ABCD_RB910 recognizes an intracellular epitope of the endogenous human adhesion G protein-coupled receptor L2 by immunofluorescence

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Abstract

The recombinant antibodies ABCD_RB910, ABCD_RB911, ABCD_RB912, ABCD_RB913, and ABCD_RB914 detect by ELISA a synthetic peptide from the intracellular domain of the Latrophilin-2 (LPHN2). ABCD_RB910 recognizes the endogenous LPHN2 by immunofluorescence.

Introduction

Latrophilin-2 (LPHN2, *ADGRL2*, UniProt # O95490) is an adhesion G-protein-coupled receptor best known for its role in neuronal development and synapse formation (Anderson *et al*., 2017). Emerging evidence indicates that it is widely distributed across tissues and versatile in various pathological contexts (Lee *et al.,* 2022). LPHN2 structure typically comprises a C-terminal signaling domain and an extended N-terminal extracellular domain. This latter domain plays a pivotal role in mediating complex interactions with cell surface molecules and extracellular matrix components, thus influencing its functional roles beyond traditional GPCR signaling. There are few commercial antibodies against LPHN2 and they are not well characterized. Indeed, developing antibodies to identify LPHN2 is a challenge due to the protein's complex structure and its potential of changing conformations depending on the protein's active state. Here we describe the ability of five recombinant antibodies, ABCD_RB910-914, to detect by ELISA a synthetic biotinylated peptide from the human LPHN2 protein. This peptide is part of the C-terminal intracellular domain of the protein. One of these antibodies is further characterized for its capacity to detect endogenous LPHN2 in the Calu-3 airway epithelial cell line.

Materials & Methods

Antibodies: The antibodies ABCD_RB910, ABCD_RB911, ABCD_RB912, ABCD_RB913 and ABCD_RB914, (ABCD nomenclature, https://web.expasy.org/abcd/) were generated by the Geneva Antibody Facility (http://unige.ch/medecine/antibodies/). Briefly, a semi-synthetic phage display library (Novimmune) was panned against the biotinylated peptide corresponding to amino acids 1293 to 1331 of the human LPHN2 protein (P2, sequence: GSSSEDDAIVADASSLMHSDNPGLELHHKELEAPLI PQR, cytoplasmic region). After three rounds of panning, selected phage vectors were isolated using a plasmid preparation kit (Qiagen), and the scFv inserts were subcloned into custom-made expression vectors and sequenced. The selected antibodies were expressed as mini-antibodies, consisting of the antigen-binding scFv domain fused to a rabbit IgG Fc region. HEK293 suspension cells, cultured in HEK TF medium (Sartorius #861-0001) supplemented with 0.1% Pluronic F68 (Sigma #P1300), were transiently transfected with the vectors encoding the scFv-Fc constructs. Supernatants containing the mini-antibodies were harvested three days post-transfection. Estimated production yields were approximately between 42–128 mg/L.

Antigen: The same peptide used for phage selection was used as the antigen for ELISA detection. For negative control, an alternative N-biotinylated peptide derived from the extracellular domain of the human LPHN2 protein was utilized (P1, residues 335-357, sequence: TGKNSIDYIYNTRLNRGEYVDVP).

ELISA: The whole procedure was carried out at room temperature. Biotinylated peptides at saturating concentration (10 pmol/well) were immobilized on streptavidin-coated ELISA plates (Pierce #15124) for 30 min. Each well was rinsed three times with 100 µl of washing buffer (PBS + 0.5% (w/v) BSA + 0.05% (w/v) Tween20), then incubated for 1 hour with 50 µl of RB antibody-containing supernatant diluted in washing buffer (Fig. 1). After rinsing 3 times (100 µl washing buffer), wells were incubated with horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma #A8275, dilution 1:1000, 50 µl per well) for 30 min. After 3 rinses, Tetramethylbenzidine (TMB) substrate (Sigma #T5569) was added (50 µl per well). The reaction was stopped by the addition of 25 µl of 2 M H_2SO_4. The absorbance (OD) was measured at 450 nm, and the absorbance at 570 nm was subtracted.

