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Molecular characterization and expression of the low-density lipoprotein receptor-related protein-10, a new member of the LDLR gene family

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ABSTRACT

We report the characterization of a new member of the low-density lipoprotein receptor (LDLR) gene family designated LRP10. Human LRP10 cDNA encodes a 1905 amino acid type I membrane protein consisting of five functional domains characteristic of the LDLR gene family. CHO-*ldlA*7 cells transfected with human LRP10 cDNA bound LDLR-associated protein, but not β-VLDL and HDL. Human LRP10 transcripts were primarily found in the brain, muscle and heart. *In situ* hybridization of the rat brain showed that the transcripts were intensely present in the cerebral cortex, hippocampus, choroid plexus, ependyma and granular layer. In the developing rat brain, transcript levels gradually increased from postnatal day 1 to 20. Immunofluorescence analysis indicated that LRP10 was observed in the ventricular zone of the embryonic day 14.5 mouse cerebral cortex. The present studies suggest that LRP10 may play a significant role in the brain physiology other than lipoprotein metabolism.

Introduction

The low-density lipoprotein receptor (LDLR) is a well-known cell surface receptor that mediates lipoprotein metabolism in the body [1]. Genetic deficiencies of the LDLR gene give rise to familial hypercholesterolemia, one of the most common genetic diseases in humans [2]. The LDLR gene encodes a single transmembrane protein that consists of five functional domains [3]: a ligand binding domain composed of multiple cysteine-rich repeats; an epidermal growth factor (EGF) precursor homology domain with the sequence Tyr-Trp-Thr-Asp (YWTD) that forms a β-propeller structure; an *O*-linked sugar domain; a transmembrane domain; and a cytoplasmic domain with a coated pit targeting signal. In mammals, the following receptors are considered to be part of the LDLR gene family in mammals: LDLR [3]; very low-density lipoprotein receptor (VLDLR) [4]; apolipoprotein E receptor 2 (ApoER2 also known as LRP8) [5]; LDLR-related protein-1 (LRP1 also known as CD91, or α2macroglobulin receptor, α2MR) [6]; LRP2 (LRP1 also known as megalin, or glycoprotein 330, GP330) [7]; LRP3 (closely resembles ST7 and LRP9) [8,9]; LRP4 (also known as corin) [10,11]; LRP5 (also known as LRP7) [12,13]; LRP6 [14]; and LR11 (also known as sorLA) [15]. Because all of the LDLR gene family is structurally similar to LDLR, it has been thought that all members of this family play a primary role in lipoprotein metabolism. However, recent studies have provided evidence indicating that other members of the LDLR gene family are involved in diverse biological functions distinct from that of LDLR. VLDLR and ApoER2 transmit the extracellular Reelin signal to migrating neurons, which governs neuronal layering of the brain during embryonic brain development [16]. LRP1 regulates cellular entry of viruses and toxins and protects from atherosclerosis by modulating plate-derived growth factor receptor-β signaling in the vascular wall [17]. LRP2 acts as an endocytic receptor that mediates the availability of several extracellular signaling molecules such as vitamin D, vitamin A and sex steroids [18]. LRP3 modulates cellular uptake of β-VLDL [8]. LRP4 serves as a type II transmembrane serine protease, and as a pro-atrial natriuretic peptide-converting enzyme that regulates blood pressure [19]. LRP5 and LRP6 bind Wnt and Frizzled proteins, and activate the Wnt signaling pathway involved in cell proliferation, cell polarity and cell fate determination [20]. LR11 may participate in the development of Alzheimer's disease by modulating endocytosis of the amyloid precursor protein, which generates the amyloid β peptide [17].

In 1998, Nakayama et al. [21] identified a partial cDNA MEGF7 by EGF motif-trap screening. We considered that MEGF7 may

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belong to the LDLR gene family because it contains a part of the functional domain of the LDLR gene family. We successfully cloned the full-length human MEGF7 cDNA and designated the cDNA as LRP10 (GenBank Accession No. AB084910). In the current paper, we describe the structure, expression and lipoprotein binding of LRP10.

