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Short Communication

Characterization and Immunohistochemical Localization of α_2-Macroglobulin Receptor (Low-density Lipoprotein Receptor-related Protein) in Human Brain

Beni B. Wolf,* M. Beatriz S. Lopes,† Scott R. VandenBerg,† and Steven L. Gonias*†

From the Departments of Biochemistry and Pathology,† University of Virginia Health Sciences Center, Charlottesville, Virginia*

Proteinase inhibitors have been implicated in brain development and in degenerative processes such as Alzheimer's disease. Low-density lipoprotein receptor-related protein (LRP) is a multifunctional cell-surface receptor that binds activated forms of the proteinase inhibitor, α_2-macroglobulin (α_2M) and apolipoprotein E. Solubilized plasma membranes of human cerebral cortical gray matter were subjected to affinity chromatography on α_2M-methylamine-sepharose. A single receptor was purified; this protein was LRP as determined by molecular mass, peptide structure, and immunoreactivity with monoclonal and polyclonal antibodies. In adult human brain, LRP immunoreactivity was abundant on neuronal cell bodies and proximal processes. Other cells within the neuropil, including glia and microvascular cells (endothelium and pericytes), were immunonegative. Weak LRP immunoreactivity was identified in a perivascular pattern corresponding to the location of astrocytic foot processes. The distribution of LRP in the central nervous system is consistent with the potential function of this receptor in the regulation of proteinase activity, cytokine activity, and cholesterol metabolism. (Am J Pathol 1992, 141: 37–42)

α_2-Macroglobulin (α_2M) is a homotetrameric glycoprotein ($M_r \sim 718{,}000$) and an inhibitor of proteinases from all four major classes.[1] When α_2M reacts with proteinase, the inhibitor undergoes a major conformational change that has been termed "proteinase trapping."[2] Conformational change in α_2M exposes a specific domain at the C-terminus of each subunit that binds to cellular receptors.[3,4] The exposure of receptor-recognition domains in α_2M is referred to as activation. The structure of each α_2M subunit includes a single β-cysteinyl-γ-glutamyl thiol ester bond; direct aminolysis of the thiol esters with methylamine causes α_2M activation equivalently to proteinases.[1]

The α_2M receptor was purified from rat liver and human placenta.[5–7] By limited-sequence analysis, the receptor was identified as low-density lipoprotein receptor-related protein (LRP),[8,9] a membrane component that bears structural similarities to the low-density lipoprotein (LDL) receptor.[10] LRP was cloned and sequenced by Herz et al.[10] This multifunctional receptor is synthesized as a 600-kda precursor and modified intracellularly into the two-chain form.[11] The 85-kda subunit includes a transmembrane domain whereas the 515-kda subunit includes binding sites for activated α_2M and apolipoprotein E (apo E) enriched chylomicron remnants. Lipopro-

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Address reprint requests to Dr. Steven L. Gonias, Departments of Pathology and Biochemistry, University of Virginia Health Sciences Center, Box 214, Charlottesville, VA 22908.

tein lipase is another ligand for LRP; binding of lipoprotein lipase to LRP causes LRP to bind increased amounts of apo-E containing lipoproteins.[12] A 39-kda protein associates with LRP at the cell surface and usually copurifies with LRP. This factor modulates binding of ligands to the 515-kda subunit.[13–15]

Herz et al[10] identified mRNA for LRP in a number of organs including the brain. The same group also identified a mouse brain plasma membrane factor that reacted with an antiserum raised against the carboxyl-terminal 13 amino acids of LRP. Apo E is synthesized in the brain by astrocytes,[16,17] suggesting a possible role for LRP in cholesterol metabolism in this organ. Whether LRP or any other macromolecule represents a functional α_2M receptor in the central nervous system has not been addressed.

