A New Low Density Lipoprotein Receptor Homologue with 8 Ligand Binding Repeats in Brain of Chicken and Mouse*

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The blood-brain barrier necessitates disparate macromolecular transport systems in the brain and central nervous system. We now report the discovery of a new member of the low density lipoprotein receptor (LDLR) family whose expression is highly restricted to the brain. The full-length cDNA specifying the chicken receptor (open reading frame, 2754 base pairs) as well as a cDNA for the major portion of its murine homologue have been obtained. The novel receptor shows the greatest similarity to the group of LDLR relatives with 8 tandemly arranged ligand binding repeats, in chicken termed LR8 and in mammals, very low density lipoprotein receptors. Thus, in addition to 8 tandemly arranged ligand binding repeats, the five-domain receptor contains an O-linked sugar region and the internalization signal, Phe-Asp-Asn-Pro-Val-Tyr, typical for all LDLR gene family members. In chicken, the 6.5-kb receptor transcript is present at high levels in brain and at much lower levels in extraocular cells of the ovary; in mouse, the same transcript of 6.5 kb was detected in brain, but not in heart (the major site of very low density lipoprotein receptor expression), lung, liver, kidney, and ovary. An antibody directed against the predicted carboxyl terminus of the avian receptor detected a 130-kDa protein in brain extracts. The apparent size of the immunoreactive protein is compatible with extensive glycosylation of the 894-residue mature form of the receptor. The presence of this novel receptor in brain of a bird and a rodent suggests an important and evolutionary conserved function.

Since the molecular characterization of the low density lipoprotein receptor (LDLR) (1), an ever increasing number of related proteins have been discovered. The members of the LDLR family are characterized by distinct functional domains present in characteristic numbers. These modules are (i) the "type A binding repeats" of ~40 residues each, displaying a triple-disulfide-bond-stabilized negatively charged surface; certain head-to-tail combinations of these repeats are believed to specify ligand interactions (1); (ii) "type B repeats," also containing six cysteines each; (iii) modules of ~50 residues with a consensus tetrapeptide, YWTD, found in the epidermal growth factor precursor; (iv) a transmembrane domain, and (v) the cytoplasmic region with a (signal) for receptor internalization via coated pits, containing the consensus tetrapeptide Asn-Pro-xaa-Tyr (NPXY).

The members of this protein family characterized to date are the LDLR (2), the LDLR-related protein (LRP) (3), gp330/megalin (4), and the VLDL receptor (5). At least in vitro, these receptors bind a large number of unrelated ligands, suggesting overlapping ligand specificities in vivo (6–11). One of these common ligands is the small intracellular "receptor-associated protein" (RAP), which acts as a chaperone preventing intracellular ligand-induced receptor aggregation (12, 13). Interestingly, the most specific family member, the LDLR, which binds only apoB and apoE with high affinity, has the lowest affinity for RAP (14). In the chicken, we have characterized 4 members of the LDLR family; two of these receptors mediate the massive transport of yolk precursors into growing oocytes. These are (i) the oocyte-specific LDLR relative with 8 binding repeats termed LR8 (15), a homologue of the mammalian VLDL receptors (16), and (ii) the oocyte-specific LRP (17). The other two receptors, expressed in somatic cells, are avian homologues of the mammalian LDLR (18) and of LRP, respectively (19).

It has been proposed that apoE, which is highly expressed in the mammalian central nervous system and is a high affinity ligand for the above-mentioned receptors, may serve as mediator of local lipid transport in the brain. Such transport would be expected to be independent of systemic lipoprotein metabolism, due to separation via the blood-brain barrier (20). To date, it has been assumed, but not proven experimentally, that in the central nervous system of mammals LDLR and/or LRP could function as corresponding receptor(s) for the uptake of apoE containing, possibly brain-specific, lipoproteins. However, brain abnormalities in humans with homozygous LDLR defects or in mice homozygous for the knock-out of the LDLR gene have not been observed.

Here we report the molecular characterization of LR8B, a new LDLR homologue with 8 ligand binding repeats from chicken and mouse. Since both species express this receptor predominantly in the brain, we propose that it represents a new candidate receptor for brain-specific transport processes.