Materials and methods

cDNA cloning. Total RNA of the human brain was purchased from Clontech (Mountain View, CA). 5′ RLM-RACE (RNA ligase-mediated rapid amplification of cDNA ends) was performed by a commercial kit (Ambion, Austin, TX) with the following oligonucleotides corresponding to the reported DNA sequence of MEGF7 (GenBank Accession No. AB011540): 5′-AACAG CAGCA CAGGC TCTGG-3′ (reverse); 5′-ATACC CCTCC TCGGC ACATTC-3′ (reverse); and 5′-CCACA GCAGA ATTGC CG-3′ (forward). The RACE PCR analysis was performed by two rounds of PCR for 30 cycles under the following conditions: initial denaturing at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min and a final extension at 72 °C for 10 min. The fragment was ligated into the pGEM-T vector (Promega, Madison, WI), transformed into competent *Escherichia coli* DH5α and sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Cell culture. HEK293 cells, a cell line derived from human embryonic kidney, were cultured in Dulbecco's Modified Eagle's medium supplemented with antibiotics and 10% fetal bovine serum at 37 °C in 5% CO_2. CHO-*ldlA*7 cells [22], a mutant Chinese hamster ovary cell line lacking LDLR, were cultured in Minimum Essential medium supplemented with antibiotics and 10% fetal bovine serum at 37 °C in 5% CO_2.

Receptor-associated protein (RAP) binding assay. Recombinant GST-fusion proteins of RAP were prepared as previously described [23]. CHO-*ldlA*7 cells were transfected with ligand binding domain of human LRP10 or ApoER2 cDNAs. The cells were also transfected with PSA (prostate-specific antigen) cDNA as a control. The ligand binding domains of human LRP10 and ApoER2 cDNAs were amplified by PCR and inserted into pSecTag2 (Invitrogen, Carlsbad, CA). pSecTags contains the murine IgM kappa chain leader sequence to allow protein secretion at the N-terminus and the c-myc epitope at the C-terminus. After 5 days of incubation, 50 ml of conditioned medium was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reduced or non-reduced conditions, and the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. Each protein was detected by immunoblotting with the anti-c-myc antibody. The binding assay of RAP to LRP10 was performed by blocking the PVDF membrane of non-reduced SDS–PAGE with 5% skim milk, 0.05% Tween-20 and 2 mM $CaCl_2$ in PBS and incubating in the presence of 2 μg/ml of RAP at room temperature (RT) for 4 h. After being washed, bound RAP was detected by immunoblot analysis with the anti-RAP antibody.

Lipoprotein binding assays. HDL (1.063–1.21 g/ml) and β-VLDL ($d \leqslant 1.006$ g/ml) were prepared from the plasma of rats and 1% cholesterol-fed rabbits, respectively [24]. Fluorescently labeled β-VLDL and HDL were prepared using 1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine perchlorate as previously described [25]. Mammalian expression vectors for human LRP10, human ApoER2 or mouse scavenger receptor BI (SR-BI) were constructed in pcDNA3.1(+) (Invitrogen). CHO-*ldlA*7 cells transfected with the expression vectors were incubated for 3 h at 4 °C with fluorescently labeled β-VLDL or HDL (20 μg/ml each). ApoER2 and SR-BI are known to bind β-VLDL [5] and HDL [26], respectively; therefore, they can be used as controls. After the cells were washed three times with PBS, fluorescence was measured using an IX-70 fluorescent microscope (Olympus, Tokyo, Japan).

Northern blot analysis. Commercially available northern blots containing mRNA from various human tissues (Clontech) were hybridized with [^{32}P]-labeled probes primed with random hexanucleotides. The probes used for northern blot analysis included the human LRP10 cDNA (nt 875–1715) and the human LDLR cDNA (nt 54–718).

Quantitative real-time PCR. Various tissues were obtained from 5-week-old male Wistar rats (Japan SLC, Hamamatsu, Japan). Whole brains were taken from male Wistar rats of the mid-late embryonic stage (E12, E16, E18, and E20) and the early postnatal stage (P1, P4, P8, P12, P16, P20, P24, and P27). Total RNA was isolated using TRIzol reagent™ (Invitrogen) for quantitative real-time PCR to determine the expression of LRP10 mRNA in various rat tissues. Reverse transcription was performed with 5 g of total RNA, random primer, and Superscript III RNase H$^-$ RT (Invitrogen) according to the manufacturer's instructions. Quantitative real-

