The Low Density Lipoprotein Receptor Gene Family

DIFFERENTIAL EXPRESSION OF TWO α2-MACROGLOBULIN RECEPTORS IN THE BRAIN*

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LR7/8B is a member of the low density lipoprotein receptor gene family that is specifically synthesized in the brain. Here we have functionally expressed in 293 cells the splice variant harboring eight ligand binding repeats (LR8B). As assessed by confocal microscopy, the expressed receptor is localized to the plasma membrane. Importantly, in cell binding experiments, we demonstrate that this protein is a receptor for activated α2-macroglobulin. Because to date low density lipoprotein receptor-related protein (LRP) has been shown to be the only α2-macroglobulin receptor in brain, we became interested in the expression pattern of both proteins at the cellular level in the brain. LR7/8B is expressed in large neurons and Purkinje cells of the cerebellum and in cells constituting brain barrier systems such as the epithelial cells of the choroid plexus, the arachnoidea, and the endothelium of penetrating blood vessels. Anti-LR7/8B antibody stains the plasma membrane, dendrites, and vesicular structures close to the cell membrane of neurons, especially of Purkinje cells. In contrast, LRP is present in patchy regions around large neurons and most prominently in the glomeruli of the stratum granulare of the cerebellum. This suggests that, contrary to LR7/8B, LRP is expressed in synaptic regions of the neurons; furthermore, there is a striking difference in the expression patterns of LR7/8B and LRP in the choroid plexus. Whereas LRP shows baso-lateral and apical localization in the epithelial cells, LR7/8B is restricted to the apical cell aspect facing the cerebrospinal fluid. Finally, these studies were extended to cultured primary rat neurons, where double immunofluorescence labeling with anti-LR7/8B and anti-microtubuli-associated protein 2 (MAP2) confirmed the somatodendritic expression of the receptor. Based upon these data, we propose that LR7/8B is involved in the clearance of α2-macroglobulin-proteinase complexes and/or of other substrates bound to α2-macroglobulin from the cerebrospinal fluid and from the surface of neurons.

The LDL receptor gene family specifies a group of highly related composite membrane proteins engaged in receptor-mediated endocytosis of a variety of independent ligands. The members of the family are, listed in the order of their discovery, LDL receptor (LDLR), LDL receptor-related protein (LRP), gp330/megalin, VLDL receptor (or LR8), LR11, apolipoprotein E receptor 2 (apoER2), and LRSB (1, 2). Common features of these proteins are structurally and functionally distinct modules that are defined by distinct exons in the corresponding genes. These modules are (i) the type A binding repeats of ~40 residues, each harboring 6 paired cysteines; (ii) type B repeats, also containing 6 cysteines each; (iii) modules of ~50 residues with a consensus tetrapeptide, Tyr-Trp-Thr-Asp (YWTD) (together with the type B repeats they constitute the epidermal growth factor precursor homology domain); (iv) a short stretch containing many serines and threonines carrying O-linked sugars (in the case of LR8 this domain is 30 amino acids long and is encoded by a single exon (3)); (v) a short transmembrane domain of approximately 20 amino acids, and (vi) the cytoplasmic region with one or more signals for receptor internalization via coated pits. The structural hallmark of the members of this family is the presence of specific arrangements of exactly these domains. For example, the extracellular part of LRP, which is one of the largest members of the family, is highly complex and contains 31 type A repeats organized in four clusters of 2, 8, 10, and 11 of these repeats, respectively. These clusters are separated from each other by epidermal growth factor precursor domains. LRP is a multifunctional receptor involved in a variety of independent processes such as lipoprotein metabolism and extracellular proteinase homeostasis (for review see Refs. 4 and 5). The latter function is mediated by α2-macroglobulin (α2M), a multifunctional proteinase inhibitor (for review see Ref. 6). α2M forms complexes between a variety of proteinases that are rapidly removed from the circulation by specifically binding to hepatic LRP. The receptor binding site on α2M is exposed by a conformational change which is the result of a cleavage in the “bait region” of α2M by proteinases that subsequently become cross-linked to α2M via an internal thiolester. Because α2M also binds a broad spectrum of cytokines and growth factors, the LRP/α2M system might even function as a clearing or

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‡‡ The abbreviations used are: LDL(R), low density lipoprotein receptor; VLDL, very low density lipoprotein; LR7/8B, brain-specific LDLR relative with 7 or 8 ligand binding repeats; LRP, LDLR-related protein; RAP, receptor-associated protein; apo, apolipoprotein; apoER2, apoE receptor 2; αM, α2-macroglobulin, α2M*, activated α2M; GST, glutathione S-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MAP2, microtubuli-associated protein 2.
targeting mechanism for these cytokines (for review see Refs. 7 and 8). A recent report by Hughes et al. (9) linked α-M-mediated transport processes in the brain to Alzheimer’s disease by showing that α-M associates with β-amylloid peptide and prevents fibril formation.

