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The LDL receptor-related protein can form homo-dimers in neuronal cells

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Abstract

The ability of the low density lipoprotein receptor-related protein (LRP) to form homo-dimers was studied in mouse neuroblastoma and human neuroglioma cells as well as in primary cortical cultures from adult mouse brain. Homo-dimerization of LRP light chain (LC) was shown by several methods including co-immunoprecipitation, fluorescence lifetime imaging microscopy, and bimolecular fluorescence complementation assay. The requirement of intact NPXY motifs of LRP LC for homo-dimerization was ruled out by co-immunoprecipitation assay.

Keywords

LRP; low density lipoprotein receptor-related protein; dimer; homo-dimerization

Introduction

Many transmembrane proteins exist in cells as dimers; examples include: lectin-like oxidized low density lipoprotein receptor [16], low density lipoprotein receptor (LDLr) [26], transferring receptor [2], amyloid precursor protein (APP) [6,22], and others. Dimerization of transmembrane proteins is known to be involved in signal transduction and protein processing regulation; thus it is an important mechanism for transforming a ligand binding to intracellular events as well as for proteolytic cleavage.

LRP is a large receptor from the LDLr gene family expressed in various cell types. This type I transmembrane protein is synthesized as a precursor protein, which is further cleaved in the trans-Golgi compartment by furin to two subunits that remain non-covalently bound. A processed LRP consists of C-terminal 85 kD light chain (LC) and N-terminal 515 kD heavy chain (HC). HC located outside the cell contains several ligand-binding repeats and interacts with numerous LRP ligands including apoE-containing lipoproteins, plasminogen activators, activated α 2-macroglobulin (α 2M*), lipoproteins and others, as well as with APP. LC is

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composed of a short ectodomain, a transmembrane domain, which anchors LRP to the cell membrane, and an intracellular domain. The intracellular domain in turn contains two NPXY and two di-leucine motifs and is responsible for interaction with intracellular adaptor proteins such as Dab1, Shc, Fe65 and with endocytic machinery [5,7,8]. LRP participates in the internalization and degradation of its ligands as well as in signal transduction (reviewed in [9,14]). This receptor also undergoes regulated intramembrane proteolysis and its released intracellular domain participates in transcriptional modulation [12,17].

Multivalent LRP ligands $\alpha 2M^*$ and an anti-LRP antibody have been shown to modulate Ca^{2+} influx in cultured neurons in contrast to Fab fragments of the same antibody (monovalent ligands) [4,20]. Thus, dimerization of LRP may be important for its signaling functions. The ability of bivalent ligands of VLDLr or ApoER2 to mimic Reelin signal in neuronal cells [23] suggests that clustering of VLDLr and ApoER2 is important for signal transduction via these receptors. LDLr clusters were revealed by electron microscopy in fibroblasts and hepatocytes [21], and self-association of LDLr via its cytoplasmic domain was reported in bovine adrenal cortex membranes and in hamster fibroblasts [26]. LDLr family members are structurally related; hence LRP dimerization was a feasible opportunity to be explored.

In the present investigation we demonstrated an ability of LRP LC to form homo-dimers, which may be important for LRP-mediated signal transduction and endocytosis.

Materials and Methods

Expression constructs, cell lines and transfection

Previously described [15] LC constructs encode LRP light chain (also called β chain) with two myc tags on the C-terminus (LC-myc) or N-terminus (myc-LC). Mutations were introduced with the QuickChange kit (Stratagene, La Jolla, CA) as described earlier [19] into the first (NPXY $\rightarrow$ APTA) and second (NPVY $\rightarrow$ APVA) NPXY motifs of the myc-LC to make the myc-LC double NPXY mutant construct (myc-LC_[NPXY]). The LC-GFP construct was created by insertion of LC, which was PCR-generated (with addition of KpnI and AgeI restriction sites) from LC-myc to pEGFP-N1 vector (BD Biosciences Clontech, Palo Alto, CA). BiMC constructs were prepared using PCR-generated fragments of eGFP protein: eG1 and eG2 (encoding 1–158 and 159–239 amino acids, respectively). eG1 and eG2 were cloned to the XbaI site of myc-LC construct described earlier [15]. Further, a fragment (GGC GGG GGA GGC TCC)₂ encoding a linker was cloned into the construct between LC and eG1 or LC and eG2 sequences using NotI site, thus producing LCeGFP1 and LCeGFP2 constructs (myc tags remain intact on the N-termini of both constructs). Synuclein-eGFP1 and -eGFP2 constructs used for negative controls were previously described [18].