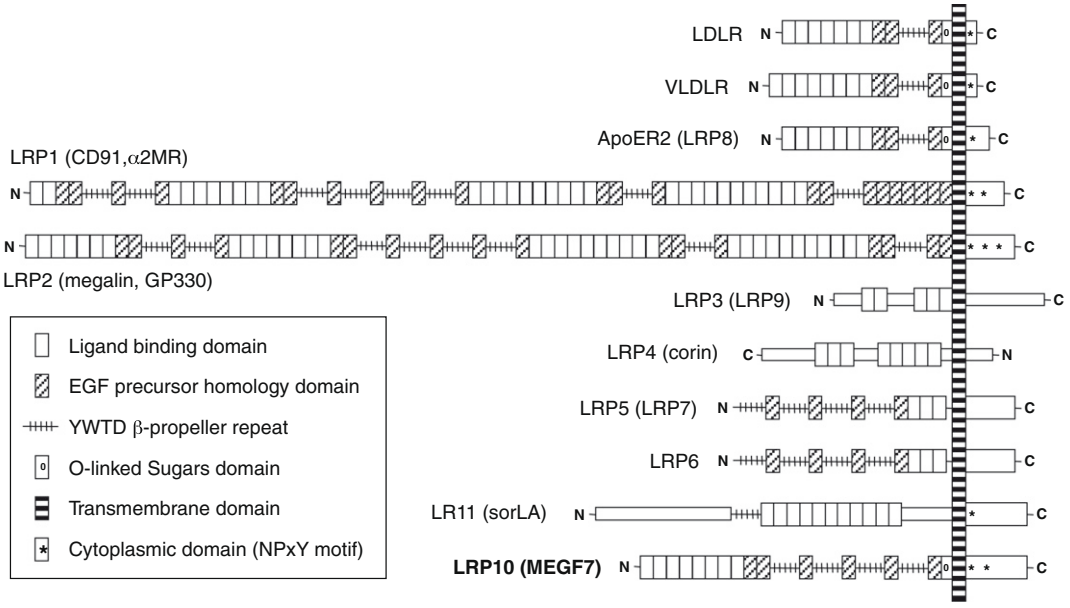

Fig. 1. Comparison of the structural organization of LRP10 with the LDLR gene family.

time PCR was performed using Absolute QPCR Master Mixes (THERMO Scientific, Waltham, MA) with an ABI PRISM 7000 Sequence Detection system (Applied Biosystems). All animal experiment procedures were approved by the Regulation on Animal Experiments in Osaka City University.

In situ hybridization. *In situ* hybridization was performed with digoxigenin (DIG)-labeled RNA probes. DIG-labeled RNA probes were synthesized from linearized rat LRP10 cDNA (nt 4807–5727) and rat LDLR cDNA (nt 1092–2095) inserted into pBluescript KS(+) using T3 and T7 RNA polymerase (Roche, Basel, Switzerland) to create sense and anti-sense RNA probes, respectively. Frozen rat brain tissue was sectioned on a cryostat (20 μm thickness), thaw-mounted on 3-aminopropyltriethanoxysilane-coated slides, and stored at −80 °C until used. Hybridization, washing, and development were performed as previously described [27].

Generation of a rat monoclonal antibody and immunohistochemistry. We constructed a recombinant GST fusion fragment containing the cytoplasmic domain of human LRP10 (GST-LRP10) to prepare the immunogen against LRP10. The entire cytoplasmic tail sequences (nt 5485–5958 bp) of human LRP10 were amplified by PCR and cloned into pGEX-6p-1 vector (GE Healthcare, Piscataway, NJ). The fusion protein that was expressed in BL21 bacteria was purified with glutathione agarose and used as the immunogen. The antibody was prepared by the rat lymph node method [28]. Finally, we obtained the monoclonal antibody, MAb 3H5 (rat IgM(κ)). For immunohistochemistry, HEK293 cells transfected with human LRP10 cDNA and frozen brain sections of an E14.5 ICR mouse embryo were pretreated with blocking solution (5% normal goat serum, 0.2% bovine serum albumin, 0.1% Triton X-100, 5 mM NaN_3, 0.1 M PBS) for 1 h at RT and then incubated with MAb 3H5 at 4 °C overnight. Immunofluorescence staining was detected with Alexa488-conjugated goat anti-mouse IgG (1:400, Invitrogen). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

Immunoblot analysis. Total proteins were prepared from HEK293 cells transfected with or without human LRP10 cDNA by M-PER (BioRad, Hercules, CA). Following SDS–PAGE, proteins were transferred onto PVDF membranes and blocked with 5% skim milk, 0.05% Tween-20 in PBS and blotted with the specific antibodies. The membranes were washed with 0.05% Tween-20 in PBS for 30 min and incubated for 1 h with secondary antibody. After washing with 0.05% Tween-20 in PBS, membranes were subjected to enhanced chemiluminescence detection analysis (ECL Plus; GE Healthcare) using horseradish peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA).