The latest additions to the family of LDL receptor related proteins, apoER2 (10), and LR7/8B (11, 12) have in fact been shown to be differentially spliced products from the same gene. The structure of these proteins is reminiscent of the smaller members of the family, LDLR and VLDL receptor, in that they harbor only one cluster of either 3, 4, 7, or 8 type A repeats (11, 13). The most interesting feature of apoER2 and LR7/8B is their site of expression. Whereas human apoER2 is found in brain and placenta, LR7/8B in chicken is expressed in brain only. This is in sharp contrast to other members of the LDLR family, which are all expressed to a certain extent in the brain but most prominently in a variety of other organs and cells. Lately, considerable interest has focused on LDLR, LRP, and VLDL receptors in respect to their expression in the central nervous system, because these receptors bind apoE isoforms, which are thought to play a role in the development of late onset Alzheimer’s disease (for review see Ref. 14). Because apoER2 binds apoE-rich β-VLDL in vitro (13), this receptor was proposed to be involved in apoE-mediated transport processes in the brain.

Here we demonstrate that LR7/8B is a receptor for activated α-M; expression of LR7/8B at the cellular level is compared with that of LRP and receptor-associated protein (RAP). LR7/8B is expressed in the perikaryon of all neurons throughout the brain, with a marked specificity for proximal dendrites. This localization was confirmed in cultured primary rat neurones, where the receptor was found in the cell body and dendrites. In addition, there is significant expression in the arachnoid membrane and, most notably, on the apical aspect of the epithelium of the choroid plexus. These results strongly suggest that LR7/8B is involved in the clearance of α-M-proteinase complexes or other substrates that bind to α-M from the cerebrospinal fluid and in particular from the surface of neurons.

**MATERIALS AND METHODS**

**Antibodies**—The antibody against the carboxy-terminal 14 amino acids of LR7/8B was described previously (12). Another antibody was produced against the entire intracellular domain of LR7/8B, expressed as a glutathione S-transferase (GST) fusion protein as follows. The cDNA coding for the cytoplasmic domain of the receptor was generated by polymerase chain reaction from a single-stranded cDNA produced by Superscript Reverse Transcriptase (Life Technologies, Inc.) using brain poly(A)+ RNA and random hexamers. The primers used for the polymerase chain reaction were (1) 5'-CGGGATCCGCCGGAACACCAAAGC and (2) 5'-GGATATGATCAGGAGCCATACATC. The polymerase chain reaction product was cleaved with BamHI and EcoRI and cloned into the pGEX-2X vector (Amerham Pharmacia Biotech). Production of the GST fusion protein in DH5α cells was induced with isopropyl-1-thio-β-D-galactopyranoside for 5 h at 30 °C. Cells from 1 liter of culture were pelleted and washed with buffer A (50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.5). Cells were lysed in 20 ml of buffer A containing 1 mM dithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 0.2% Triton X-100 by sonication for 10 min on ice. After centrifugation at 35,000 × g for 30 min, the supernatant was incubated with 2 ml of GST-Sepharose at 4 °C for 30 min. Finally the matrix was packed into a column and washed with lysis buffer, and the fusion protein was eluted in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 25 mM glutathione. Fractions containing the pure protein were used for immunization of female New Zealand White rabbits (15). A polyclonal antibody against chicken LRP-515 was obtained by immunizing female New Zealand White rabbits with purified chicken LRP-515, obtained as follows. Chicken liver membrane extract was prepared as described (16) using CHAPS as detergent at a final concentration of 30 mM. The extract was diluted with three volumes of buffer A (50 mM Tris HCl, pH 8.0, 2 mM CaCl2, 16 mM CHAPS, 5 μM leupeptin, and 0.1 mg/ml phenylmethylsulfonyl fluoride). RAP-GST coupled to Sepharose 4B according to the manufacturer’s instructions (5 mg of RAP-GST/ml of wet gel) was added, and the suspension was rotated end over end at 4 °C for 20 h. The RAP-GST-Sepharose was packed into a column and washed with 25 column volumes of buffer A. Bound LRP was eluted in two column volumes of a linear gradient from 1 mM ammonium bicarbonate to 1 M ammonium bicarbonate containing buffer A. LRP-containing fractions were subjected to 4.5% polyacrylamide gel electrophoresis under nonreducing conditions. Bands were visualized by negative staining with 4 M sodium acetate and excised from the gel, and the protein was eluted in the Bio Trap separation system (Schleicher & Schuell). The polyclonal anti-peptide antibody against chicken RAP was described previously (17).

**Expression of Chicken LRSB, LR8, and LRP**—Chicken LRSB and LR8 were expressed in the human embryonic kidney cell line 293. The full-length cDNA of chicken LRSB was cloned into the Nhel and SalI sites of the eukaryotic expression vector pCIneo. The construct used for expression of LR8 in 293 cells has been described previously (18). Transfection of the cells was performed using Lipofectin reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Stable transformants were selected by the addition of 500 μg/ml G418 to the medium (Dulbecco’s modified Eagle’s medium; Life Technologies, Inc., 10% fetal calf serum, 584 mg/liter glutamine). Expression of LRP in CHO-K1 cells, a mutated CHO cell line lacking endogenous LRP, was described previously (19).

**Preparation of Membrane Detergent Extracts, Electrophoresis, and Western and Ligand Blotting**—Cellular membranes from total brain and liver from laying hens were prepared and extracted with 1% Triton X-100 as described previously for chicken ovarian follicles (20). Total cell extracts from 293 cells expressing LRSB were prepared as described for chicken embryo fibroblasts (21). Electrophoresis, transfer to nitrocellulose membranes, Western blotting, and ligand blotting with 125I-RAP were performed as described previously (20). Recombinant human RAP was produced as a GST fusion protein using a pGEX 2T-derived (Amerham Pharmacia Biotech) expression plasmid in DH5α bacteria (22). Rabbit β-VLDL was prepared as described in a previous publication (21).

