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INTERACTION OF THE APOLIPOPROTEIN E RECEPTORS LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN AND sorLA/LR11

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Abstract—In this study, we examined protein–protein interactions between two neuronal receptors, low density lipoprotein receptor-related protein (LRP) and sorLA/LR11, and found that these receptors interact, as indicated by three independent lines of evidence: co-immunoprecipitation experiments on mouse brain extracts and mouse neuronal cells, surface plasmon resonance analysis with purified human LRP and sorLA, and fluorescence lifetime imaging microscopy (FLIM) on rat primary cortical neurons. Immunocytochemistry experiments revealed widespread co-localization of LRP and sorLA within perinuclear compartments of rat primary neurons, while FLIM analysis showed that LRP-sorLA interactions take place within a subset of these compartments.

Key words: LRP, sorLA, LR11, LDL receptor.

Low density lipoprotein receptor-related protein (LRP) is a large (600 kDa) multifunctional receptor that plays roles in binding and clearance of multiple ligands, including apolipoprotein E (apoE) (Herz and Strickland, 2001), and in trafficking of the Alzheimer's disease–related protein amyloid precursor protein (APP), which impacts amyloid-beta peptide (Aβ) production in cells (Ulery et al., 2000; Pietrzik et al., 2002; Cam et al., 2005). Furthermore, strong evidence indicates that LRP is involved in signal transduction pathways initiated by *N*-methyl-D-aspartate receptors as well as platelet-derived growth factor (PDGF) BB (Bacskai et al., 2000; Loukinova et al., 2002; Boucher et al., 2003). Furin cleavage of LRP within the trans-Golgi network during its transit through the secretory pathway leads to formation of a heterodimer consisting of an 85 kDa light chain, containing the transmembrane domain and an intracellular domain with two NPXY motifs, and a 515 kDa heavy chain that constitutes the majority of the extracellular domain and contains four distinct cysteine-rich complement-type repeats necessary for ligand binding.

Another member of the low density lipoprotein (LDL) receptor family expressed in neurons that has also been shown to bind apoE in the nervous system is sorLA (Jacobsen et al., 2001; Taira et al., 2001). SorLA contains a unique combination of modules and may be regarded as a member of a novel family of vacuolar protein sorting 10 protein (Vps10p) receptors, which contain a domain with high homology to the yeast sorting protein Vps10p (Hermans-Borgmeyer et al., 1998). In addition to a Vps10p domain, the luminal domain of sorLA also comprises a cluster of low density lipoprotein receptor (LDL-r) class A complement-type repeats and a β-propeller, similar to other LDL-r family members, as well as six fibronectin type III repeats. Like LRP, sorLA is known to influence APP trafficking and generation of Aβ (Andersen et al., 2005; Offe et al., 2006; Spoelgen et al., 2006). LRP and sorLA are also both known to interact with BACE and modulate its trafficking to and from perinuclear compartments (von Arnim et al., 2005; Spoelgen et al., 2006).

Since these two receptors share ligands, we reasoned that they might form multimeric complexes analogous to those observed with other members of the LDL-r family, such as very low density lipoprotein receptor (VLDL-r)/apolipoprotein E receptor-2 (ApoER2) heterodimers (Hiesberger et al., 1999), LRP5/LRP6 interactions implicated in Wnt-signaling (He et al., 2004), and LRP/integrin interactions in the presence of protease/protease inhibitor complexes (Cao et al., 2006). We therefore sought to test the hypothesis that LRP and sorLA interact. Using co-immunoprecipitation experiments and surface plasmon resonance analysis, we demonstrate that LRP and sorLA in-

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Abbreviations: apoE, apolipoprotein E; ApoER2, apolipoprotein E receptor-2; APP, amyloid precursor protein; Aβ, amyloid-β; FBS, fetal bovine serum; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; LBD, ligand-binding domain; LC, low density lipoprotein receptor-related protein light chain; LDL-r, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; mLRP, mini–low density lipoprotein receptor-related protein receptor; NDS, normal donkey serum; N2a, Neuro2a; PDGF, platelet-derived growth factor; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing 0.05% Tween-20; Vsp10p, vacuolar protein sorting 10 protein.

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teract at endogenous levels within neurons as well as when overexpressed within neuroblastoma cells, and that interaction occurs through both their respective luminal domains and cytoplasmic tails. Immunocytochemistry and fluorescence resonance energy transfer (FRET) imaging of endogenous proteins further showed that LRP–sorLA interactions localize to intracellular perinuclear compartments.

EXPERIMENTAL PROCEDURES

Cell culture conditions and treatment of cells

Mouse neuroblastoma Neuro2a (N2a) cells and primary rat cortical neurons were used in this study. N2a cells were maintained in Opti-MEM (Gibco, Gaithersburg, MD, USA) with 5% fetal bovine serum (FBS) at 5% FBS at 37 °C, 5% CO_2. Primary cortical cultures were generated from Sprague–Dawley rats at embryonic day 18 as described (Hallett et al., 2006; Spoelgen et al., 2006). After dispersing the neurons in Neurobasal medium (Gibco) containing 10% FBS, the cells were plated onto poly-D-lysine- and laminin- (Sigma, St. Louis, MO, USA) coated four-well glass slides (Nalge Nunc International, Naperville, IL, USA). One hour after plating, the medium was replaced with Neurobasal medium containing 2% B-27 supplement (Gibco), within which neurons were maintained for 8 days at 37 °C, 5% CO_2.