Results and discussion

Isolation and characterization of human LRP10 cDNA

We successfully cloned the full-length human MEGF7 cDNA included a 5715 bp open reading frame from the brain, and designated the cDNA as LRP10 (GenBank Accession No. AB084910). This cDNA codes for a protein of 1905 amino acids with a calculated M_w of 209,122, including a predicted signal peptide composed of 17 amino acids N-terminal to the mature human LRP10. The mature human LRP10 consists of 1888 amino acids with a calculated M_w of 207,720. The deduced amino acid sequence of the cDNA reveals that human LRP10 consists of five functional domains resembling the LDLR gene family, suggesting that LRP10 is a new member of the LDLR gene family.

Fig. 1 (see also Supplementary Fig. S1) shows the structural organization of members of the LDLR gene family. The ligand binding domain of LRP10 contains eight copies of the ligand binding repeat. The EGF precursor homology domain of the LRP10 consists of six cysteine-rich repeats with four clusters of five YWTD β-propeller repeats. The putative N-terminal domain of LRP10 closely resembles the second cluster in the N-termini of LRP1 [6] and LRP2 [7]. The second cluster in the N-terminus of LRP1 has been reported to be an important region that binds various ligands [29]. The *O*-linked sugar domain of LRP10 contains 72 amino acid residues including 15 serine and threonine residues. The transmembrane domain of LRP10 contains 24 amino acid residues. The cytoplasmic domain of LRP10 consists of 158 amino acids, which contains two typical internalization signal sequences, Asn-Pro-Xxx-Tyr (NPxY). It has been recently elucidated that the NPxY motif interacts with various cytoplasmic adapters and scaffold proteins involved in signal transduction such as disabled protein-1, FE65, JIP1, PSD-95, Shc, and CED-6 [30–32]. The structural organization predicts that LRP10 may bind ligands similarly to the LDLR gene family, and that LRP10 acts as an endocytic receptor or a signal transducer.