**Binding and Internalization of RAP and α-M by Receptor-expressing Cells**—Recombinant RAP-GST was prepared and radiolabeled with 125I as described to a specific activity of 450 cpn/mg (20). α-M was isolated from chicken plasma as described previously (23). Native α-M was radiolabeled using Iodo-Gen precoated iodination tubes according to the manufacturer’s recommendation (Pierce) to specific activities of 300–400 cpn/mg. Labeled α-M-trypsin complexes (α-M*) were generated as described (23). Complete activation of labeled α-M by trypsin was monitored by native gel electrophoresis using the Tris/borate system described by van Leuven et al. (24).

**Binding and internalization of 125I-RAP-GST and 125I-α-M** by LRSB- and LR8-expressing 293 cells were measured according to the standard protocol for LDL (25). In brief, 293 cells were cultured in poly(tyline)-treated 3-cm dishes and grown to a confluence of 70%. LRP-deficient CHO-K1 cells and CHO-K1 cells expressing LRP were grown on 6-well plates and grown to 80% confluence. Three washes with PBS, cells were incubated with Dulbecco’s modified Eagle’s medium containing 2% bovine serum albumin, 584 mg/liter glutamine, and 125I-labeled ligand at the indicated concentrations for 3 h at 37 °C. After washing, the cells were lysed by adding 1 ml of 0.1 M NaOH and incubating at 23 °C for 10 min. The radioactivity in the lysate was determined with a γ counter (COBRA II, Packard), the protein concentration was determined, and values were expressed as cell-associated activity. These values represent the sum of bound and internalized ligand.

**Immunofluorescence**—Cells stably expressing LRSB were grown to a confluence of 50–70%. After washing twice with PBS at 23 °C, cells were fixed for 15 min using 3% paraformaldehyde in PBS, washed again, and permeabilized with PBS containing 0.2% Triton X-100 for 15 min. The permeabilized cells were washed again with PBS, incubated with the primary antibody at a dilution of 1:100 in PBS for 1 h, washed, and incubated with a Texas red-conjugated F(ab)2 fragment of goat anti-rabbit IgG (ACCUSPECS). After a final wash, microscopy was performed on a Zeiss Axioshot microscope with a Bio-Rad MRC-600 confocal equipment.

**Immunohistochemistry**—Chickens were anaesthetized with 2 ml of Nembutal and perfused via the left ventricle with 300 ml of PBS followed by 300 ml of a solution containing 75 mM t-lysine, 75 mM sodium phosphate, pH 7.8, 2% paraformaldehyde, and 2.4 mg/ml sodium meta-periodate. The brain was then removed, cut sagitally, and stored in the fixation solution for 4 h at 4 °C. Specimens were embedded in paraffin using a Tissue-Tek VIP (Miles Scientific) embedding ma...
chine, and 4 μm slices were cut on a Microm HM335E microtome. Slices were deparaffinized in xylene exchange medium XEM-200 (Vogel), rehydrated by consecutive washes in 96, 70, 50% ethanol and pure water. Endogenous peroxidase was blocked by incubating the slices in 3% H2O2 for 5 min. Unspecific binding of antibodies was inhibited by blocking slides with a solution of 1% milk powder and 3% total goat serum in PBS for 1 h at room temperature. Incubation with the primary antibodies (dilutions as indicated in the figure legends) was performed at 4°C for 20 h in blocking solution. After 5 washes in PBS, the following incubations were performed at 23°C: goat anti-rabbit biotinylated IgG (Sigma) diluted 1:500 in blocking solution for 1 h, five washes with PBS, peroxidase-labeled avidin (Sigma) for 1 h diluted 1:2000 in 0.1 M Tris pH 9.5, 100 mM NaCl, and 50 mM MgCl2. The slides were then washed five times with TBS. For the color reaction, slices were incubated in 0.1 M sodium acetate, pH 5.1, containing 150 μl of 30% peroxide and 20 mg of 3-amin-9-ethylcarbazole/100 ml of buffer. The staining process was followed under the microscope (Zeiss Axiosvert 135) and stopped by immersing the slides in water. Nuclei were counterstained with Harris modified hematoxylin solution (Sigma). To control for the results obtained by immunohistochemistry, the experiment was performed (i) without the primary antibody and without hematoxylin for nuclear counterstain and (ii) in the presence of an excess of the GST fusion protein, used as antigen to produce the anti-LR7/8B antibody.