EXPERIMENTAL PROCEDURES

cDNA Preparation and PCR Analysis—First-strand cDNA synthesis was performed with 1 μg of poly(A)* RNA (16), using SuperScript Reverse Transcriptase (Life Technologies, Inc.) and random hexamers. The degenerated oligonucleotides used corresponded to regions of high homology (epidermal growth factor precursor domain) within all members of the LDLR gene family: primer A, 5′-AAATT(T/C)AC(T/C/A/G)TG(T/C)GCT(C/A/G)TG(T/G)CC and primer B, 5′-GGACA(A/G/T/C)GGCA(T/C/G)A/CG(T/G)/AAATT-3′.
CGCA(A/G)CA(A/G/T)GT(A/G)AA(T/G)TT. As positive control for the RT-PCR experiments, we used primers specific for chicken ferritin (21). PCR (100 µl) was performed in the presence of 1.5 mM MgCl₂, 1 unit of Taq DNA polymerase (Perkin Elmer), and 0.5 µM of the primers at 94°C (1 min), 55°C (1 min), and 72°C (3 min) for 35 cycles. Products were subcloned into the pGEMT vector (Promega), and inserts were sequenced from both ends using Sequenase U. S. Biochemical Corp. (Cleveland).

Isolation of Chicken LR8B—The 550-bp RT-PCR product obtained with chicken brain mRNA was used as probe to screen a chicken brain cDNA library (Clontech). Hybridization conditions were as follows: 5 × NET (0.5 mM NaCl, 75 mM Tris, 20 mM EDTA), 5 × Denhardt’s solution, 0.2% SDS, and 100 µg/ml salmon sperm DNA for 20 h at 65°C. The membranes were washed in 0.1% SDS and 0.1 × SSC at 65°C; positive clones were subcloned into Bluescript KS− and sequenced on both strands. The sequence at the internal EcoRI site was verified by RT-PCR using the primers 5′-AGTTCCAGTGGCATA and 5′-CAGTTAACTGCTTGGG. The missing 5′-end of the cDNA was cloned by using the 5′-RACE kit from Clontech and the nested specific primers, 5′-TCAGGCAGCAGCCACGCGG and 5′-TCACGCAACAAGTCTTGTGG. Cloning of the Mouse LRB8 Homologue—RT-PCR was performed as described above using mRNA isolated from brains of adult female Balb/c mice with the pair of degenerate primers used to amplify chicken LRB8. The amplified ~700-bp product was subcloned into the pGEMT vector (Promega), and the resulting 2-fragment was subcloned independently from both ends into the pGEMT vector; thus, it was used to screen a mouse testis 10-bp chicken cDNA library under the conditions used for screening the chicken library. The longest insert (1755 bp) was amplified by PCR using universal primers and the product subcloned into pGEMT vector and sequenced on both strands.

Northern Analyses—Fifteen µg of total RNA (16) prepared from various tissues of a laying hen (Brown Delco) and a female Balb/c mouse were separated by electrophoresis on a 1.5% agarose gel and transferred onto nylon membranes (Amersham). After UV cross-linking (Stratagene), hybridizations were performed under the same conditions as in library screening. The 1675-bp insert of clone D2 (nucleotides 1346 to 3021 of the full-length cDNA) and the original PCR product derived from murine brain mRNA were used as probes. Blots were standardized by hybridizing with a 1.3-kb cDNA fragment of rat glyceraldehyde-3-phosphate dehydrogenase.

Preparation of Membrane Detergent Extracts, RAP-Sepharose Chromatography, Electrophoresis, and Western and Ligand Blotting—Membranes were prepared from whole brain, liver, and muscle of a laying hen and extracted with 1% Triton X-100 in buffer A (125 mM Tris/HCl, pH 7.2, 1 mM CaCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, pH 6) (14). Recombinant human RAP was produced as a glutathione S-transferase (GST) fusion protein (22) and coupled to CNBr-activated Sepharose (Pharmacia Biotech Inc.). The Triton X-100 was washed with buffer A adjusted to pH 7.2, and bound proteins were eluted from the matrix by incubation with 1 µl of glutathione loading buffer without heating and applied to 4.5- to 18% gradient SDS-polyacrylamide gels (23). Electrophoresis, transfer to nitrocellulose membranes, Western blotting, and ligand blotting with 125I-RAP were performed as described (14).

Preparation of Antibodies—A sequence-specific antibody directed against chicken LRB8 was obtained using a synthetic peptide corresponding to the 14 carboxyl-terminal amino acids (881–894) deduced against chicken LR8B. The amplification product was used to screen a mouse gt10 brain cDNA library (Clontech). Hybridization conditions were as follows: 5 × NET (0.5 mM NaCl, 75 mM Tris, 20 mM EDTA), 5 × Denhardt’s solution, 0.2% SDS, and 100 µg/ml salmon sperm DNA for 20 h at 65°C. The membranes were washed in 0.1% SDS and 0.1 × SSC at 65°C; positive clones were subcloned into Bluescript KS− and sequenced from both ends, revealing homology to chicken LRB8; thus, it was used to screen a mouse testis 10-bp chicken cDNA library under the conditions used for screening the chicken library. The longest insert (1755 bp) was amplified by PCR using universal primers and the product subcloned into pGEMT vector and sequenced on both strands.