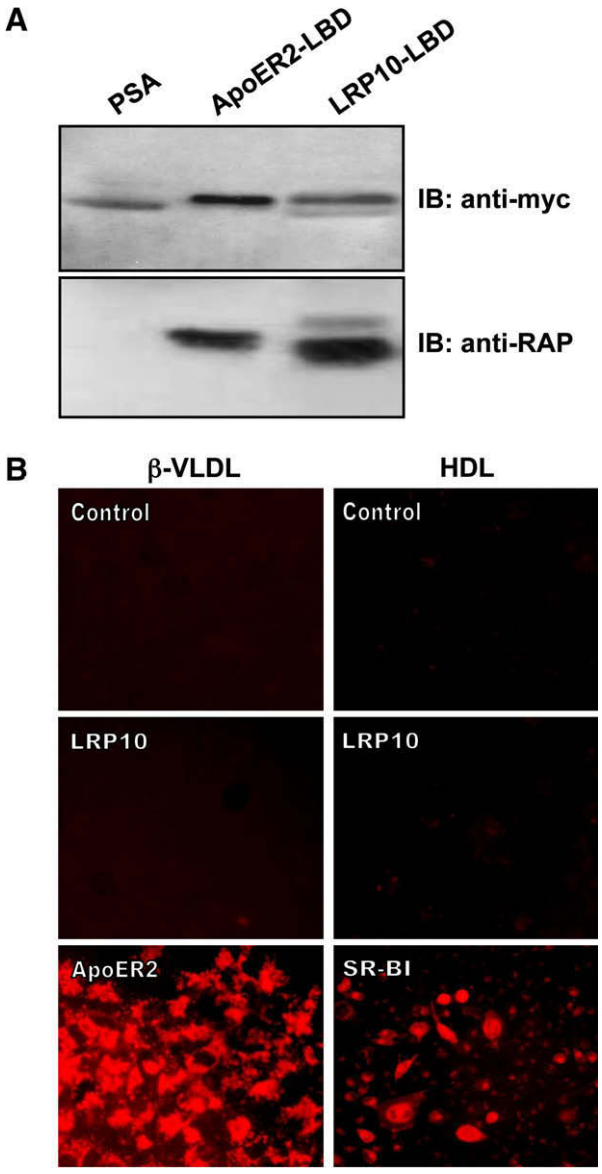

Fig. 2. Ligand binding assay of LRP10 in CHO-*ldlA*7 cells. LRP10, ApoER2, and PSA proteins were detected by immunoblotting with an anti-myc antibody (A, *upper panel*). RAP-bound proteins were detected by immunoblotting analysis with an anti-RAP antibody (A, *lower panel*). The LRP10, ApoER2, or SR-BI cDNAs were introduced into LDL-deficient CHO-*ldlA*7 cells (B). Control cells were transfected with pcDNA3.1(+). Cells were incubated with fluorescent β-VLDL (B, *left panels*) and HDL (B, *right panels*). Magnification: 80×.

Binding of RAP, β-VLDL, and HDL to LRP10

Members of the LDLR gene family are known to bind to RAP, an endoplasmic reticulum protein that associates with newly synthesized receptors [23]. We examined the interaction of RAP with LRP10 that is expressed in CHO-*ldlA*7. Indeed, LRP10 sufficiently bound RAP as did ApoER2 (Fig. 2A). However, no accumulation of fluorescent β-VLDL or HDL was observed in cells transfected with LRP10 (Fig. 2B), although fluorescent β-VLDL and HDL were found in cells transfected with ApoER2 and SR-BI, respectively. Our results do not support the prediction that all of the LDLR gene family with the ligand binding domain would bind to apoE-containing lipoproteins. We suggest that LRP10 may have other distinct physiological functions than predicted.

Expression of LRP10 transcripts in human and rat tissues

We assessed the tissue distributions of LRP10 mRNA in humans and rats. Northern blot analysis of LRP10 mRNA from various human tissues revealed that the LRP10 probe hybridized to a major 8.1 kb transcript (Fig. 3A). The pattern of tissue distribution of LRP10 mRNA in humans was completely different from the pattern of LDLR mRNA: the LRP10 mRNA was highly expressed in the brain, muscle and heart, whereas LDLR mRNA was expressed in various tissues including the liver (Fig. 3A). Fig. 3B shows the relative mRNA expression levels of LRP10 from various rat tissues. LRP10 mRNA was expressed intensely in the olfactory bulb, cerebral cortex, brain stem, hippocampus, and cerebellum and moderately in muscles, heart, kidney, liver, and adipose tissue.

In situ hybridization analysis of the adult rat brain showed that LRP10 transcripts were expressed the most intensely in the cerebral cortex, hippocampus, choroid plexus, ependyma, granular layer, cerebellar nuclei and, to a much lesser extent, in the cerebrum and meninges (Fig. 3C). Alternatively, the LDLR transcripts were detectable in the cerebellar cortex and hippocampus, but significant signals were not detected in the choroid plexus, ependyma or olfactory bulb, suggesting that the role of LRP10 in the CNS is different from that of the LDLR. No significant signals were detected in any brain regions when hybridized with sense RNA probes for LRP10 and LDLR as controls (data not shown).

We found that the expression of the LRP10 mRNA showed ontogenetic changes during brain development (Fig. 3D). Expression of the LRP10 mRNA in the brain gradually increased from P1 to P20. These results suggest that LRP10 may play a significant role in brain development.

Immunohistochemical analysis of LRP10 expression in the brain

We generated a rat monoclonal antibody, MAb 3H5, against LRP10 to investigate the cellular localization of LRP10 in the brain. MAb 3H5 densely stained the cell membrane by immunofluorescence analysis and detected approximately 250 kDa proteins by immunoblotting (Fig. 4A). These results indicate that mature LRP10 is a membrane protein of approximately 250 kDa.

Furthermore, we performed immunofluorescence staining with E14.5 brains to clarify the distribution of LRP10. In the cerebral cortex, LRP10 proteins were mainly deposited in the ventricular zone (VZ), but the proteins were not found in the intermediate zone (IZ) and the cortical plate (CP) (Fig. 4B).