Mouse neuroblastoma cells N2a (ATCC; Manassas, VA) were maintained in Opti-MEM (Gibco, Gaithersburg, MD) with 5% heat-inactivated fetal bovine serum (FBS) (Gibco). Human neuroglioma cells H4 (ATCC) were maintained in Opti-MEM with 10% FBS. Cells were transiently transfected using FuGENE6 reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. Primary cortical cultures were isolated from embryonic day 15 CD-1 mouse embryos (Charles River, Wilmington, MA) as described in [1]. Primary cultures were transiently transfected after 5 – 6 days *in vitro* using Lipofectamine2000 (Invitrogen, Gaithersburg, MD) according to the manufacturer's protocol. Transfected cells were used 48 hours after a transfection.

Preparation of cell lysates

N2a cells were scraped into lysis Tris-HCl buffer (pH 7.4) with 150 mM NaCl, 0.5% NP-40 and 2 mM EDTA, and protease inhibitor tablets (Roche). Cells were passed through a 27.5 gauge needle, centrifuged 10 min at 12,000 × g, and the supernatant was collected.

Immunoprecipitation

μMACS myc and GFP isolation kits (Miltenyi Biotec, Auburn, CA) were used according to the manufacturer's protocol. The samples eluted from μMACS beads with SDS sample buffer (Miltenyi) were analyzed by Western blotting.

Western blotting

Immunoprecipitates in SDS sample buffer after incubation for 3 min at 95°C were separated on 4–12% Tris-Glycine polyacrylamide gels (Invitrogen). Samples were then transferred from the gel to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, CA). Membranes were blocked in Odyssey blocking buffer (LI-COR, Lincoln, NE), probed with rabbit anti-GFP (Abcam; Cambridge, MA), rabbit anti-myc (Upstate, Lake Placid, NY) antibodies, or mouse anti-LRP 11H4 antibody directed to the LRP LC (described in [13]). Then the proteins were visualized by goat anti-rabbit and goat anti-mouse antibodies conjugated to infrared dyes IRDYE800CW (Rockland Immunochemicals, Gilbertsville, PA) on a two-channel Odyssey infrared imaging system (LI-COR). Alternatively, the membranes were blocked in 5% non-fat milk in TBS with 0.05% Tween 20, probed with 11H4 mouse anti-LRP and HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch), visualized using SuperSignal West Femto Maximum Sensitivity substrate (Pierce, Rockford, IL) and Kodak BioMax MR film (Kodak, Rochester, NY), which was scanned to obtain image files. Brightness/contrast adjustments were performed on the whole images using Adobe Photoshop 7.0.

Fluorescence Lifetime Imaging Microscopy (FLIM)

FLIM was used in this study to reveal colocalization of two proteins in cells. This assay is based on fluorescence resonance energy transfer (FRET) from a donor fluorophore to an acceptor fluorophore. FRET occurs if an acceptor is situated within 10 nm from the donor, and it can be detected by the decrease of the donor's fluorescence lifetime. In this study GFP was used as a donor and Cy3 – as an acceptor. GFP was attached to a protein of interest directly, and Cy3 was attached using a Cy3-conjugated antibody. Immunostaining was performed as described in [19]. Briefly, transfected N2a cells were fixed in paraformaldehyde, permeabilized with Triton X-100, blocked with goat serum (Jackson ImmunoResearch), incubated with mouse anti-myc antibody (Upstate) and Cy3-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch). The FLIM acquisition system [3] and FLIM procedure [19] were described previously.

Bimolecular Fluorescent Complementation Assay

H4 cells plated on 35 mm glass bottom tissue culture dishes (MatTek, Ashland, MA) were transfected with constructs described above using Superfect (Qiagen, Valencia, CA) following the manufacturer's protocol. After two hours of incubation with cells, DNA-Superfect complex was replaced with 10% FBS in OptiMEM. Six to eight hours post-transfection, the dishes were placed into a 30°C incubator overnight, and the images were acquired on a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany) using 488 nm excitation laser.

Statistical analysis

Statistical analysis was performed using a two-sample t-test in StatView 5.0.1 (SAS Institute, Inc). Differences between samples were considered significant if $p < 0.05$.

Results

Differentially tagged LRP LCs interact in neuroblastoma cells

Co-immunoprecipitation experiments were used to assess an interaction between LRP LCs in neuronal cells. For that purpose differentially tagged LRP LCs (LC-GFP and LC-myc) were co-expressed in N2a cells and immunoprecipitated by anti-GFP or anti-myc magnetic beads. Immunoprecipitates were subjected to SDS-PAGE and Western blotting analysis with anti-GFP and anti-myc antibodies. Myc-tagged LC was present in a sample immunoprecipitated with anti-GFP beads from N2a cells co-transfected with LC-GFP and LC-myc (Fig 1A, lane 3). Negative controls proved the specificity of the interaction. Namely, no LC-myc band was present in immunoprecipitates from cells transfected with only LC-GFP (Fig. 1A, lane 1) or LC-myc (Fig. 1A, lane 2).

