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Functional lysophosphatidic acid receptors expressed in *Oryzias latipes*

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ABSTRACT

Lysophosphatidic acid (LPA) signaling is known to play biological and pathophysiological roles in many types of animals. Medaka (*Oryzias latipes*) is an experimental fish that can be easily maintained, propagated, and analyzed, and whose genome has been completely sequenced. However, there is limited information available regarding medaka LPA receptors. Here, using information from the medaka genome database, we examine the genomic structures, expression, and functions of six LPA receptor genes, *Lpar1–Lpar6*. Our analyses reveal that the genomic structures of *Lpar1* and *Lpar4* are different from those deduced from the database. Functional analyses using a heterologous expression system demonstrate that all medaka LPA receptors except for LPA_{5b} respond to LPA treatment with cytoskeletal changes. These findings provide useful information on the structure and function of medaka LPA receptor genes, and identify medaka as a useful experimental model for exploration of the biological significance of LPA signaling.

1. Introduction

Lysophosphatidic acid (LPA) is an intercellular lipid mediator that exerts a wide variety of cellular effects through G protein-coupled LPA receptors (Choi and Chun, 2013; Choi et al., 2010; Lin et al., 2010; Tsujiuchi et al., 2014; Yanagida et al., 2013). Thus far, six LPA receptor subtypes (*Lpar1–Lpar6*) have been identified in various vertebrates including human, mouse, rat, chick, frog, and zebrafish. Phylogenetic analyses have revealed that two distinct families, namely, the endothelial differentiation gene (edg) family consisting of *Lpar1–Lpar3* and the non-edg family consisting of *Lpar4–Lpar6*, are clearly separated in human, mouse, frog, and zebrafish (Masse et al., 2010; Yanagida et al., 2013; Yukiura et al., 2011). In addition, each member is highly related to its orthologs identified in other species. These analyses suggest that LPA receptor-mediated signaling has been conserved across multiple species during evolution.

Since the first LPA receptor gene *Lpar1* was identified, many investigators including ourselves have examined the functions of LPA receptors using cultured cells and tissues as well as the spatio-temporal expression of LPA receptor genes in adult tissues during development using mice (Choi and Chun, 2013; Choi et al., 2010; Ishii et al., 2004; Lin et al., 2010). Over the last decade, the biological and pathophysiological roles of each LPA receptor have been explored in vivo using genetically engineered mice as well as in zebrafish and human genetic studies. For example, LPA_1-mediated signaling is involved in neurogenesis, brain formation, initiation of neuropathic pain, and formation of pulmonary fibrosis (Herr et al., 2011; Ueda et al., 2013; Yung et al., 2011). LPA_2 affects synaptic transmission and tumorigenesis, and LPA_3 plays an important role in implantation of oocytes (Trimbuch et al., 2009; Ye et al., 2005). LPA_4 has been shown to mediate blood and lymphatic vessel formation (Lin et al., 2012; Sumida et al., 2010). Like LPA_1, LPA_5 is involved in neuropathic pain (Lin et al., 2012). *Lpar6* was first identified as a gene responsible for autosomal recessive hypotrichosis (Pasternack et al., 2008).

Medaka fish (*Oryzias latipes*) are commonly used for experimental purposes, similar to zebrafish. Medaka belongs to a different taxonomic group from zebrafish in teleost phylogeny and can be easily maintained and propagated in a wider range of water temperatures than zebrafish (Kinoshita et al., 2009; Naruse et al., 2004; Wittbrodt et al., 2002). Moreover, females spawn 20–30 eggs per day and embryos hatch after 8–10 days. Adult medaka is sexually mature 3 months after hatching and shows fast vestibulo-ocular reflexes, optomotor tracking activities,

Abbreviations: LPA, lysophosphatidic acid; RT-PCR, reverse transcription-polymerase chain reaction; cDNAs, complementary DNA; RACE, rapid amplification of cDNA ends; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ANOVA, analysis of variance; AA, amino acid; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; BAP, bacterial alkaline phosphatase; PTX, pertussis toxin; Ola, *Oryzias latipes*; Dre, *Danio rerio*.

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and sophisticated social behaviors that have been closely analyzed (Kagawa, 2013, 2014; Kinoshita et al., 2009). The medaka genome has been determined by the whole genome shotgun method (Kasahara et al., 2007; Kobayashi and Takeda, 2008), and nine distinct sequences identified as LPA receptor-like genes were found in the GenBank database. However, whether all of these genes are expressed and functional in medaka remains to be determined. Here, we report the structures and expression of LPA receptor genes in medaka, and the functions of cloned LPA receptors.

2. Materials and methods

2.1. Maintenance of medaka

Medaka (d-rR) was maintained with a 14:10-h light:dark cycle at 25 °C and fed daily with brine shrimp eggs (A&A Marine, Salt Lake City, Utah). Water was changed twice a week. All experiments were performed in accordance with the guidelines and regulations established by the Animal Research Committee of Kinki University.