The VZ plays a significant role in development of the cerebral cortex [33]. The first neurons destined to settle in the cortex are born in the VZ. After their last mitotic divisions, neurons migrate from the VZ across the IZ into the CP and stop at their appropriate destinations. Reelin secreted by Cajal–Retzius cells has been proposed to act as a stop signal, which is mediated by VLDLR and ApoER2 belonging to the same gene family as LRP10 [16]. VLDLR is expressed in the IZ and ApoER2 is expressed throughout the developing neocortex with the exception of the VZ [16]. Although the expressions of VLDLR and ApoER2 in the cortex are different from that of LRP10, our results suggest that LRP10 may potentially act in neuronal migration during brain development. Further study is necessary to elucidate the interaction of LRP10 with Reelin.

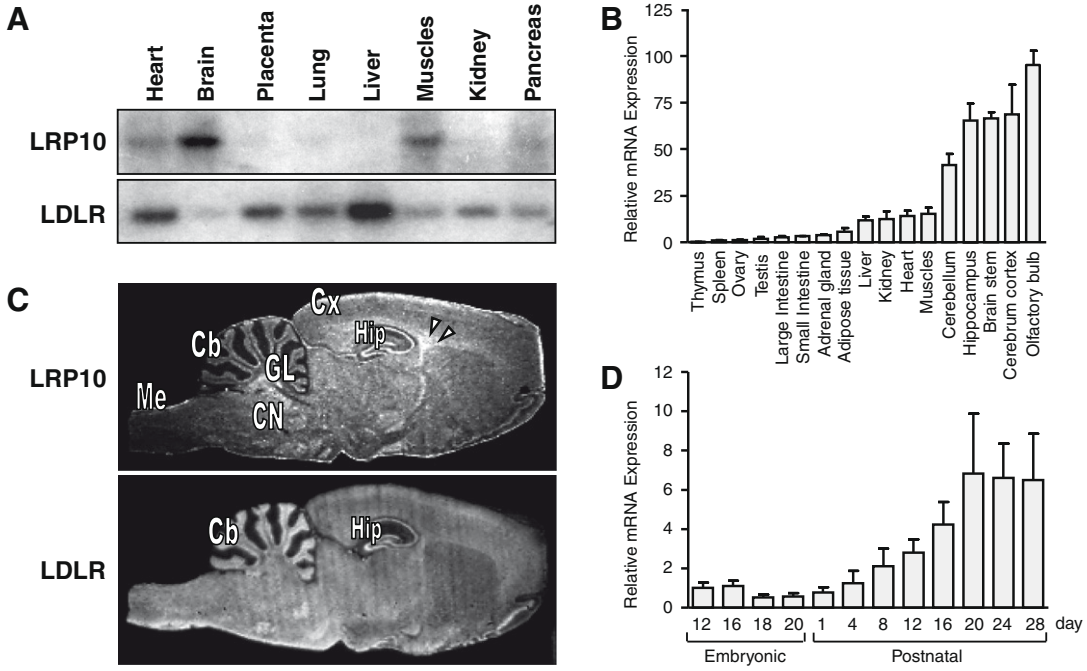

Fig. 3. Expression of LRP10. Commercially available northern blots containing mRNA from various human tissues (Clontech) were hybridized with [^{32}P]-labeled LRP10 and LRLR cDNAs (A). The expression of LRP10 mRNA in various rat tissues was measured by quantitative real-time PCR analysis (B). *In situ* hybridization was performed with digoxigenin DIG-labeled RNA probes synthesized from linearized rat LRP10 and LDLR cDNAs (C): the expression pattern of LRP10 mRNA in the rat brain was measured at the mid-late embryonic stage (E12, E16, E18, and E20) and the early postnatal stage (P1, P4, P8, P12, P16, P20, P24, and P27) by quantitative real-time PCR analysis (D). Hip, hippocampus; arrowheads, choroid plexus, and ependyma; GL, granular layer; CN, cerebellar nuclei; Cx, cerebral cortex; Cb, cerebrum; and Me, meninges.