In Situ Hybridization—Paraffin-embedded slices were prepared, deparaffinized with xylol, and rehydrated as described above. After post-fixation in 4% paraformaldehyde for 20 min, slices were washed three times in TBS and incubated at room temperature in the following solutions: 10 min in 0.2 M HCl, 3 washes with TBS, 0.5% acetic anhydride in 0.1 M Tris, pH 8.0, for 10 min, three washes with TBS, 20 μg/ml protease K and 2 mM CaCl2 in TBS at 37°C for 20 min, 3 washes in TBS, and finally consecutively in 30, 50, 75, and 96% ethanol. The slides were kept for 30 min in a humid chamber at 55°C. Digoxigenin-labeled RNA (antisense and sense) probes (Boehringer Mannheim digoxigenin RNA labeling kit) were added in 25-μl hybridization mix (10% 20× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 50% formamide, 10% dextran sulfate, 0.01% salmon sperm DNA, 0.02% SDS) and covered by a coverslip. The RNA was then denatured by heating to 94°C for 4 min and hybridized by incubating for 4 to 6 h at 65°C in a humid chamber. After hybridization, the coverslips were removed by immersing the slides for 1 h in 2× SSC. Stringent washes were performed by incubating the slides 3 times in a solution containing 50% formamide and 50% 1× SSC at 55°C, then twice for 15 min in 1× SSC at 23°C and finally once in TBS. After blocking of unspecific binding sites with a solution containing 0.5% blocking reagent (Boehringer Mannheim) and 10% fetal calf serum in TBS, the hybridized probe was detected by incubating the slide for 60 min with an affinity-purified anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:500 in the blocking solution. Slides were then washed five times with TBS. As a color substrate, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used (45 μl of a solution containing 100 mg of 4-nitro blue tetrazolium chloride/ml of 70% dimethylformamide and 35 μl of a solution containing 50 mg of 5-bromo-4-chloro-3-indolylphosphate/ml of dimethylformamide with 1% H2O2 added to 10 ml of a buffer containing 100 mM Tris, pH 9.5, 100 mM NaCl, and 50 mM MgCl2). The reaction was performed at 4°C for up to 3 days. For nuclear stain see “immunohistochemistry.”

Culture of Primary Rat Neurons and Laser Scanning Confocal Microscopy—Hippocampal neurons and astroglia were prepared from brains of 18-20-day-old rats according to Bartlett and Banker (26). Neurons grown on coverslips were cocultured facing glia cells cultured on microscope slides, separated by a 1-mm spacer for 11 to 18 days in serum-free medium (minimum essential medium supplemented with 1 g/liter ovalbumin, 0.6% glucose, 50 mg/liter insulin, 63 g/liter d-glucoses, 161 mg/liter putrescine, 100 mg/liter transferrin, and 33 g/liter selenium dioxide). Cells were fixed with 1% formaldehyde, rinsed, and dehydrated with acetone. For immunocytochemistry, anti-LR7/8B antibody and a monoclonal anti-MAP2 antibody (generously provided by Stefanie Kaech from the Friedrich Miescher Institute, Basel, Switzerland) were used. Localization of primary antibodies were visualized by biotin-labeled anti-rabbit IgG antibody (Amersham Pharmacia Biotech) followed by avidin-horseradish peroxidase for 3,3-diaminobenzidine tetrahydrochloride staining technique or by fluorescein isothiocyanate-labeled anti-rabbit and tetramethylrhodamine isothiocyanate-labeled anti-rabbit IgG antibodies for fluorescent microscopic visualization. Fluorescent preparations were examined using a Carl Zeiss laser scan microscope equipped with an argon laser (488- and 514-nm excitation) and two HeNe lasers (543 and 633 excitation). The tetramethylrhodamine isothiocyanate fluorescence was excited with the 543-nm laser. The emitted light was detected on photomultiplier 2 with a 557-640 band-pass filter. Fluorescein isothiocyanate was excited with the 488-nm laser and was detected on photomultiplier 3 with a 515-565 band-pass filter. Scanning with the 543 and 488 laser was performed sequentially. In this mode 3 sections (with a distance of 1–1.2 μm) in the Z direction were made with the 543 and 488 laser, respectively. Subsequently, sections made with the 543 and 488 laser lying in the same Z plane were merged to produce a single picture.

RESULTS

To characterize potential function(s) of LR7/8B, we stably expressed one of the splice variants harboring 8 ligand binding repeats (LR8B) in 293 cells. Northern and Western blot analyses show strong expression of LR8B in these cells (Figs. 1, A and B). Confocal microscopical studies of immunofluorescence stained 293 cells expressing the receptor demonstrated the presence of LR8B on the cell surface and in vesicular structures within the cell (Fig. 1C). To functionally assess the competence of the recombinant receptor to interact with ligands, we measured LR8B-mediated binding and internalization of RAP, which has recently been shown to bind to native LR7/8B (12). Triton X-100 extracts of LR8B-expressing cells were subjected to ligand blot experiments with 125I-labeled RAP, and as seen in Fig. 2A, LR8B expressed in 293 cells indeed binds RAP (lane 2), demonstrating binding competence of the recombinant receptor. The binding of 125I-RAP can be competed for with an excess of unlabeled RAP but not with rabbit β-VLDL, which

