lipoprotein Binding Assay—Rabbit β-VLDL (*d* 1.006 g/ml) was prepared from the plasma of 1% cholesterol-fed animals (*24*). ^{125}I-labeled β-VLDL was prepared (*25*) and its binding by the transfected cells was assayed according to the procedure described previously (*2*).

RESULTS

Isolation and Characterization of Murine LRP4 cDNA—A near full-length cDNA encoding a new member of the LDLR family, designated LDLR-related protein 4 (LRP4),

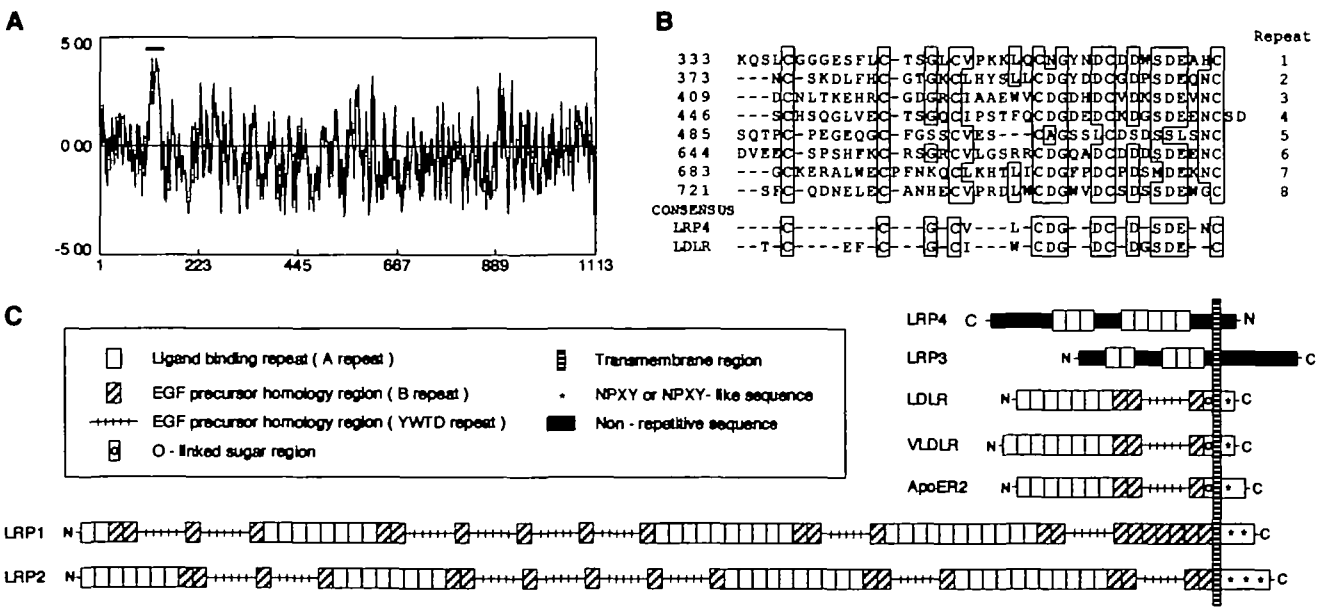

Fig. 2. **Functional regions in LRP4.** (A) Hydropathy plot analysis of the murine LRP4 protein. The numbers on the *x*-axis correspond to the positions of the amino acid residues in the protein. The putative transmembrane (TM) region is shown by a thick line. (B) Comparison of the amino acids in the eight ligand-binding repeats of murine LRP4. Amino acid alignment was optimized and gaps were introduced to match the six cysteine residues in each repeat. Amino acid residues conserved in more than 50% of the repeats are boxed and shown below as a consensus sequence. The consensus sequence of the ligand-binding repeats of human LDLR (*1*) is also represented. (C) Schematic representation of LRPs1-4, apoER2, LDLR, and VLDLR.

was isolated from a murine heart cDNA library by using a mixture of degenerative oligonucleotides corresponding to the highly conserved amino acid sequence WRCDGD among the ligand-binding domains of LDLR, VLDLR, and apoER2. Figure 1 shows the nucleotide and deduced amino acids sequences of the cDNA, which has an open reading frame of 3,339 bp corresponding of 1,113 amino acids with a calculated molecular mass of approximately 123 kDa. The putative initial methionine was preceded by an in-frame termination codon present 87 nucleotides upstream.

A hydropathy plot (*26*) of the deduced amino acid sequence of murine LRP4 shows the presence of a hydrophobic region at amino acid residues 113-133 (boxed in black in Fig. 1 and identified with thick lines in Fig. 2A). This hydrophobic sequence of 21 amino acids strongly resembles the transmembrane region of membrane proteins, being flanked by a positively charged amino acid (arginine) on the N-terminal side. This structural feature suggests that LRP4 has a type II transmembrane protein structure (amino terminus in the cytosol).