RESULTS

In order to detect brain-specific members of the LDLR gene family in the chicken, several degenerate primer pairs corresponding to regions highly conserved among members of the gene family were used in RT-PCRs with mRNA from different chicken tissues. Sequence analysis of a 700-bp product obtained from brain mRNA and absent in all other tissues tested revealed, depending upon the domains used for comparison, 40–50% identity with chicken LRB8 (16) and somatic LRP (19). Thus, we used this fragment to screen a chicken brain cDNA library and obtained 2 independent overlapping clones (D1, D2).

LRB8 from Chicken and Mouse

Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA for chicken LRB8. Amino acid sequence numbering starts from the putative signal sequence cleavage site; negative numbers refer to the N-terminal signal peptide. Cysteine residues are encircled. Potential O-linked glycosylation sites (threonine clusters) are marked by asterisks, and N-linked glycosylation sites by circles, respectively. In the O-linked sugar domain, single proline residues following clusters of threonines are boxed. The transmembrane domain is underlined. The internalization signal sequence is boxed in, and the internal EcoRI site (see text) is underlined.

covering about 2.9 kb. D1 contained an internal EcoRI site (Fig. 1), and the resulting 2 fragments were subcloned independently. Using primers flanking the EcoRI site, we confirmed by RT-PCR that we had not missed a small EcoRI/EcoRI fragment. The 5′-end of the coding region was cloned by 5′-RACE yielding an additional 100 bp containing a single start codon.

The complete cDNA sequence contained one open reading frame of 2754 bp coding for a 918-residue protein (Fig. 1). The single ATG codon at the 5′-end of the cDNA most likely represents the translation initiation site, since it is in a sequence context fulfilling the rules of Kozak (27). The initiator methionine is followed by a stretch of 23 predominantly hydrophobic residues defining a cleavable signal sequence (28). The mature protein consists of 5 domains, starting with a domain of 8
cysteine-rich binding repeats of about 40 amino acids in length and 6 cysteines each. This structure is very reminiscent of the ligand binding domains of LR8 in chicken and the VLDL receptor in mammals, but differs significantly from that of LDLR containing only 7 such repeats. A "linker" region connecting repeats 4 and 5 in the LDLR (29) is found between repeats 5 and 6, and therefore in identical location as in the VLDL receptor and LR8 (5, 16). The remaining 4 domains of the protein are entirely analogous to those of the LDLR or of LR8+, the splice variant form of LR8 containing an O-linked sugar region (15). The 8 ligand binding repeats are followed by the epidermal growth factor precursor domain consisting of 3 type A (Y/F)W (3) repeats and five repeats containing the signature tetrapeptide, (Y/F)W (31). In summary, alignment of the amino acid sequence with the corresponding region of chicken LR8B showed an overall identity of 73% (Fig. 4), with most of the differences in the extracellular domain containing a perfect internalization signal (FDNPVYY) (31). In summary, alignment of the amino acid sequence of the brain protein with that of LR8 (16) and mammalian VLDL receptors from different species (4, 5, 32, 33) establishes that this newly discovered member of the LDLR family is a novel 8-ligand binding repeat receptor.

Using D2 as probe for Northern blotting, we re-evaluated the expression pattern found in the original RT-PCR experiment. Indeed, the corresponding transcript had a size of ~6.5 kb (Fig. 2), indicating untranslated 5' and 3' regions totalling at least 3.5 kb, and was detected only in brain. Upon prolonged exposure (3 days) of the blot, faint signals in granulosa cell sheets as well as in cultured chicken embryo fibroblasts could be detected. In contrast to the LDLR, transcript levels in fibroblasts were not regulated by extracellular cholesterol (18).