FIG. 1. Expression of LR8B in 293 cells. A, total RNA was isolated from pCIneo mock-transfected and LR8B-transfected 293 cells (lanes 1 and 2 respectively, 20 μg of RNA/lane), denatured, and separated on a 1.5% agarose gel. Hybridization was performed with 32P-labeled full-length DNA probe for LR8B. B, Triton X-100 membrane extracts from mock-transfected and LR8B-expressing 293 cells were prepared as described under “Materials and Methods” and subjected to electrophoresis under nonreducing conditions on a 4.5–18% SDS-polyacrylamide gel. Proteins were blotted overnight to a nitrocellulose membrane. Lane 1, 0.3 μg of 293-pCIneo mock extract; lane 2, 0.3 μg of 293-LR8B extract. The blot was incubated with anti LR7/8B (20 μg/ml), and bound IgG was visualized with protein A-horseradish peroxidase (1 μg/ml) and a chemiluminescence system. C, 293 cells stably expressing LR8B were processed for immunofluorescence as described under “Materials and Methods” using anti-LR7/8B antibody at a dilution of 1:100 and a Texas red-conjugated Fab1 fragment of goat anti-rabbit IgG. Cells were viewed with a Zeiss Axioshot microscope with a Bio-Rad MRC-600 confocal equipment. kb, kilobases.
has been shown to bind to human apoER2 (13). In this particular ligand blot experiment, \(\beta\)-VLDL appears to enhance RAP binding; this effect, however, was not seen in cell binding experiments.

We used these cells to study RAP binding and internalization. Fig. 2B shows the results of a representative experiment comparing LR8B- and LR8-mediated cell association at a RAP concentration of 1 \(\mu\)g/ml. This concentration is close to the \(K_d\) of RAP binding to LR8B, which was determined in a separate experiment (data not shown). Cells expressing LR8, which is the chicken homologue of the mammalian VLDL receptor, were chosen as positive control because we have recently established RAP as ligand for this receptor (20). Mock-transfected cells bind and internalize, possibly via low levels of endogenously expressed LR8. LR8-mediated RAP binding and uptake amounts to 82 ng of RAP/mg of cell protein/h.

The fact that \(\alpha_2M\) not only binds to LRP but also to LR8, which mediates vitellogenesis in egg-laying species and harbors eight ligand binding repeats just like LR8B (23), prompted us to test the possibility that LR8B might be a receptor for \(\alpha_2M\) (\(\alpha_2\)-M* in brain. As shown in Fig. 3A, LR8B-expressing cells bind and internalize \(\alpha_2M^*\) with a \(K_d\) of 76 nm. This binding is specific because it is reduced to background levels by a 40-fold molar excess of RAP. Mock-transfected 293 cells bind small amounts (6 times less than LR8B-expressing cells) of \(\alpha_2M^*\) with a \(K_d\) of 41 nm. This binding is also inhibited by RAP and is most likely mediated by low endogenous levels of LRP in these cells (see above, RAP binding). Most significantly, as evaluated with a mutant CHO cell line expressing recombinant chicken LRP (19), the binding affinities of \(\alpha_2M^*\) for LR8B and LRP, the prototype \(\alpha_2M\) receptor, are similar (76 versus 30 nm) (Fig. 3B). Specific binding of \(\alpha_2M^*\) to mock-transfected cells was absent, because CHO-K1 do not express endogenous LRP (19).

Having established that LR8B functions as an additional receptor for \(\alpha_2M\), we studied the differential expression of both receptors in the brain at the cellular level by cytobhistochemistry and in situ hybridization. We prepared antibodies against the entire cytosolic domain of chicken LR7/8B (12) produced in Escherichia coli as GST fusion protein. To compare expression of LR7/8B with the expression of LRP, an antibody against purified chicken LRP-515 was prepared. Fig. 4 shows a Western blot characterizing both new antibodies. As expected, anti-LR7/8B detects the antigen in a crude chicken brain membrane extract (lane 3); as control we used recombinant LR8B expressed in 293 cells (lane 1). Most importantly, this antibody does not cross-react with LRP in brain (lane 5) and liver (lane 4). Anti-LRP also fulfills the criteria needed in this study, in that it is specific for LRP and does not cross-react with LR7/8B, as demonstrated with transfected 293 cells (lane 7, 10 times as much protein loaded as in lane 1).

Fig. 5 displays an overview of sites and cells in chicken brain expressing LR7/8B. Representative for all regions of the brain, large neurons of the brain stem are prominently stained by anti-LR7/8B (Fig. 5A). Inspection of other regions of the brain revealed a hierarchy among neurons based on the expression of...
and extracts from 293 cells were prepared as described under "Materials and Methods" and subjected to electrophoresis under nonreducing conditions on a 4.5–18% SDS-polyacrylamide gel. Proteins were blotted overnight to a nitrocellulose membrane. Lane 1, 0.3 μg of 293-LR8B extract; lane 2, 3 μg of 293-pCIneo mock extract; lanes 3 and 6, 50 μg of chicken brain membrane extract; lanes 4 and 5, 50 μg of rooster liver membrane extract; lane 7, 3 μg of 293-LR88 extract. Lanes 1–4 were incubated with anti LR7/8B (20 μg/ml), and lanes 5–7 were incubated with anti LRP515 (20 μg/ml). Bound IgG was visualized with protein A-horseradish peroxidase (1 μg/ml) and a chemiluminescence system.