The C-terminal side of the putative transmembrane domain contains two clusters of cysteine-rich repeats that resemble the ligand binding repeats (class A motifs) of LDLR: one cluster contains three repeats and the other has five (Fig. 2, B and C). Each repeat has six completely conserved cysteines and a highly conserved C-terminal SDE tripeptide, which forms a part of the ligand-binding site of LDLR (Fig. 2B). Unlike LDLR, VLDLR, apoER2, LRP1, and LRP2, there are neither YWTD repeats nor growth factor repeats (class B motifs) in the murine LRP4 sequence (Fig. 2C).

The cytoplasmic domains of LDLR, VLDLR, apoER2, LRP1, and LRP2 contain one or two copies of a highly conserved coated pit signal, FXNPXY (*23*). In the putative cytoplasmic region (N-terminus), we found neither a typical FXNPXY sequence nor a similar tyrosine-based sequence (*27*). Further studies are required to determine whether LRP4 may function as an endocytic receptor.

Southern Blot Analysis of the LRP4 Genes in Various Species—To test the possibility that LRP4 homologue genes might also be present in nonmammalian vertebrates (known to lack the apoE gene), Southern blot analysis of genomic DNA from several different organisms was carried out. This "zoo blot" (containing DNAs of humans, mouse, chicken, and frog) was hybridized with the entire coding region of the murine cDNA under relatively stringent conditions (see "MATERIALS AND METHODS"). As shown in Fig. 3, intense hybridization signals are present in mouse, and fainter but significant signals can also be detected in human and chicken DNAs. These data suggest the presence of LRP4 homologues in chicken lacking the gene encoding apoE, which is recognized by the ligand-binding repeats of mammalian LDLR, VLDLR, and apoER2.

Expression of LRP4 Transcripts—Northern blot analysis of RNA from various murine tissues revealed hybridization of the LRP4 probe to a major transcript of 5.0 kb in mouse, with the highest expression in heart, relatively high levels in testis, and much lower levels in kidney and lung (Fig. 4A). Figure 4B shows a blot hybridization of RNA from various human tissues probed with the murine cDNA. In human tissues, major transcripts of 5, 2.6, and 2.3 kb and a minor transcript of 4 kb are detected almost exclusively in heart. A fainter but significant signal of 2 kb can also be detected in skeletal muscle and testis. The transcripts of 2.0, 2.3, 2.6, and 4 kb detected in human tissues may be a consequence of alternative splicing.

β-VLDL Binding—To test the possibility that LRP4 might bind apoE-rich β-VLDL (as do LDLR, VLDLR, and apoER2), an expression plasmid containing the entire coding region of murine LRP4 cDNA was constructed and introduced into COS-7 cells, and ligand-binding activity was measured using ^{125}I-labeled β-VLDL. As shown in Fig.

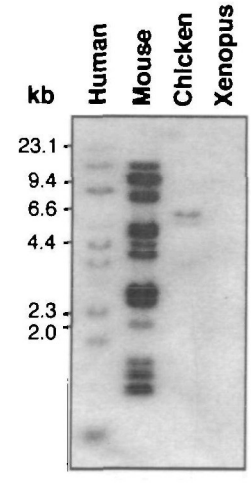

Fig. 3. **Genomic Southern blot analysis of LRP4-related sequences in various eukaryotic species.** A blot containing 10 μg of *Eco*RI-digested DNA from the indicated species was hybridized with the entire coding region of murine LRP4 cDNA under the conditions described in "MATERIALS AND METHODS" and exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 16 h.

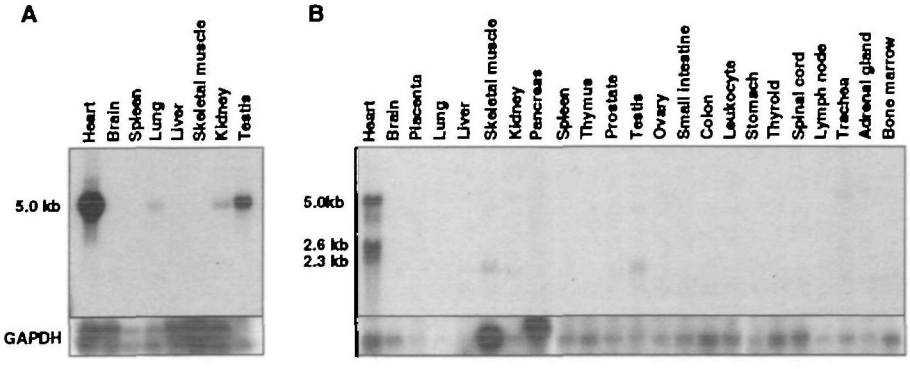

Fig. 4. **Expression of LRP4 transcripts in mouse (A) and humans (B).** Poly(A) RNA (2 μg) from the indicated murine (A) and human (B) tissues was probed with ^{32}P-labeled murine LRP4 cDNA. The filters were exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 14 h. Control hybridization with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown in the lower portion.

