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Molecular regulation of lysophosphatidic acid receptor 1 trafficking to the cell surface

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ABSTRACT

The lysophosphatidic acid receptor 1 (LPA1), a G-protein coupled receptor, regulates cell proliferation, migration, and cytokine release. Here, we investigate the molecular signature of LPA1 trafficking to the cell surface. The overexpressed LPA1 with a C-terminal V5 tag (LPA1-V5) is majorly expressed on the cell surface, while two deletion mutants (C320 and Δ84–87) failed to be trafficked to the cell surface. Further, site-directed mutagenesis analysis of the LPA1 revealed that Ile325, Tyr85, and Leu87 within these two fragments regulate LPA1 maturation and trafficking to the cell surface. Over-expression of Sar1, a component of coat protein complex II (COPII), enhances glycosylation of LPA1 wild type, but not these mutants. The mutants of LPA1 are majorly localized in the endoplasmic reticulum (ER) and exhibit a higher binding affinity to heat shock protein 70 (Hsp70), when compared to the LPA1 wild type. Further, we found that all these mutants failed to increase phosphorylation of Erk, and the cytokine release in response to LPA treatment. These results suggest that Ile325, Tyr85, and Leu87 within LPA1 are essential for LPA1 protein properly folding in the ER.

1. Introduction

Lysophosphatidic acid (LPA) is a naturally occurring bioactive phospholipid that triggers intracellular signals including the increase of intracellular calcium, and the activation of protein kinases and transcriptional factors [1–4]. The biological effects of LPA are dependent on LPA receptors on the cell surface, which are members of G-protein coupled receptors (GPCRs) [5]. Among them, LPA receptor 1 (LPA1) is highly expressed in the most cell types including lung epithelial cells [6–8]. LPA1 is considered to be a pro-inflammatory receptor [9–11]. LPA1 deficient mice reduced intratracheal instillation of lipopolysaccharide (LPS)- or bleomycin-induced lung injury [9,11]. LPA1-mediated signals regulate tumor cell migration and proliferation and contribute to the tumorigenesis [12,13]. LPA1 is a potential target in the development of new therapeutic strategies to lessen lung inflammation and reduce tumor growth and metastasis [9,10,14–19].

GPCRs belong to the seven transmembrane receptors which are localized on the cell surface. The expression levels of GPCRs on the cell surface determine the effects of ligand-induced cellular responses. Receptors are synthesized on the rough endoplasmic reticulum (ER). Receptors trafficking from the ER/Golgi to the cell surface regulate the GPCR levels on the cell surface. Once the properly folded receptor has passed the ER quality control, it is transported to the Golgi for glycosylation and maturation [20]. Sar1, a member of coat protein complex II (COPII), is known to regulate protein trafficking from the ER to the Golgi [21,22]. Misfolded receptors within the ER are recognized by heat-shock protein 70 (Hsp70) family members and destroyed by the ER-associated degradation pathway (ERAD) [23–25].

The PDZ protein GIPC (GAIP-interacting protein, C terminus) promotes LPA1 internalization and attenuates LPA responses [26]. However, the regulation of LPA1 trafficking to cell surface has not been studied. Here, we show that three amino acids play a vital role in the regulation of LPA1 glycosylation, trafficking to the cell surface, and LPA1-mediated signaling.

2. Materials and methods

2.1. Cell culture and reagents

Murine lung epithelial cells (MLE12) and human bronchial epithelial cell (Beas2B) [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured with HITES medium containing 10% FBS. All the cells were cultured at 37 ºC in 5% CO2. V5 antibody, mammalian expressional

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plasmid pcDNA3.1D/His-V5 TOPO, *Escherichia coli* Top 10 competent cells were from Invitrogen (Carlsbad, CA, USA). β-Actin and Flag antibodies and LPA were from Sigma Aldrich (St. Louis, MO, USA). Hsp70 antibody was obtained from StressMarq Bioscience (Victoria, Canada). Immunobilized protein A/G beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All materials in highest grades used in the experiments are commercially available.

2.2. DNA construction

Human LPA1 cDNA was inserted into pcDNA3.1D/His-V5 TOPO vector. C-terminal or intracellular domain deletion mutants of LPA1 were generated by PCR with specific primers designed to target LPA1 cDNA sequence. Site directed mutagenesis was performed to generate LPA1 mutants according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA).

2.3. Immunoblotting and immunoprecipitation

Cells were washed with cold PBS and collected in cell lysis buffer. An equal amount of cell lysates (20 μg) was subjected to SDS-PAGE without boiling, electrotransferred to membranes and immunoblotted as standard protocol. For immunoprecipitation, equal amounts of cell lysates (1 mg) were incubated with a V5 tag antibody overnight at 4 ºC, followed by the addition of 40 μl of protein A/G agarose and incubation for additional 2 h at 4 ºC. The immunoprecipitated complex was washed 3 times with 1% Triton X-100 in ice cold phosphate-buffered saline and analyzed by immunoblotting with an Hsp70 antibody.

2.4. Immunostaining

MLE12 cells were plated on 35 mm glass-bottom culture dishes. After LPA treatment, cells were fixed in 3.7% formaldehyde for 20 min, followed by permeabilization with 0.1% Triton X-100 for 2 min. Cells were incubated with 1:200 dilution of V5 tag antibody, followed by a 1:200 dilution of fluorescence-conjugated secondary antibody sequentially for immunostaining.