Materials and Methods

Materials

α_2M was purified from human plasma as previously described.[18] Immobilon P membranes were from Millipore. Monoclonal antibody 8G1 directed against the LRP heavy chain and polyclonal antibody R777 directed against the intact receptor were provided by Dr. Dudley Strickland, American Red Cross, Rockville, MD. Monoclonal antibody SMI-33, directed against neurofilament epitope, NF-H/M (phosphorylation independent), was from Sternberger, Inc., Baltimore, MD. Polyclonal antibody against glial fibrillary acidic protein (GFAP) was from the Dako Corporation, Carpinteria, CA. Peroxidase-conjugated antibodies specific for mouse and rabbit IgG were from Sigma, St. Louis, MO. Avidin-biotin immunoperoxidase reagents were from Vectastain, Burlingame, CA.

Preparation of Plasma Membranes

Plasma membranes were prepared from the gray matter of human cerebral cortex by the method of Hubbard et al.[19] or by the method of Fleischer and Kervina.[20] Equivalent results were obtained with either preparation. Plasma membranes were prepared from whole human liver by the method of Fleischer and Kervina.[20] Protein content was estimated by the Amidoschwartz dye technique.[21]

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

Plasma membranes and purified proteins were subjected to SDS-PAGE using a Hepes-imidazole, pH 7.4, buffer system as previously described.[22] Resolved components were electrotransferred to Immobilon-P membranes. The membranes were blocked with 5.0% low-fat milk and incubated with primary antibody (diluted 1:1,000) in 20 mmol/l Tris-HCl, 150 mmol/l NaCl, 0.1% Tween 20, 2.0 mmol/l $CaCl_2$, pH 7.5. After washing, the membranes were incubated with peroxidase-conjugated secondary antibody (1:1,000) and then with 3-3′-diaminobenzidine tetrahydrochloride (DAB) (0.3 mg/ml) in 50 mmol/l Tris-HCl, pH 7.2, and 0.01% H_2O_2.

Affinity Chromatography

Cerebral cortex plasma membrane preparations were analyzed by affinity chromatography, adapting methods previously applied with other tissues.[5–7] To form the affinity matrix, α_2M-methylamine was covalently coupled to sepharose CL-4B. The membranes were solubilized in 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonic acid (CHAPS) (1.0%) and subjected to chromatography in 20 mmol/l Tris-HCl, 150 mmol/l NaCl, 0.6 mmol/l $CaCl_2$, 1.0% CHAPS, pH 7.8. The elution buffer included 2.0 mmol/l ethylenediamine tetraacetic acid (EDTA) instead of $CaCl_2$ (pH 6.0). EDTA reverses the binding of activated α_2M to purified LRP and to all cell types examined to date.[1,5–7] For some experiments, native α_2M was coupled to sepharose CL-4B instead of α_2M-methylamine. Solubilized plasma membranes were then subjected to chromatography in an equivalent manner.

Immunohistochemistry

Normal adult brain obtained 4 hours after death and surgical specimens of temporal cortex from patients with epilepsy were analyzed. Ten percent formalin-fixed, paraffin-embedded sections (6 μm) were deparaffinized in xylene for 10 minutes and rehydrated through graded ethanols. After suppressing endogenous peroxidase activity with H_2O_2 (0.5% in methanol for 30 min, 22°C), tissue sections were treated with primary antibodies for 15–18 hours at 4°C. The primary antibodies were detected using the avidin-biotin immunoperoxidase technique.[23] The chromogen was DAB, DAB and $NiCl_2$, or DAB-intensified with 0.2% osmium tetroxide. Surgical specimens of liver served as the positive control. Normal nonimmune serum was substituted for primary antibody as a negative control. Slides were counterstained with hematoxylin or light green. The primary antibodies were: 1) 8G1 (1:1,000); 2) SMI-33 (1:1,000); 3) anti-GFAP (1:1,400).