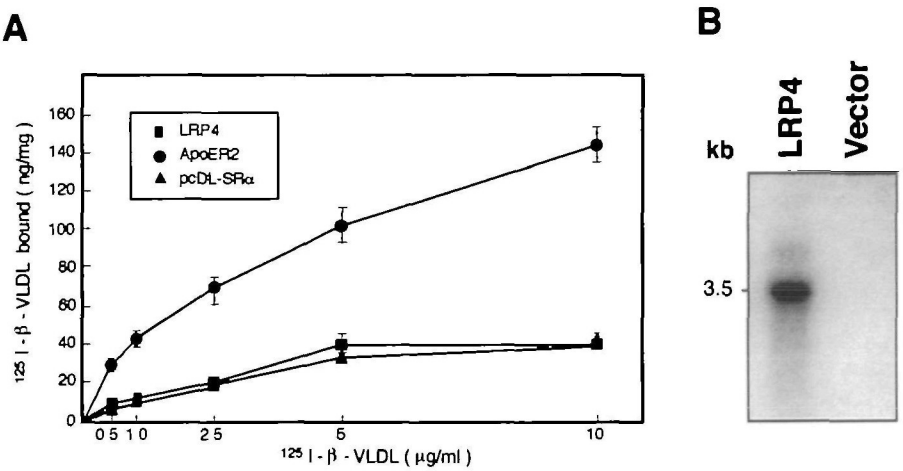

Fig. 5. **Transient expression of LRP4 in COS cells.** (A) Surface binding of ^{125}I-labeled β-VLDL. COS cells transfected with an expression plasmid encoding murine LRP4 (pLRP4-SRα), human apoER2 (pNR1), or the parental vector of pLRP4-SRα (pcDL-SRα296) were incubated for 2 h at 4°C with the indicated concentrations of ^{125}I-β-VLDL (540 cpm/ng), after which the values for surface-bound β-VLDL were determined as described under "MATERIALS AND METHODS." (B) Northern blot analysis of LRP4 transcripts in COS cells transfected with murine LRP4 expression plasmid (LRP4), or the parental vector (pcDL-SRα296). Total RNA (10 μg) from the indicated transfected cells was probed with ^{32}P-labeled murine LRP4 cDNA. The filter was exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 12 h.

5A, the level of surface bound β-VLDL in LRP4-transfected cells was similar to those in cells transfected with equal amounts of the parental vector, despite the high levels of accumulation of 3.0-kb LRP4 mRNA (lacking approximately 2.0 kb in the 3′-untranslated region) in the LRP4-transfected cells (Fig. 5B). In control experiments, marked induction of ^{125}I-β-VLDL binding was observed in cells transfected with human apoER2.

DISCUSSION

In the present study, we have shown the structure and expression of a novel member of the LDLR family termed LRP4. The most interesting feature of LRP4 is that, unlike other members of the LDLR family, this protein has a type II membrane protein-like structure. The hydropathy plot analysis shows the presence of a hydrophobic region at amino acid residues 113-133 of murine LRP4. There are eight ligand-binding repeats clustered into two regions in the C-terminal side of this putative transmembrane region. Based on the presence of ligand-binding repeats in the extracellular regions of other LDLR family members, it seems reasonable to predict that the C-terminal side of the putative transmembrane region constitutes the extracellular region of the protein.

Despite the presence of eight ligand-binding repeats, COS cells transfected with LRP4 failed to bind β-VLDL, suggesting that LRP4 does not function in lipoprotein metabolism. Of the four clusters of ligand-binding repeats in LRP2, the recognition site for apoE has been mapped to the second cluster (*28*). This suggests that these clusters are not functionally equal, despite their structural similarity. Therefore, the ligand-binding repeats in LRP4 may be functionally different from those in other family members that bind β-VLDL.

Although the exact function and ligands of LRP4 remain unclear, the abundant expression of LRP4 transcripts in heart is noteworthy. Based on the structural features of LRP4 and its almost exclusive expression in the heart, LRP4 may play a role as a surface receptor that is related to cardiac function. Further studies are necessary to elucidate the exact role of this structurally interesting molecule.

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