In turn, co-immunoprecipitation experiments performed from cells co-transfected with LC-GFP and LC-myc using anti-myc beads showed a similar result. LC-GFP protein was present in a sample immunoprecipitated with anti-myc beads (Fig. 1B, lane 3). Negative controls (Fig. 1B, lanes 1 and 2) proved the specificity of the immunoprecipitation. Thus, the co-immunoprecipitation experiment (Fig. 1) reveals interaction of LRP LCs in transiently transfected N2a cells.

Differentially tagged LRP LCs are situated in close proximity in neuroblastoma cells and primary mouse cortical neurons

Interaction between LCs revealed by co-immunoprecipitation experiments was further supported by fluorescence lifetime imaging microscopy (FLIM) data. FLIM is used as a “nano-ruler” as this method reveals proximity of two proteins labeled with donor and acceptor fluorophores by detection of the donor’s fluorescence lifetime decrease [10]. For the FLIM assay, N2a cells plated on glass chamber slides were transfected with LC-GFP (donor fluorophore), or co-transfected with LC-GFP and LC-myc. LC-myc protein was then immunostained with Cy3 (acceptor fluorophore). Average GFP (donor) fluorescence lifetime calculated for LC-GFP in the presence of Cy3-stained LC-myc (acceptor) was significantly shorter than in the absence of LC-myc (Fig 2A). FLIM data, depicted as representative pseudocolored images, are shown in Fig. 2B. These images were built on a pixel-by-pixel basis, where donor lifetime is represented by color. Colors corresponding to short lifetimes are located towards the red area of the spectrum. Representative GFP lifetime distributions clearly show a shortening of the lifetime (shift to the red area of the spectrum) upon introduction of Cy3-labeled LC-myc to the system. Cells co-transfected with LC-GFP and LC-myc, and immunostained omitting primary anti-myc antibody were used as negative controls. GFP fluorescence lifetime in these cells did not differ significantly from GFP lifetime in the absence of transfected LC-myc (Fig. 1A), and lifetime distribution in the representative image does not show any shift of the lifetime. The results of this FLIM experiment reveal a close proximity of differentially tagged LRP LCs in N2a cells, which supports the co-immunoprecipitation experiments, described earlier (Fig. 1) by demonstrating an LRP-LRP interaction in the setting of intact cells. Moreover, these results show the subcellular localization of LRP-LRP interactions to be not only at the cell surface, but throughout the cell (excluding the nucleus). Similar results were obtained using primary mouse cortical neurons culture transfected with LC-GFP (donor fluorophore), or co-transfected with LC-GFP and LC-myc (Supplementary Fig 1).

LC forms homo-dimers in transiently transfected H4 cells

FLIM assay provides information about protein-protein interactions in fixed cells. To further test the hypothesis that LRP-LRP dimers occur in living cells, we utilized a Bimolecular Fluorescence Complementation (BiFC) assay. This assay allows direct visualization of protein

complexes in living cells without removing interacting proteins from their natural intracellular environment [11]. In this assay two potentially interacting proteins fused to non-fluorescent fragments of a fluorescent protein are utilized. These fusion constructs are expressed in cells, and if the proteins of interest interact, the fragments of the fluorescent protein form a fluorescent complex, which can be detected in living cells (Fig. 3A).

To reveal LC homo-dimerization, we performed BiFC assay on live H4 cells co-expressing a LC fused to a non-fluorescent fragment of eGFP protein (LC-eGFP1) and a LC fused to a complementary fragment of eGFP protein (LC-eGFP2). The schematic view of the experiment is shown in Fig. 3A. Co-transfection with LC-eGFP1 and LC-eGFP2 resulted in reconstitution of eGFP fluorescence (Fig. 3B). To confirm that reconstitution of eGFP fluorescence was dependent on LC-LC homo-dimerization, LC-eGFP1 was also co-transfected with eGFP2 fused to the protein α -synuclein (syn-eGFP2) (previously described in [18], which did not result in reconstitution of eGFP fluorescence (Fig. 3C). Complimentary co-transfection with syn-eGFP1 and LC-eGFP2 also did not yield significant eGFP fluorescence (data not shown). Western analysis confirmed expression of the corresponding constructs in each condition (Supplementary Fig. 2). These control experiments confirm that formation of fluorescent eGFP protein from eGFP fragments is driven by dimerization of LCs fused to eGFP1 and eGFP2. Again, fluorescence is observed throughout multiple intracellular compartments, sparing the nucleus.