Results

The cerebral cortex plasma membrane preparation was analyzed by Western blotting. A single prominent high-

molecular weight band was demonstrated with monoclonal antibody 8G1 suggesting the presence of the 515-kda subunit of LRP (Figure 1A). A band with comparable mobility was detected in plasma membranes from whole human liver (Figure 1B). Polyclonal antibody R777 detected the heavy chain of LRP in the cerebral cortex membranes together with the 39-kda receptor-associated protein (Figure 1C). An 85-kda band was identified in some immunoblots but always faint. Small amounts of immunoreactive material near 130 kda were also identified in cerebral cortex and liver preparations with monoclonal and polyclonal antibodies (Figure 1A–C). This band probably represents a digestion product of the LRP heavy chain.

To identify receptor(s) that bind activated α_2M in the cerebral cortex plasma membrane preparation, affinity chromatography was performed using α_2M-methylamine coupled to sepharose CL-4B. A single peak of protein eluted from the column after application of EDTA. By SDS-PAGE, two major bands with apparent molecular masses of 500 kda and 85 kda were demonstrated, consistent with the reported mass of the LRP subunits. The 39-kda protein was demonstrated in separate gels (not shown). From a single preparation with 45 mg of plasma membrane protein, the total yield of receptor and associated protein in the EDTA eluent was 320 μg. The 500-kda band in the affinity chromatography-purified preparation bound monoclonal antibody 8G1, confirming the identity of this peptide as the LRP heavy chain.

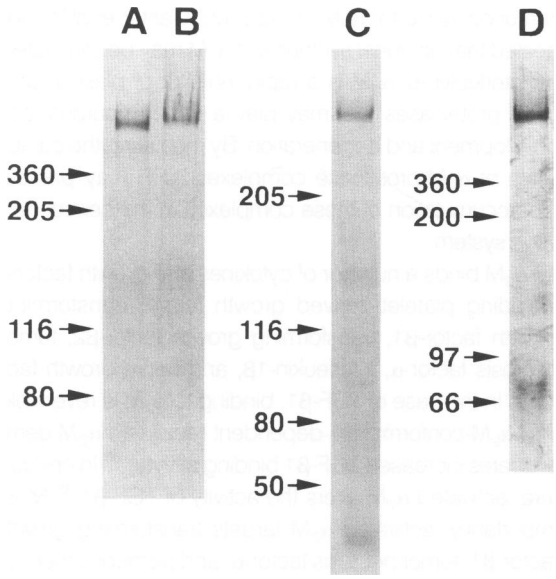

Figure 1. *SDS-PAGE and Western blotting of cerebral cortex plasma membranes and purified LRP.* **A**: *Immunoblot of cerebral cortex plasma membranes using monoclonal antibody 8G1.* **B**: *Immunoblot of whole human liver plasma membranes using monoclonal antibody 8G1.* **C**: *Immunoblot of cerebral cortex plasma membranes using polyclonal antibody R777.* **D**: *Silver-stained polyacrylamide gel of protein purified from cerebral cortex plasma membranes by affinity chromatography.*

To demonstrate that LRP in the cerebral cortex plasma membrane preparation specifically binds activated α_2M, chromatography was performed using native (nonactivated) α_2M coupled to sepharose CL-4B. The yield of receptor (μg per mg of plasma membrane) was decreased by greater than 95%.

The immunoblotting and affinity chromatography experiments demonstrate that 1) LRP is expressed in the brain; 2) LRP isolated from the brain is a functional receptor for activated α_2M, and 3) LRP is the only major receptor for activated α_2M in cerebral cortex gray matter.

Human temporal cortex, frontal cortex, thalamus, and cerebellum were examined by immunohistochemical analysis using monoclonal antibody 8G1. Intense immunoreactivity was identified in the pyramidal neurons of the cerebral cortex, thalamus, and dentate nucleus (Figure 2A–D). Staining was restricted to neuronal cell bodies and proximal processes. Myelinated axonal processes in the adjacent white matter were immunonegative. When normal nonimmune serum was substituted for the primary antibody, staining was not detected in any section.