Immunofluorescence: To detect the ability of RB910 to recognize endogenous LPHN2 in a human cell line, Calu-3 epithelial cells, cultured in Minimum Essential Medium GlutaMAX supplemented with 1% non-essential amino acids, 1% HEPES, 1% sodium pyruvate, 10% heat-inactivated Fetal Bovine Serum (ThermoFisher #10270-

106) and Penicillin/Streptomycin/Fungizone (Bioconcept #4-02F00-H) at 37°C in humidified 5% CO2 atmosphere, were fixed for 30 min with 4% paraformaldehyde (Sigma #158127). Cells were permeabilized for 15 min with 0.2% Triton X-100 (Sigma #T8787) buffer before being blocked for an hour with the PBS 1% BSA saturation buffer. Cells were then incubated with RB910 primary antibody at 1/10 dilution for 2 hours at room temperature, washed and then incubated with secondary goat anti-Rabbit IgG antibody conjugated with AlexaFluor-647 (ThermoFisher #A21245) for 1 hour in the dark. Cells were then washed and mounted with Vectashield (Vector Laboratories #H-1000). Images were captured using a stellaris 63x oil objective. Specificity of the antibody was tested in a Calu-3 cell line knockdown for ADGRL2 by CRISPR-Cas9 using Lentiviral particles for sgRNA and Cas9 nuclease (GeneCopoeia). mRNA expression of ADGRL2 was determined by RT-qPCR using the following primers: forward 5'_acaggcaagaactcaattgatta_3' and reverse 5'_cacttgggcaggatcaggtggacca_3'.

Results

The chosen peptide sequence is predicted to be unstructured and has no homology to any known protein.
Antibodies RB910, RB911, RB912 and RB914 bound in a concentration-dependent manner to the intracellular LPHN2 P2 peptide, against which they were generated, but did not bind to the negative control P1 peptide (Fig. 1). Antibody RB913 exhibited only a slight signal at the highest concentration. Although these antibodies recognize specifically the intracellular LPHN2 P2 peptide by ELISA, we sought to study their ability to bind the full-length endogenous protein by immunofluorescence.

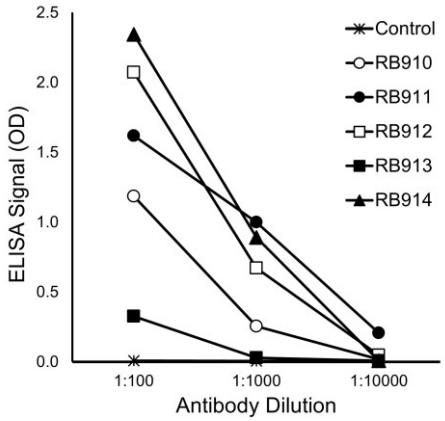

Fig. 1. Specific binding of RB antibodies to the target intracellular LPHN2 peptide, as detected by ELISA. 'Control' indicates the binding of RB910 to the negative control peptide (all other control curves were superimposed).

RB910 detected LPHN2 in Calu-3 epithelial cell line (Fig.2A). Labeling appears to be mainly localized underneath the plasma membrane and in the cytoplasm. The staining was significantly decreased in cells with LPHN2 knockdown (Fig.2B). No signal was found in the absence of the primary antibody (Fig.2C). Fig.2D shows LPHN2 mRNA expression after knockdown in Calu-3 cells.
Future studies should assess whether the other antibodies characterized in this study can bind endogenous LPHN2 using immunofluorescence.

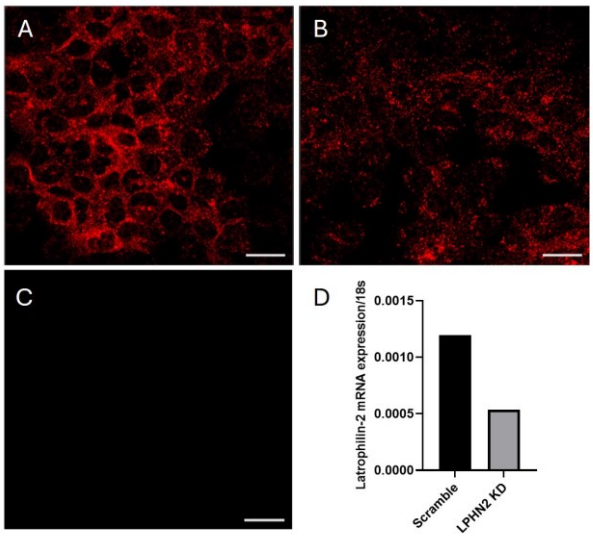

Fig. 2. RB910 recognizes LPHN2 (red) in Calu-3 cells (A). A significant decrease of the labeling was observed in Calu-3 with LPHN2 knockdown (B). No signal was detected when the first antibody was omitted (C). LPHN2 mRNA expression decreases in Calu-3 with LPHN2 knockdown compared to scrambled cells by qRT-PCR (D). Scale bars: 20 µm.

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Conflict of interest

The authors declare no conflict of interest.