Polyclonal rabbit antibodies against a synthetic peptide corresponding to the deduced carboxyl-terminal 14 amino acids of the novel receptor recognized a protein with an apparent relative molecular mass of ~130 kDa in membrane extracts of brain, but not of liver and muscle (Fig. 3A). The additional low molecular weight bands in lanes 1-3 most likely represent unidentified cross-reactive proteins. Upon affinity chromatography on RAP-Sepharose of brain membrane extract, the same protein which reacts with the antibody was present in the bound fraction (Fig. 3B, lane 1). This protein represents 1 of 6 bands reacting with a single chain antibody fragment, ScFv (26), which recognizes the ligand binding domain of all known members of the LDLR family (Fig. 3B, lane 2). The double band at about 95 kDa most likely corresponds to very small amounts of the two splice variants of LR8 (15) present in brain and detectable after enrichment on the RAP-matrix. The other 3 membrane proteins which bound to RAP-Sepharose and reacted with ScFv7 might be yet unidentified proteins belonging to the LDLR family. As control for the reciprocity of ScFv7 and RAP in identification of LDLR family members, we blotted the RAP-Sepharose eluates with 125I-RAP (Fig. 3B, lane 3). The double band at 95 kDa was also visualized using the anti-myc-tag antibody 9E10 (Fig. 3B, lane 3).

To determine whether this newly characterized brain-specific member of the LDLR family is also expressed in mammals, we searched by PCR, performed exactly as described for the chicken receptor, for the corresponding murine mRNA. A 700-bp PCR-amplified product was 90% identical with the corresponding product from chicken brain. We used this fragment to screen a mouse brain cDNA library and sequenced the longest positive clone identified; it contained a continuous open reading frame of 1755 bp including the carboxyl terminus of the protein. Protein sequence alignment of the murine receptor portion with the corresponding region of chicken LR8B showed an overall identity of 73% (Fig. 4), with most of the differences observed in the putative O-linked sugar region. Northern blot analysis revealed that in the mouse the same transcript of 6.5 kb is found in brain, but not in heart, lung, liver, kidney, and ovary (Fig. 5).
new receptor LR8B, where LR stands for LDLR-relative, the number specifies the number of ligand binding repeats, and the B indicates the brain-specific expression of this receptor. This nomenclature appears preferable over one that refers to ligands, which might be misleading since the list of proposed ligands grows (14, 34). The new receptor shows the highest homology, both in modular structure and primary sequence, to the so-called VLDL receptors. It has 8 ligand binding repeats, and the linker, a short stretch between binding repeats 4 and 5 in LDLRs (29), is located at the same position as in VLDL receptors, i.e. between repeats 5 and 6. The presence of a perfect internalization signal, FDNPVY, in the cytoplasmic domain of the receptor strongly suggests endocytic competence (31). Deduced from the sequence, the receptor might be heavily glycosylated, in agreement with its apparent Mr of \(130,000\) on SDS gels versus a calculated Mr of \(-101,000\).

An anti-peptide antibody detected the 130-kDa protein in brain fractions, but not in other tissues tested. Due to the expected binding of RAP to the new receptor, we were able to partially purify the receptor by affinity chromatography of brain membrane extracts on immobilized RAP. Deduced from the extremely high conservation of the ligand binding domain with that of LR8 and mammalian VLDL receptors, we assume that these receptors potentially recognize a very similar set of ligands: apoB (24), apoE (35), \(\alpha_2\)-macroglobulin (34), lactoferrin, and, as demonstrated here, RAP (14). With respect to a possible function of LR8B, we must consider that LR8B is the first member of the LDLR gene family predominantly expressed in brain of such diverse species as chicken and mouse. It is widely appreciated that in mammals apoE might serve as mediator of brain-specific lipid transport, which operates separated from the general circulation by the blood-brain barrier (20). ApoE is synthesized by astrocytes, and apoE-containing lipoprotein particles are found in cerebrospinal fluid. The involvement of the LDLR and LRP, both expressed in mammalian brain (36, 37), in apoE-mediated brain lipid transfer is not defined, however. The fact that patients with a total absence of functional LDLRs lack abnormalities in the brain suggests that LDLR is not essential for such pathway(s). LRP may play a crucial role in the brain, since a certain proportion of mice homozygous for the disruption of the LRP gene do implant into the uterus, but die early showing failure of neuronal tube closure and incomplete expansion of brain vesicles (38, 39). In addition to a role in normal brain physiology, LR8B, if expressed in man, may also be a new candidate for involvement in the pathophysiology of neurodegenerative disorders due to the association of the apoE4 allele with late onset Alzheimer's disease (40). At first sight, the situation in chicken is different, as birds are not known to synthesize apoE (41, 42). However, they do synthesize apoAI in brain (43) and, importantly, at sites of peripheral nerve injury and regeneration (44, 45). In view of these observations, it has been proposed that apoAI in chickens may function in analogy to apoE in mammals (44, 45). It remains to be tested whether chicken apoAI is the pendant to mammalian apoE in brain metabolism, or whether this novel receptor's physiological ligand is a yet unidentified gene product common to chicken and mammals.

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