Glial cell bodies that were intermixed with the 8G1-immunopositive neurons did not bind detectable levels of 8G1. Choroid plexus, ependyma, endothelium, and vascular smooth muscle were also immunonegative. Focal weak immunopositivity was identified in a perivascular distribution corresponding to the location of astrocytic foot processes as demonstrated by glial fibrillary acidic protein immunoreactivity (Figure 2E,F). In a previous study, Choi et al[24] demonstrated that the proteinase inhibitor, protease nexin-1, is also present in the astrocytic foot processes that form a physiologic component of the blood-brain barrier. At this site, LRP and protease nexin-1 may function to regulate the activity of extravasated proteinases. Boyles et al[16] demonstrated dense apo-E immunoreactivity in the region of the astrocytic foot processes. The foot processes have also been implicated in the regulation of cytokine activity and the immune response in the central nervous system.[25,26]

Discussion

The studies presented here demonstrated significant levels of LRP in the human central nervous system, where it is the only major membrane-associated protein that binds activated α_2M. The distribution of LRP antigen in the brain, as demonstrated by immunohistochemical analysis was highly restricted; antigen was limited to the gray matter and primarily associated with neuronal cell populations.

One potential ligand for LRP in the central nervous system is apo E. In addition to its primary role in choles-

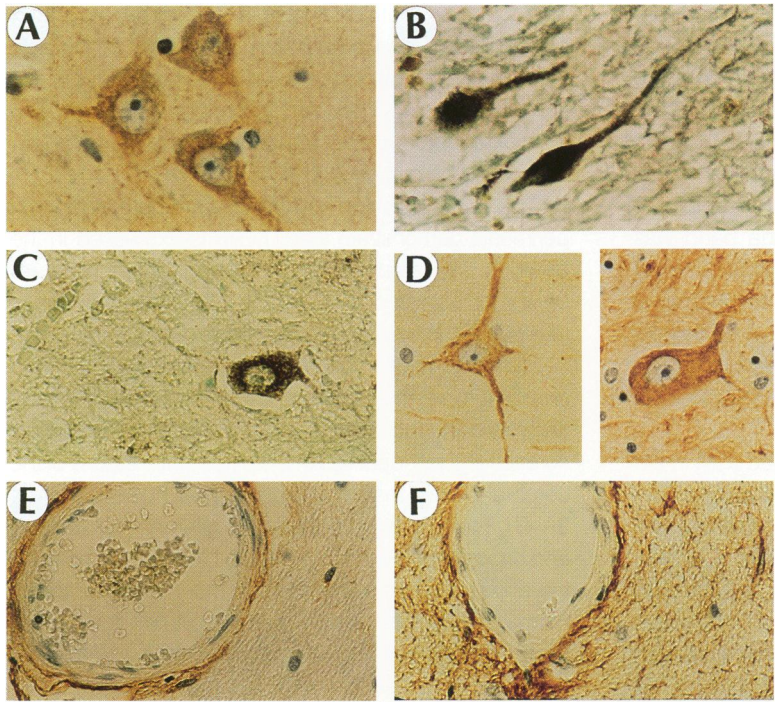

Figure 2. *Immunohistochemical localization of LRP in human brain.* **A–C:** *Neuronal cell bodies and proximal processes demonstrated 8G1 immunoreactivity. In the temporal cortex* **(A)**, *neuronal LRP staining was localized to the cell bodies and proximal axonal segments of pyramidal cells. Note the absence of immunoreactivity in the adjacent oligodendrocytes and astroglia [8G1 immunoperoxidase, 3-3'-diaminobenzidine tetrahydrochloride (DAB) with hematoxylin counterstain; ×600]. In the dentate nucleus* **(B)**, *an equivalent pattern of LRP immunoreactivity selectively defined the neuronal cell population [8G1 immunoperoxidase, DAB/OsO_4 with light green counterstain; ×400]. In the thalamus* **(C)**, *the cell bodies of the large neurons were intensely immunoreactive. This strong staining contrasted with the complete absence of immunoreactivity in the glial cells and microvascular endothelium [8G1 immunoperoxidase, DAB/OsO_4 with light green counterstain; ×400]. In adjacent sections from the temporal cortex and thalamus* **(D)**, *monoclonal antibody 8G1-positive neurons were also immunoreactive for neurofilament epitopes [SMI33 immunoperoxidase, DAB with hematoxylin counterstain, ×400]. Perivascular processes immunoreactive for LRP were present in some brain regions, including the cerebellum shown here* **(E)**. *Note that the endothelial and smooth muscle cells were immunonegative [monoclonal antibody 8G1 immunoperoxidase, DAB with hematoxylin counterstain; ×400]. Tissue section adjacent to* **(E)** *demonstrating that the 8G1 immunopositive region is also GFAP-immunoreactive* **(F)** *[GFAP immunoperoxidase, DAB with hematoxylin counterstain].*

