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Functional characterization of lysophosphatidic acid receptor 1 mutants identified in rat cancer tissues

Shoichi Ishii [a], Toshifumi Tsujiuchi [b], Nobuyuki Fukushima [a, *]

[a] Division of Molecular Neurobiology, Department of Life Science, School of Science and Engineering, Kindai University, Higashiosaka, Japan
[b] Division of Molecular Oncology, Department of Life Science, School of Science and Engineering, Kindai University, Higashiosaka, Japan

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ABSTRACT

Lysophosphatidic acid (LPA), an extracellular lipid mediator, exerts various cellular effects through activation of LPA receptors, LPA_1–LPA_6, in many types of cells including cancer cells. We recently found several missense mutations of *Lpar1* in rat cancer tissues. One of these mutations is located at the extracellular tip of the seventh transmembrane domain of LPA_1, and another three mutations are found within the NPXXY motif in the seventh transmembrane domain. These mutants are designated F295S LPA_1 and P308S, I310T, and Y311H LPA_1, respectively. Here, we examined the functions of these LPA_1 mutants. Compared with wild-type (WT) LPA_1, F295S, P308S, and I310T LPA_1 showed decreased maximal responses in inhibition of cAMP formation, Ca^{2+} mobilization, and cytoskeletal changes. Y311H LPA_1 failed to show LPA-induced cellular responses. However, these LPA_1 mutants were internalized in response to LPA exposure. Finally, while WT and F295S LPA_1 showed a similar, broad distribution throughout the cell, P308S, I310T, and Y311H LPA_1 displayed a restricted cellular distribution and co-localized with the endoplasmic reticulum. These data suggest that the LPA_1 mutants perturb LPA signaling in cancer tissues.

1. Introduction

Lysophosphatidic acid (LPA) is an extracellular lipid mediator that exerts various cellular effects, including enhancement or inhibition of cell proliferation, differentiation, migration, or motility [1,2]. These effects are mediated through G protein-coupled LPA receptors, LPA_1–LPA_6, and dysregulated LPA receptor-mediated signaling is thought to account for the occurrence or progression of some diseases, such as cancer, fibrosis, and neuropathic pain [1,2]. Indeed, many types of cancer show aberrant expression of LPA receptor genes or abnormal LPA signaling. Moreover, an artificial LPA_1 lacking the Ser-Val-Val sequence at the carboxyl terminal is constitutively active and has oncogenic activity [3]. Therefore, if they arise, missense mutations in LPA receptor genes might be involved in carcinogenesis or cancer progression.

A missense mutation in *LPAR1* has been reported in human neuroblastoma, which results in substitution of Arg with Trp at codon 163 to produce R163W LPA_1, leading to increased cellular motility [4]. More missense mutations in *LPAR1* are deposited in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database, although they have not yet been reported to be closely related to carcinogenesis or cancer progression. In rat liver and lung cancer tissues, we have identified seven missense mutations in the *Lpar1* gene [5,6]. Interestingly, four of these seven mutations are at codon 295, where Phe is substituted with Ser (F295S); at codon 308, where Pro is substituted with Ser (P308S); at codon 310, where Ile is substituted with Thr (I310T); and at codon 311, where Tyr is substituted with His (Y311H) (Fig. 1A). Codon 295 is adjacent to Lys at codon 294, which is involved in LPA recognition by LPA_1 [7]. The latter three mutations are located within the NPXXY motif, corresponding to codons 308–311 of LPA_1, in the putative seventh transmembrane domain (Fig. 1A).

The NPXXY motif is well conserved in G protein-coupled receptors (GPCRs) and plays a role in their internalization, as demonstrated by mutagenesis studies of many artificial GPCR mutants harboring single mutations within the NPXXY motif [8–11]. These studies have demonstrated that the mutants showed varied functions, depending not only on the receptor types but also

Abbreviations: COSMIC, Catalogue Of Somatic Mutations In Cancer; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FAFBSA, fatty acid-free bovine serum albumin; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; WT, wild type.

* Corresponding author. Division of Molecular Neurobiology, Department of Life Science, Kindai University, 3-4-1 Kowakae, Higashiosaka 577-8502, Japan.
E-mail address: nfukushima@life.kindai.ac.jp (N. Fukushima).