NPXY domain is not required for LC homo-dimerization in transiently transfected neuroblastoma cells

The LC intracellular domain contains two NPXY motifs that are important for LRP interaction with intracellular adaptor proteins. Adaptor proteins could facilitate LC dimer formation by holding LCs in close proximity. The importance of LC NPXY domains for formation of LC dimers was assessed by co-immunoprecipitation experiment. N2a cells were transiently transfected with LC-GFP and either wild type myc-LC or double NPXY-mutated myc-LC (myc-LC_[NPXY]), in which both NPXY motifs were mutated (NPTY→APTA and NPVY→APVA). Cell lysates were prepared, and LC-GFP was immunoprecipitated using anti-GFP magnetic beads. Samples were subjected to SDS-PAGE and immunoblotting with mouse anti-LC antibody. Both myc-LC and myc-LC_[NPXY], as well as endogenous untagged LC co-immunoprecipitated with LC-GFP (Supplementary Fig. 2). This result shows that intact LC NPXY domains are not required for LC dimerization.

A cytoplasmic adaptor protein, engulfment-associated protein (GULP), was previously shown to bind LC via the LC NPXY domain. GULP forms homo dimers via its leucine zipper domain [24,25]. Thus, GULP dimer formation could facilitate LC homo-dimerization by holding LC in close proximity. However, overexpression of GULP did not increase co-immunoprecipitation of LC-GFP with LC-myc from transiently transfected N2a cells (data not shown). This result further supports the lack of importance of the LC NPXY domain for LC homodimerization.

Discussion

Current results indicate that LRP can form homo-dimers in neuronal cells and in brain tissue. Interaction of LCs in transiently transfected cells was shown by co-immunoprecipitation experiments performed on cell lysates. These data were supported by detection of FRET between fluorescently-labeled LCs in fixed cells, which occurs when donor and acceptor fluorophores are situated within 10 nm from each other. Additionally, LC dimerization was demonstrated by BiFC assay performed in unfixed live cells thus being an independent detection method supporting LC interaction in intact cells. The role of intact NPXY motifs

(and thus the role of intracellular adaptor proteins that interact with LRP via its NPXY domains) in LC dimers formation seems to be insignificant according to our experiments.

In summary, our current investigation revealed an ability of LRP to form homo-dimers. The interaction occurs via LC of LRP; the interacting domains still remain to be elucidated, but intact NPXY motifs of LC do not seem to be required. It still remains to be elucidated whether *in vivo* the dimers can be formed by LRP molecules present on adjacent cells (*trans*-dimerization) as well as by LRP molecules within one cell (*cis*-dimerization). Homodimerization of LRP may play a role in signaling or endocytic functions of this receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1. Differentially tagged LRP LCs co-immunoprecipitate from neuroblastoma cells
 N2a cells were transiently transfected with GFP-tagged LC (LC-GFP) and myc-tagged LC (LC-myc) constructs, and cell lysates were prepared. LC-GFP (A) or LC-myc (B) was immunoprecipitated using anti-GFP or anti-myc magnetic beads, respectively. The samples were subjected to SDS-PAGE, transferred to PVDF membrane, and probed with primary rabbit anti-GFP and anti-myc antibodies. Primary antibodies were then visualized with secondary goat anti-rabbit infrared dye-conjugated antibody.

Figure 2. Differentially tagged LRP LCs are situated in close proximity in neuroblastoma cells N2a cells were transfected with LC-GFP (donor), or co-transfected with LC-GFP and LC-myc. Cells were fixed, permeabilized, and LC-myc was labeled with primary mouse anti-myc antibody, which was visualized by secondary donkey anti-mouse antibody conjugated with Cy3 (acceptor). Cells co-transfected with LC-GFP and LC-myc, and immunostained omitting primary anti-myc antibody were used as a negative control. Average lifetimes of GFP fluorescence were determined by FLIM (A). Representative GFP fluorescence intensity and lifetime images are shown on panel B. Pseudocolored FLIM images (B, middle column) represent donor fluorescence lifetimes on pixel-by-pixel basis, where shorter lifetimes are located towards red area of the spectrum, and longer lifetimes – towards blue area. Fluorescence lifetime distributions (B, right column) are shown for corresponding representative images. * $p < 0.00001$ vs. donor only.

Figure 3. Co-expression of eGFP fragments fused to LRP LCs induces bimolecular fluorescence complementation in H4 cells

(A) Principle of BiFC assay: association of non-fluorescent eGFP fragments fused to two proteins produces bimolecular fluorescent complex, formation of which is driven by interaction of proteins fused to eGFP fragments. (B–C) H4 cells were co-transfected with LRP LC fused to non-fluorescent partial eGFP1 (LC-eGFP1) and LRP LC fused to non-fluorescent partial eGFP2 (LC-eGFP2), or co-transfected with LC-eGFP1 and α -synuclein fused to eGFP2. Fluorescence of bimolecular fluorescent complex of eGFP fragments brought together by interacting LCs was detected using fluorescence microscopy.