terol metabolism, apo E has been implicated in peripheral nerve regeneration, cellular growth regulation, differentiation, and the immune response.[17,27,28] In the brain, apo E is secreted primarily by astrocytes and not by neurons,[16] opposite to the pattern of expression of LRP in cerebral cortex. This difference in cellular expression of ligand and receptor may provide a pathway for intercellular transport of apo-E–containing lipoproteins and cholesterol in the central nervous system.

mRNA for the LDL receptor has been identified diffusely throughout the brain of mature and immature rabbits in both neurons and glia by *in situ* hybridization.[29] By contrast, an immunohistochemical analysis of adult monkey and rat brain by Pitas et al[30] demonstrated minimal LDL receptor antigen in most of the cerebral cortex. Cellular localization of LDL receptor was not always possible in the study by Pitas et al[30] due to low antigen density; however, at sites with significant staining (such as adjacent to the arachnoid space), LDL receptor expression was restricted primarily to glial cell populations. The respective roles of LRP and the LDL receptor in cholesterol and lipoprotein metabolism in the central nervous system is a topic for future investigation.

A second potential ligand for LRP in the central nervous system is activated α_2M. Although α_2M reacts with many proteinases in the circulation, an essential role for α_2M as a proteinase inhibitor in the blood has not been shown. Dziegielewska et al[31] demonstrated α_2M mRNA in fetal and adult human brain. Using immunohistochemical analysis, Bauer et al[32] did not identify α_2M in normal adult brain; however, these same investigators showed that senile plaques in Alzheimer's disease are strongly immunopositive for α_2M. In addition, Ganter et al[33] suggested that neuronal synthesis of α_2M may be stimulated by interleukin-6. α_2M is a rapid inhibitor of plasmin and other proteinases that may play a role in neuronal cell development and degeneration. By mediating the catabolism of α_2M-proteinase complexes, LRP may prevent the accumulation of these complexes in the central nervous system.

α_2M binds a number of cytokines and growth factors, including platelet-derived growth factor, transforming growth factor-β1, transforming growth factor-β2, tumor necrosis factor-α, interleukin-1β, and nerve growth factor.[34] In the case of TGF-β1, binding to α_2M is reversible and α_2M-conformation–dependent (activated α_2M demonstrates increased TGF-β1 binding affinity).[35] In cell culture, activated α_2M alters the activity of TGF-β1.[35] Most importantly, activated α_2M targets transforming growth factor-β1, tumor necrosis factor-α, and perhaps other cytokines to cells expressing LRP.[36,37] The localization of LRP on neuronal cell bodies and proximal processes suggests an additional role for LRP in the central nervous system. By interacting with α_2M, LRP may regulate the assortment and concentration of cytokines and growth factors available at the neuronal cell surface.

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