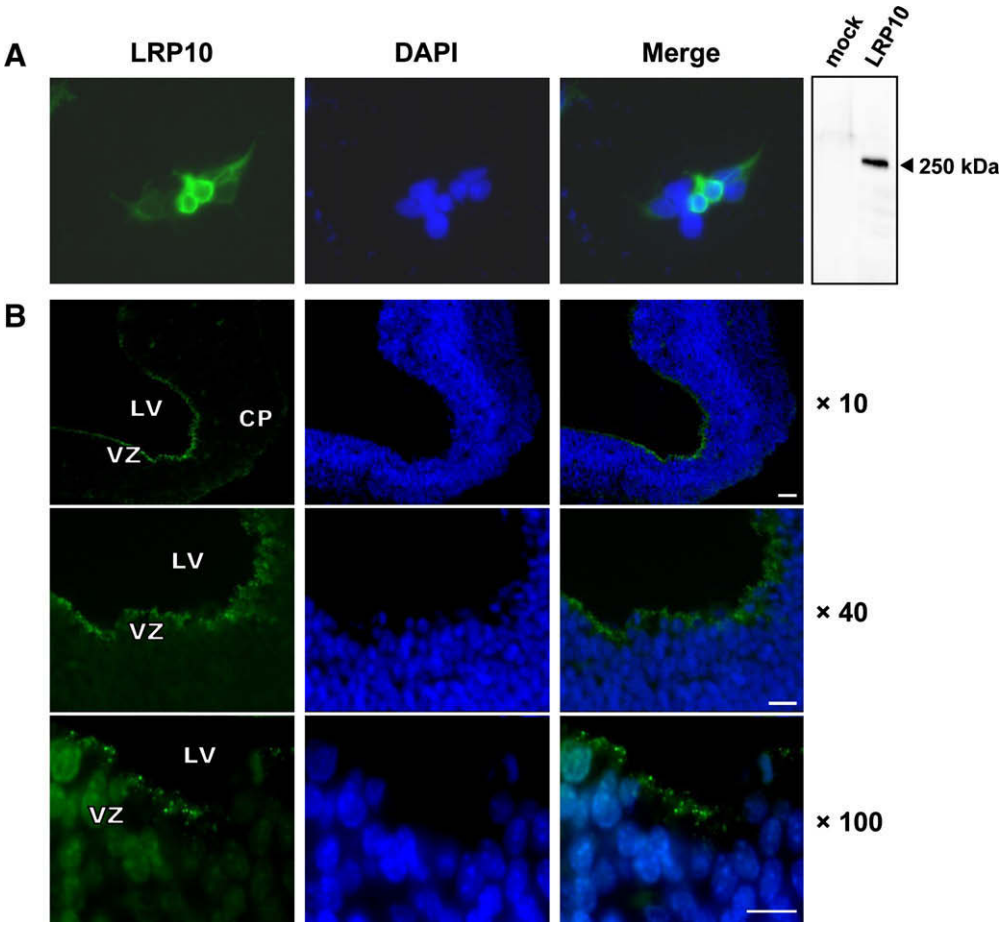

Fig. 4. Immunohistochemical analysis of LRP10 expression. HEK293 cells were transfected with LRP10 and stained with an anti-LRP10 antibody, MAb3H5 (A). LRP10 and nuclei shown in green and blue, respectively. Magnification: 200×. The membrane proteins were extracted from HEK293 cells transfected with LRP10 or pcDNA3.1(+) and were analyzed by immunoblotting analysis with an anti-LRP10 antibody MAb 3H5 (A, *right panel*). Coronal sections of E14.5 mouse brains were immunostained with an anti-LRP10 antibody, MAb3H5 (B). LRP10 and nuclei shown in green and blue, respectively. Scale bars indicate 300 nm (*top panels*), 1.2 nm (*middle panels*), and 3 nm (*bottom panels*). CP, cortical plate; LV, lateral ventricle; and VZ, ventricular zone.

Conclusion

We have described the structure, expression and lipoprotein binding of a new member of the LDLR gene family designated LRP10. LRP10 consists of five functional domains characteristic of the LDLR gene family. However, LRP10 failed to bind β-VLDL and HDL. In the brain, the predominant expression pattern of LRP10 was different from the pattern of LDLR. In the brain, expression of LRP10 gradually increased at the early postnatal stage. Although the exact function of LRP10 remains to be elucidated, our findings will promote studies on the molecular mechanisms of LRP10 on brain development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.033.

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