FIG. 4. Analysis of specificity of anti-LR7/8B and anti-LRP515 antibodies. Triton X-100 membrane extracts from liver and total brain and extracts from 293 cells were prepared as described under "Materials and Methods" and subjected to electrophoresis under nonreducing conditions on a 4.5–18% SDS-polyacrylamide gel. Proteins were blotted overnight to a nitrocellulose membrane. Lane 1, 0.3 μg of 293-LR8B extract; lane 2, 3 μg of 293-pCIneo mock extract; lanes 3 and 6, 50 μg of chicken brain membrane extract; lanes 4 and 5, 50 μg of rooster liver membrane extract; lane 7, 3 μg of 293-LR88 extract. Lanes 1–4 were incubated with anti LR7/8B (20 μg/ml), and lanes 5–7 were incubated with anti LRP515 (20 μg/ml). Bound IgG was visualized with protein A-horseradish peroxidase (1 μg/ml) and a chemiluminescence system.

the receptor. Large neurons express significantly more of the receptor than small neurons. Glial cells, however, do not show any detectable expression. Another site of massive expression of LR7/8B is the epithelium of the choroid plexus (Fig. 5B). This cuboidal cell layer surrounds a capillary network producing most of the cerebrospinal fluid. Because the ependymal layer of the ventricles shows only very little or no staining, there is a clear demarcation between these cells and the epithelium of the choroid plexus marked by the expression of LR7/8B (arrow in Fig. 5B). However, along the lateral wall and especially on the bottom of the third ventricle some ependymal cells were stained (arrow in Fig. 5C). Another cell layer in close contact with the cerebrospinal fluid is the arachnoid membrane, which together with the pia mater and dura mater forms the three meningeal layers covering the brain. In addition to the wall of capillaries present in the subarachnoid space, strong immunoreactivity can be seen in the arachnoid membrane (Fig. 5D). The staining of the vessel wall seen in Fig. 5D is not specific for the subarachnoid space but is present in all blood vessels in the brain (Fig. 5E). Both the endothelium and the media of the blood vessels express LR7/8B. The major site of expression in the cerebellum are the Purkinje cells, which are arranged side by side in a single layer separating the molecular layer from the granular layer (Fig. 5F). As already clearly visible at this magnification, the staining extends into the proximal dendrites of the Purkinje cells, which continue into the molecular layer of the cerebellum. To control for the results presented in Fig. 5, we used the cerebellum again and performed the same experiment without the primary antibody and without hematoxylin for nuclear stain. As seen in Fig. 5G, staining of the Purkinje cells depends upon the presence of anti-LR7/8B antibody. When the immunostaining procedure was carried out in the presence of an excess of the GST fusion protein used for the production of the antibody, staining at all sites of expression was strongly reduced (data not shown). Furthermore, in situ hybridization experiments using antisense (Fig. 5H) and sense (Fig. 5I) RNA probes specific for LR7/8B confirm the expression data obtained by cytohistochemistry.

Next, we compared the expression of LR7/8B with that of LRP and RAP. The most significant differences in the expression of these proteins are shown in Fig. 6 and relate to large neurons, Purkinje cells, and the choroid plexus. As seen in Fig. 6A.1 and A.2, anti LR7/8B antibody produces a distinct punctate signal close to the cell membrane in large neurons of the brain stem and Purkinje cells, which is compatible with the staining of endocytic vesicles. Significantly, the signal obtained with anti-LRP antibody in the same area of the brain stem as seen in Fig. 6A.1 is clearly different from that obtained with anti-LR7/8B. It outlines the cell membrane of the neurons without exhibiting a punctate staining within the cells (Fig. 6B.1). Most significantly, areas surrounding the cell bodies of the neurons (arrows) exhibit prominent staining. RAP however, which is an ER-resident protein and interacts with both receptors (12, 27), is expressed in all neurons and their dendrites (Fig. 6C.1).

As mentioned above, anti-LR7/8B antibody also stains dendrites extending from the Purkinje cells into the stratum moleculare of the cerebellum (Fig. 6A.2). In sharp contrast to RAP (Fig. 6C.2), which is expressed throughout the dendritic trees of the Purkinje cells, expression of LR7/8B seems to be restricted to the proximal part of the dendrites. The molecular layer and the granular layer do not show any significant expression of LR7/8B. Again, the anti-LRP antibody produces a completely different pattern in the cerebellum (Fig. 6B.2). Expression of LRP within the Purkinje cells is much weaker than that of LR7/8B and RAP. The most intense staining with anti-LRP antibody can be seen in distinct areas of the granular layer. As evidenced by the nuclear staining with hematoxylin, these areas correspond to cerebellar glomeruli, acellular spaces within the granular layer, which is densely packed with small neurons (granule cells). Within glomeruli, these cells form complex synaptic contacts with the bulbous expansions offferent fibers. Together with the intense staining surrounding large neurons, this pattern suggests that LRP is strongly expressed in synaptic regions of the brain.

Finally, we have studied the expression of LR7/8B, LRP, and RAP in the choroid plexus. As seen in Fig. 6A.3, expression of LR7/8B is restricted to the apical side of the epithelial cells. In sharp contrast, LRP is also found on the baso-lateral side of these cells (Fig. 6B.3), whereas RAP is uniformly distributed in the epithelial cells of the choroid plexus (Fig. 6C.3).