Transient transfection and generation of expression constructs

Transient transfections were performed 1 day after cell plating using FuGENE6 (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The following constructs were used in transient transfections: pEGFP-N1 (Clontech, Mountain View, CA, USA), pcDNA3.1-sorLA, -sorLA-flag, -sorLA–green fluorescent protein (GFP), -sorLAΔcd, and sorLA-tail were described previously (Andersen et al., 2005; Spoelgen et al., 2006). The sorLA constructs are based on the sequence of accession number: NM_003105. The constructs encoding the LRP minireceptors (pSec-mini–low density lipoprotein receptor-related protein receptor (mLRP1)-myc, -myc-mLRP2, -myc-mLRP3, -myc-mLRP4) were previously described (Li et al., 2000; Mikhailenko et al., 2001), as were the pSec-myc-LC and pSec-LC-myc-his constructs (Loukinova et al., 2002).

Surface plasmon resonance analysis

Interaction between LRP and sorLA was studied by surface plasmon resonance (SPR) analysis on a Biacore 2000 instrument (Biacore, Sweden). Human LRP was purified from placenta as described (Moestrup et al., 1990), and immobilized at pH 3.0 on Biacore CM5 chip corresponding to a receptor density of 19 fmol/mm^2. A hexa-His-tagged fragment of sorLA including the YWTD-repeated β-propeller and the single epidermal growth factor (EGF)-type-domain followed by the 11 complement-type repeat (CR)-domains (residues 728–1526) was expressed and secreted from EBNA293 cells and purified using Ni^{2+}-ion affinity chromatography as previously reported (Andersen et al., 2005). Samples were analyzed in 10 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM $CaCl_2$, 1.0 mM EGTA and 0.005% tween-20 at a flow of 5 μl/min. Sample and running buffer were identical. Regeneration of the sensor chip after each analysis cycle was performed with alternating pulses of 1.6 M glycine–HCl buffer, pH 3.0 and 0.01% SDS. The instrument response is expressed in relative response units (RU), i.e. the difference in response between protein and control flow channel. Kinetic parameters were determined using the BIAevaluation 3.1 software using a Langmuir 1:1 binding model and simultaneous fitting of all curves in the concentration range from 50 to 2000 nM (global fitting).

Co-immunoprecipitation experiments

For co-immunoprecipitation of endogenous LRP and sorLA, mouse brain extracts were prepared in 1% Triton X-100 lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, supplemented with Complete Protease Inhibitor cocktail [Roche, Branchburg, NJ, USA]). Extracts were pre-cleared with Protein G Sepharose beads (Sigma-Aldrich) for 1 h at 4 °C, and then incubated with either non-immune mouse IgG or mouse anti-LRP 11H4 antibody overnight at 4 °C. Protein G sepharose beads were added and samples incubated for 1 h at 4 °C. Beads were washed three times with 1% CHAPSO buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% CHAPSO, supplemented with Complete Protease Inhibitor cocktail (Roche)) and boiled in 2× Tris–glycine SDS sample buffer. Western blots were performed as described below.

Co-immunoprecipitations of overexpressed proteins were performed on N2a cells co-transfected with the indicated constructs. Twenty-four hours to 48 h after transfection, cells were harvested and lysed in 1% Triton X-100 buffer. Lysates were incubated with μMACS magnetic beads conjugated either to mouse anti-GFP, mouse anti-myc, or mouse anti-his antibody (Miltenyi Biotec, Auburn, CA, USA) for 2–3 h at 4 °C, and then purified using μMACS magnetic separation columns (Miltenyi Biotec) according to the manufacturer's instructions.

Western blotting

Lysate and immunoprecipitation fractions were boiled for 5 min in Tris–glycine SDS sample buffer (Invitrogen, Gaithersburg, MD, USA) and electrophoresed through Tris–glycine polyacrylamide gels (Novex, San Diego, CA, USA), followed by transfer of the protein to PVDF membrane (Millipore, Bedford, MA, USA). Membranes were incubated in blocking buffer (5% nonfat dried milk in Tris-buffered saline (TBS), pH 7.4 containing 0.05% Tween-20) for 1 h prior to incubation with primary antibody diluted in fresh blocking buffer for 1 h at room temperature. The following primary antibodies were used: mouse anti-sorLA (BD Biosciences, San Jose, CA, USA), rabbit anti-N-terminal sorLA, rabbit anti-C-terminal sorLA, goat anti-sorLA (all provided by Anders Nykjaer, University of Aarhus, Denmark), mouse anti-LRP/LC 11H4 (Kowal et al., 1989), mouse anti-85 kDa LRP 5A6 (Strickland et al., 1990) (both provided by Dr. D. Strickland), mouse anti-c-myc 9E10 (Abcam, Cambridge, MA, USA), and mouse anti-GFP (Abcam). Membranes were washed with Tris-buffered saline containing 0.05% Tween-20 (TBST), and incubated with HRP-conjugated secondary antibody diluted in fresh blocking buffer for 1 h at room temperature. Membranes were washed with TBST, and proteins were detected via chemiluminescence (ECL Western Blotting Detection reagent, Amersham Biosciences, Piscataway, NJ, USA).

Immunocytochemistry

After fixation in 4% paraformaldehyde for 10 min, cells were washed twice in TBS and permeabilized in 0.5% Triton X-100 for 10 min. Cells were then blocked in 1.5% normal donkey serum (NDS), followed by incubation with primary antibodies diluted in 1.5% NDS overnight at 4 °C. The following primary antibodies were used for immunocytochemistry: mouse anti-LRP 5A6, rabbit anti-N-terminal sorLA, goat anti-sorLA. After three washes in TBS, secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen), Cy3, or Cy5 (Jackson Immunoresearch, West Grove, PA, USA) were applied, diluted in 1.5% NDS, and incubated for 1 h at room temperature followed by three washes in TBS. Slides were coverslipped using GVA mounting solution (Zymed, South San Francisco, CA, USA) and examined by confocal microscopy.