2.5. IL-8 Elisa assay

Beas2B cells were treated with LPA for 3 h, and then supernatants were collected. Human IL-8 levels in the supernatants were measured by IL-8 Elisa kit according to the manufacturer's instruction.

3. Results and discussion

3.1. Ile325 within LPA1 regulates LPA1 trafficking to the cell surface and maturation

Modulation of receptor trafficking to the cell surface regulates the receptor-mediated signals and biological functions. LPA1 is a receptor with multiple functions, belonging to the GPCR family. GPCR export to the cell surface membrane is tightly regulated by the ER quality-control system, specific protein partners, and post-translational modifications. To investigate which fragment of LPA1 regulates its intracellular trafficking, we generated plasmids of LPA1 wild type and C-terminal deletion mutants with a V5 tag in the C-termini. Immunostaining with a V5 tag antibody revealed that LPA1-V5 and c-terminal deletion mutant (LPA1C330-V5) are localized primarily on the cell surface, however, further deletion of LPA1 (LPA1C320-V5, LPA1C315-V5, and LPA1C310-V5) failed to traffic to the cell surface (Fig. 1A and B). This data indicates that amino acids between 320 and 330 play a vital role in the regulation of LPA1 trafficking. To identify which amino acid is involved, several amino acids between 320 and 330 were mutated as shown in Fig. 2A. Unlike other LPA1 mutants, mutants containing Ile 325 (LPA1I325A-V5 and LPA1I325A/L326A-V5) are majorly expressed inside the cytoplasm

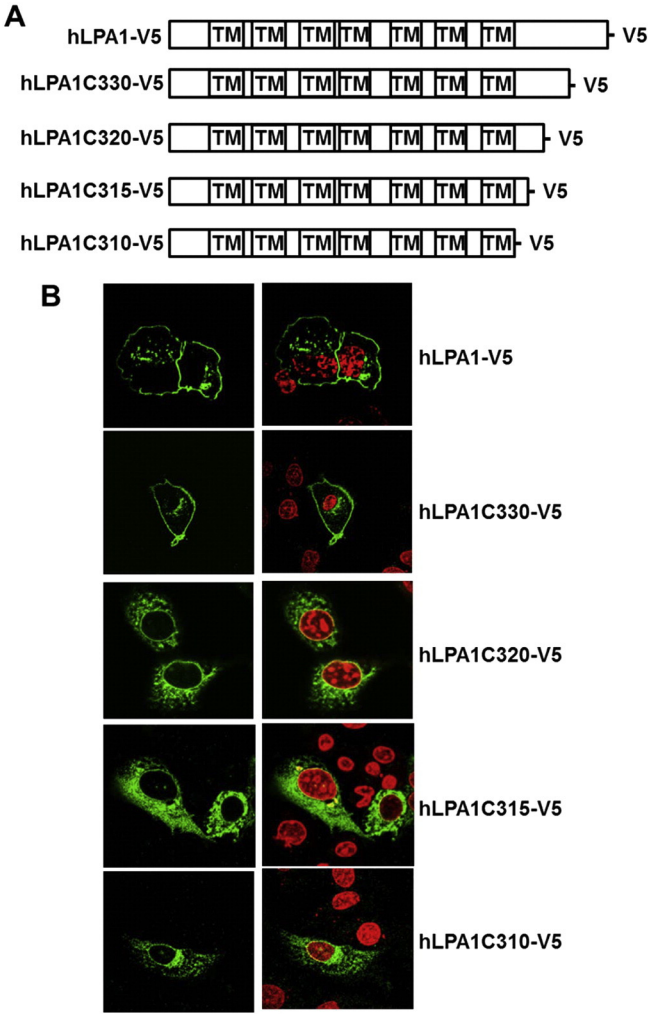

Fig. 1. Residues 320–330 are involved in LPA1 trafficking to the cell surface. A. LPA1 wild type and c-terminal deletion mutants were inserted into pCDNA3.1/Topo-V5-His plasmids. B. Ectopic expressed LPA1 wild type and c-terminal deletion mutants with V5 tag in MLE12 cells were immunostained with a V5 antibody (green). Nuclei were stained with DAPI (red).

(Fig. 2B), suggesting that Ile325 is a key amino acid in the regulation of LPA1 trafficking. Ile325 is in the dileucine like region, which has been known to regulate receptor trafficking. The dileucine motif in the vasopressin receptor regulates its trafficking from ER to the cell surface [27,28], while the dileucine motif of oncostatin M receptor mediates the receptor trafficking from the cell surface to the cytoplasm [29]. Here, we show that the dileucine like region within LPA1, plays a crucial role in the escaping the receptor from ER to the cell surface. There are double cysteine residues (C327/C328) in the fragment, which are considered as a canonical palmitoylation motif [30,31]. We found that the double cysteine residue mutation (LPA1C327S/C328S) had no effect on the alteration of LPA1 expression on the cell surface. Post-translational modification regulates GPCR trafficking to the cell surface. SDS-PAGE gel analysis with a V5 antibody shows that LPA1-V5 has more modified forms (50–70 kDa) than un-modified forms (40 kDa), while LPA1I325A-V5 has less modified forms than the un-modified forms (Fig. 2C and D). These data indicate that the LPA1I325A-V5 mutant fails to be post-translational modified and is unable to move to the cell surface.

3.2. Tyr85 and Leu87 are critical in the regulation of LPA1 trafficking to the cell surface

In addition to the c-termini of LPA1, we also found that deletion of four amino acids (84–87, IYYL) within the first intracellular loop results