To confirm the neuronal and dendritic staining seen in the cytohistochemical studies with anti-LR7/8B, we cultured primary rat hippocampal neurons (26) and stained these cells with anti-LR7/8B. Neurons cultured for 18 days, which are characterized by fully developed dendrites, show an intense staining of the cell body and dendrites (Fig. 7A). Obviously, the signal elicited by the antibody extends to the very ends of the dendrites without loosing intensity, suggesting a uniform expression of the receptor over the whole length of the outgrowing dendrites. This staining is specific, because control experiments without the first antibody completely abolished the signal (Fig. 7B). LR7/8B is already expressed to a similar extent in the cell body and outgrowing dendrites at day 11 when dendrites become first visible (data not shown). Double immunostaining of these cells with anti-LR7/8B and anti-MAP 2, a dendritic marker (28), in conjunction with confocal microscopy revealed that both proteins are indeed colocalized in dendrites (Fig. 7C). Moreover, accentuation of immunoreactivity on the surface of dendrites (Fig. 7A) indicates expression on the cell surface.

Taken together, these results demonstrate a novel α2-M receptor with a distinct expression pattern different from that of LRP.

DISCUSSION

Chicken LR7/8B is the member of the LDL receptor gene family with the most restricted expression known. Although in
mammals it seems to be present in brain, testis, ovary, and placenta (10, 13), in the female bird LR7/8B is detectable only in the brain (12). To evaluate the function(s) of this novel receptor, we examined its expression at the cellular level in distinct regions of chicken brain. Expression of LR7/8B can be allocated to two distinct types of cells: first, to large neurons.

**Fig. 5.** Immunohistochemical analysis of LR7/8B expression in chicken brain. Immunohistochemistry (A to G) was performed on sagittal sections of a brain from a laying hen as described under “Material and Methods” using anti-LR7/8B antibody (expressed LR7/8B appears in red). Nuclei are counterstained with hematoxylin (blue). A, motoneurons of the brain stem. B, choroid plexus and its connection to the ependymal layer (arrow). C, third ventricle (v) with some reactive cells (arrow) in the ependymal layer (el). D, arachnoidal membrane (am) and blood vessels (arrow). E, penetrating blood vessel (arrow) in the mid-brain. F, cerebellum with granular layer (gl), molecular layer (ml) and Purkinje cells. G, cerebellum without primary antibody and without hematoxylin for nuclear counterstain. *In situ* hybridization with a digoxigenin-labeled full-length RNA probe for LR8B (H, antisense; I, sense; cerebellum with granular layer (gl), molecular layer (ml), and Purkinje cells (arrow)).
Immunohistochemical analysis of LR7/8B, LRP, and RAP expression in chicken brain. Immunohistochemistry was performed on sagittal sections of a brain from a laying hen as described under “Material and Methods” using anti-LR7/8B antibody (A.1 to A.3), anti-LRP antibody (B.1 to B.3), and anti-RAP antibody (C.1 to C.3). Expressed proteins appear in red. Nuclei are counterstained with hematoxylin (blue). A.1, motoneuron of the brain stem with typical granular staining (arrow). A.2, Purkinje cell with staining of the proximal dendrites (arrow). A.3, choroid plexus with characteristic apical staining of epithelial cells. B.1, motoneuron of the brain stem, with typical patchy staining around cell bodies of the neurons (black arrow). B.2, Purkinje cells and characteristic staining of the glomeruli (arrow). B.3, choroid plexus with nonpolar staining of the epithelial layer. C.1, motoneuron of the brain stem; C.2, Purkinje cells with proximal and distal dendrites stained. C.3, choroid plexus with nonpolar staining of the epithelial layer.
throughout the brain including Purkinje cells of the cerebellum, and second, to cells constituting brain barriers such as endothelial cells of blood vessels, cells of the arachnoid membrane, and epithelial cells of the choroid plexus.

The pattern of the immunoreactivity in large neurons and Purkinje cells points to the presence of the receptor on the cell surface of the perikaryon and proximal dendrites as well as prominently in vesicles close to the cell membrane. These results, corroborated by analysis of brain sections, correspond well with the expression pattern of LR8B in transfected 293 cells, and most importantly, with results obtained in cultured primary rat neurons, where the protein can be seen on the cell body and throughout the dendritic tree. The granular localization close to the plasma membrane is compatible with a high endocytosis rate of the receptor, as most of it might be visible on the way from coated vesicles to endosomes and back to the cell membrane. Binding and internalization experiments with $\alpha_2$M* in LR8B-expressing 293 cells clearly show that at least one splice variant of LR7/8B, i.e. LR8B, is a receptor for $\alpha_2$M*. Direct comparison of $\alpha_2$M* binding to LRP and LR8B demonstrates that the binding kinetics of both systems are very similar.