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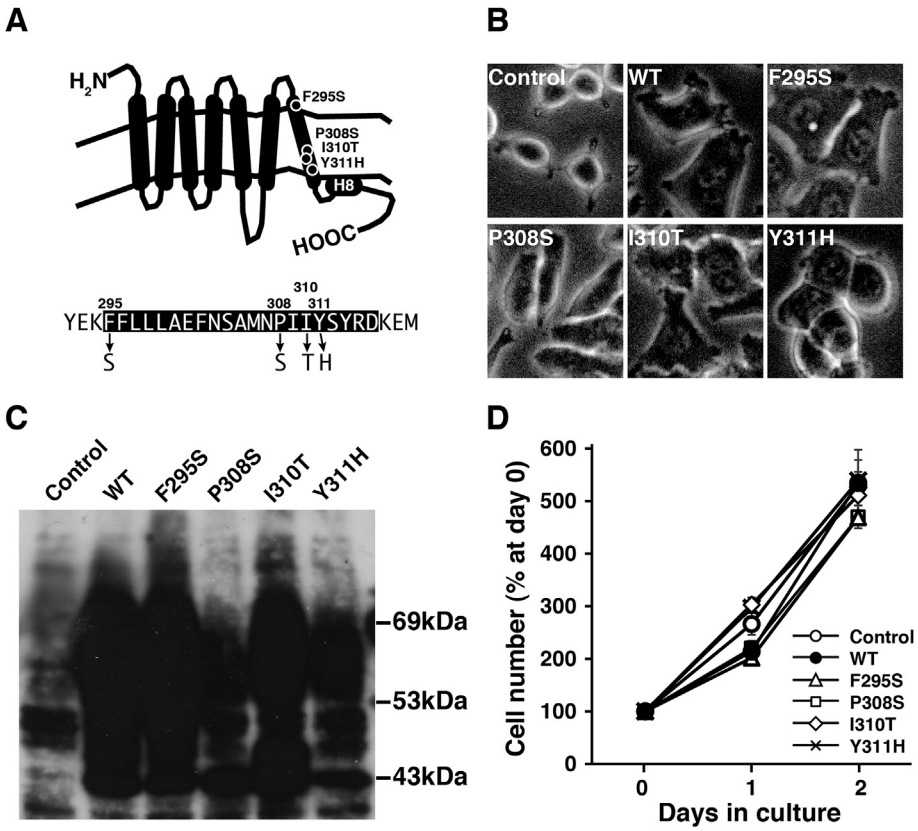

Fig. 1. Establishment of RH7777 cells expressing LPA_1 mutants. (A) Schematic illustration of LPA_1 and the positions of mutations (F295S, P308S, I310T, and Y311H) observed in cancer tissues [5,6]. H8 indicates helix 8. Part of the amino acid sequences flanking the putative seventh transmembrane domain (white on black) is shown below the illustration. The numerals denote the amino acid positions. The substituted amino acids are shown. (B) Phase contrast microscopy of clonal control, WT, F295S, P308S, I310T, and Y311H RH cells. (C) Western blot analyses of FLAG-LPA_1 in cell extracts. (D) Growth of control, WT, F295S, P308S, I310T, and Y311H RH cells. Cells were cultured in serum-containing medium for 2 days, and the cell number was determined. Data express the percentage of absorbance at day 0 and are the mean ± SD of a representative experiment.

on the properties of the substituted amino acids. For example, in experiments where Tyr at codon 368 in the 5HT2C receptor was replaced with any of 19 amino acids, some mutants demonstrated abnormal, constitutively active signaling, some retained normal signaling, and others showed lower expression on the cell surface [8]. A vasopressin receptor mutant possessing the substitution of Tyr with Phe in the NPXXY motif is normally expressed on the cell surface but is not internalized after vasopressin exposure [9]. Therefore, it needs to be resolved whether each mutation in *Lpar1* observed in rat cancer tissues affects LPA_1-mediated signaling or LPA_1 internalization. In the present study, we examined the functions of LPA_1 mutants, particularly with regard to G protein-mediated signaling.

2. Materials and methods

2.1. Cell culture

Rat RH7777 hepatoma and B103 neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemicals, Osaka, Japan) containing 10% fetal calf serum and penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan).

2.2. Generation of Lpar1-expressing cells

Four mouse *Lpar1* mutants (F295S, P308S, I310T, and Y311H) were generated using a mutagenesis kit and mouse wild-type (WT) *Lpar1* inserted into an S001-AB plasmid as a template, according to the manufacturer's manual (Takara Bio, Ohtsu, Japan). The mutations were confirmed by nucleotide sequence analyses. To generate LPA_1-expressing cells, retroviruses co-expressing green fluorescent protein (GFP) from an internal ribosomal entry site were used. Retroviruses expressing FLAG-tagged WT or mutant *Lpar1* were prepared by transfecting retrovirus producer cells with S001-AB plasmids containing WT or mutant *Lpar1*, as previously described [3]. RH7777 or B103 cells were infected with retroviruses expressing WT or mutant *Lpar1* in the presence of 5 μg/ml polybrene (Sigma, Tokyo, Japan). For cell cloning, infected RH7777 cells were harvested, and about 100 cells were sparsely replated in a 15 cm-diameter plate. Colonies in which all cells expressed GFP were picked and further propagated. Infected B103 cells were used as mixed cells for the neurite retraction assay. Retroviruses expressing FLAG-tagged WT or mutant *Lpar1*, but not the GFP gene, were also generated. Infected RH7777 cells were used as mixed cells for double labeling of FLAG-LPA_1 and the endoplasmic reticulum (ER). The WT and four LPA_1 mutant-expressing cell lines were designated WT, F295S, P308S, I310T, and Y311H RH or B103 cells, respectively. Control cells were generated by infecting empty retroviruses.

2.3. Cell proliferation assay

Cells were plated at a density of 3000 cells/well in a 48-well plate. Cell numbers were determined using a Cell Counting Kit-8 (Dojin Chemistry, Kumamoto, Japan). This assay was performed in triplicate or quadruplicate, and repeated twice.