Having established LR8B as a novel $\alpha_2$M* receptor, it was interesting to compare the expression pattern of LR7/8B with that of LRP and RAP in the brain. Immunostaining with anti-LRP is significantly different from that obtained with anti-LR7/8B. Throughout the brain, LRP is most prominently located in patchy areas around the perikaryon of large neurons. In comparison with LR7/8B and RAP, which is an ER resident protein, weaker staining of the cell bodies is evident for LRP. The staining pattern for LRP in the cerebellum shows some expression of LRP in the cell bodies of Purkinje cells but strong reaction of the cerebellar glomeruli in the granular layer. These structures are filled with synapses, where dendrites of the small granule cells, which are virtually devoid of any significant expression of LR7/8B, make intense contacts with afferent (mosaic) fibers. This finding is in agreement with a previous study of LRP transcripts in the brain, where it was shown that LRP mRNA is prominently present in the granule cells of the granular layer (29). Taken together, our results suggest that in contrast to LR7/8B, which is strongly expressed only in the perikaryon and dendrites of large neurons, LRP is also concentrated in synaptic areas likely translocated via axonal transport into synaptic terminals. However, as outlined above, LRP mRNA has also been found in the somata of cerebellar granular cells (29), from where the protein may be transported into their dendrites, located in cerebellar glomeruli. Using cultured primary hippocampal neurons from the rat, LRP expression was restricted to a somatodendritic domain, which would be compatible with a synaptic sorting of LRP through the dendrites (30). In both situations, sorting of LRP to synaptic regions most likely occurs at the protein rather than at the mRNA level. Additional immune electron microscopic studies are required to clarify axonal versus dendritic localization of LRP.

However, the situation in human brain might be different, because immunohistochemical localization of LRP in pyramidal neurons of the cerebral cortex gave intense staining of the neuronal cell bodies and proximal processes without obvious staining of the patchy areas around neurons, as found here (31, 32). Unfortunately, no data about LRP localization in the cerebellum, where the synaptic staining with anti-LRP in chicken brain is particularly evident, was presented in these reports.

The other feature of LR7/8B expression which warrants detailed inspection is its location in barriers that define the extracellular spaces of the brain and the blood-brain barrier. The most striking features of these barriers are tight junctions between the cells and the absence of fenestrations and/or channels. These tight junctions are not permeable even for small water-soluble substances, thus creating a distinct compartment filled with cerebrospinal fluid, which is metabolically set apart from the rest of the organism. Because the cerebrospinal fluid is a complex mixture of nutrients and other essential compounds, these endothelial and epithelial cells not only constitute a permeability barrier but must also produce and maintain the composition of this fluid. This is achieved by highly specific carrier-mediated transport processes which are known for hexoses, amino acids, vitamins, and ions, to name a few. Most of the cerebrospinal fluid is secreted by the choroid plexus. The presence of LR7/8B throughout the cells constituting the brain barrier systems is intriguing. In this respect it is interesting that a site of significant expression of the receptor in mammals is the placenta, which metabolically separates the fetal and the maternal circulations. Thus, the assumption that LR7/8B constitutes a transport system for $\alpha_2$M-proteinase complexes or complexes with other substances binding to $\alpha_2$M is intriguing.
Preliminary ligand binding experiments with apoER2, which is the human homologue of LR7/8B, suggest that it binds apoE (10). The mammalian brain itself produces significant amounts of apoE, which constitutes the major apolipoprotein of the cerebrospinal fluid (37). As receptors of proteinase inhibitors. Other interesting points are the presence of proteinase B in synaptic plasticity (43), and the balance between proteinases may play a role in the absence of apoE, a proposed ligand for both receptors (34, 35); moreover, birds appear not to produce apoE (36) but as shown here, express high amounts of the respective proteinase B (31). Nevertheless, in view of the fact that amyloid-β-amyloid peptide, and is also present in chicken (23). Moreover, αM is present in the brain and in particular in the cerebrospinal fluid (37). Prevalent sites of αM production in the brain appear to be the choroid plexus (38) and astrocytes (39).

Close inspection of the immunostaining of LR7/8B in the epithelial cells of the choroid plexus (Fig. 6A,3) clearly shows apical expression of the receptor. This is in sharp contrast to the expression of LRP and RAP, both of which are not expressed in a polarized fashion in these cells. The strong expression of LRP in the chicken choroid plexus is interesting, because high levels of expression at this site were also found in rat (40) but surprisingly not in man (31). Nevertheless, in addition to the baso-lateral localization, apical expression was also shown for the LDL receptor in Madin-Darby canine kidney cells (41). Although biochemically identical, LDL receptors expressed on the apical side transport their ligands to the basolateral side, whereas receptors on the basal surface endocytose their ligands in a classical way. The strictly apical expression of LR7/8B in the epithelial cells of the choroid plexus and the endothelial layer of the arachnoid might therefore point to a function of “reverse transport” or cleansing of the cerebrospinal fluid, probably by transcytosis. Such a pathway was shown for removal of organic anions from the cerebrospinal fluid. The corresponding transporter ( oatp) is also localized to the apical surface of the choroid plexus epithelial cells (42). In such a scenario, LR7/8B would remove αM-proteinase complexes from the cerebrospinal fluid or from the surface of neurons. This latter aspect is very interesting, because proteinases may play a role in synaptic plasticity (43), and the balance between proteolytic activity and its inhibition might be controlled by proteinase inhibitors. Other interesting points are the presence of αM in neuritic plaques of Alzheimer’s patients (44, 45) and the fact that amyloid β-protein can be degraded by a serine proteinase αM complex (46). Using a yeast two-hybrid system to isolate cDNAs encoding for proteins that associate with β-amyloid peptide, αM was identified (9). These results strongly link αM-mediated processes and receptors like LRP and LR7/8B to Alzheimer’s disease. However, in view of the broad ligand spectrum of LRP (4), it is conceivable that LR7/8B is a multi-ligand receptor with potentially different functions in different cell types and species.

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