2.2. Bioinformatics

Medaka LPA receptor genes were identified in the NCBI database. Homology searches were performed using a homology search tool of GENETYX MAC® (Genetyx Corporation, Japan). Amino acid sequences of LPA receptor genes of medaka, zebrafish and mouse were aligned and used to infer a neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) on the MAFFT web service (http://mafft.cbrc.jp/alignment/server/) with the setting of WAG model (Kuraku et al., 2013).

2.3. RT-PCR

The whole brain, gill, air bladder, heart, liver, gut, testis, ovary, spleen, and muscle were dissected from adult medaka. Medaka embryos were collected the day before hatching. Total RNAs from these tissues were prepared using Tri reagent (Sigma, Tokyo, Japan). Total RNAs (1 μg) were treated with RNase-free DNase (Invitrogen, Tokyo, Japan), and reverse-transcribed with oligo(dT)12–18 and AMV reverse transcriptase (Roche, Tokyo, Japan). The resultant cDNAs, which were derived from 12.5 ng total RNA, were amplified by PCR using GoTaq DNA polymerase (Promega, Tokyo, Japan). The primers used (Operon Biotechnology, Tokyo, Japan) are summarized in Table 1 and primer combinations are described in the figure legends. The cycling protocol was 60 s at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C; and 7 min at 72 °C at the end of cycling to complete extension. The amplified products were then separated on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide.

2.4. 5′ Rapid amplification of cDNA ends (RACE) analysis for Lpar1, Lpar4, and Lpar6

5′ RACE was performed using a 5′/3′ RACE kit (Roche), according to the manufacturer's protocol. Brain- or embryo-derived total RNAs were reverse-transcribed to generate cDNA using the *Lpar1*, *Lpar4*, or *Lpar6*-specific antisense primers, Lpar1 75014-as2, Lpar4 73651-as3, or Lpar6 75288-as2, respectively. PolyA-tailed cDNA was generated using terminal transferase, and amplified using Accuprime DNA polymerase HiFi (Invitrogen) or GoTaq polymerase, and Oligo dT anchor primer and Lpar1 75014-as7, Lpar4 73651-as6, or Lpar6 75288-as1 for *Lpar1*, *Lpar4*, or *Lpar6*, respectively. When using the Accuprime DNA polymerase HiFi, the cycling protocol was 60 s at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 68 °C; and finally 7 min at 68 °C to complete extension. When using GoTaq polymerase, the cycling protocol was 60 s at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and finally 7 min at 72 °C to complete extension. The resulting PCR products were further subjected to a second round of PCR using a PCR anchor primer and Lpar1 75014-as6, Lpar4 73651-as2, or Lpar6 75288-as4 for *Lpar1*, *Lpar4*, or *Lpar6*, respectively. The second sets of PCR products were used for TA cloning into pGEM-T vectors (Promega) and the DNA sequences were determined (Operon Biotechnology).

Table 1
Primers used for RT-PCR or genomic PCR.

Gene or method	Sense (5′ → 3′)	Antisense (5′ → 3′)
Lpar1	olpar1 75014-s1 tggccaatctccttgccatt	olpar1 75014-as2 ggagcagaatgaccaaaccag
	Lpar1 75014-s8 gactatcagggatccgtccaagag	Lpar1 75014-as6 ccagtccagcaaagaagtcag
	Lpar1 75014-s10 tagaaggagagacgcgccagcaca	Lpar1 75014-as7 cctcgtgtttggtccagt
	Lpar1 75014-s11 agttgcatagggcaacatggtgg	Lpar1 75014-as8 accagcctgctgaccgtattccag
	Lpar1 75014-s14 tctatgtgaggcagagga	Lpar1 75014-as9 cctctcttggacggatccct
		Lpar1 75014-as10 tcctctccaggcagcgaaa
Lpar2a	olpar1 71363-s1 gtgaatgcgacgttagcaactg	olpar1 71363-as3 ctattgccaggcagtacttctc
Lpar2b	olpar1 66101-s1 cagctgcacagtgagatgag	olpar1 66101-as2 gttgcactcagctagaacca
Lpar3	olpar3 67863-s1 cgctctggagcgatacgtct	olpar3 67863-as2 ggtccaacagatcacaaaagcc
Lpar4	Lpar4 73651-s1 ccggacacaggaaaaggagt	Lpar4 73651-as1 cagaaccgtgatcagcttca
	Lpar4 73651-s2 ggttctgctggaattcacac	Lpar4 73651-as2 ccggacacaggaaaaggagt
	Lpar4 73651-s3 gtggttgactatcgtaggtg	Lpar4 73651-as3 atccagacagcagttgag
		Lpar4 73651-as6 aggtaggtcttccaggtgt
Lpar5b	Lpar5 76013-s1 cgtgtatcctctgaggtctcgt	Lpar5 76013-as1 acccttgcagactccttcag
Lpar6	Lpar6 75288-s1 tgaacagcaccacgaatg	Lpar6 75288-as1 gcagagagcgactggatag
		Lpar6 75288-as2 ggatgggcagagggtcaca
		Lpar6 75288-as4 agccacgacgttggtgatg
5′ or 3′ RACE	Oligo dT anchor primer gaccacgactatcgatgtcgactttttttttttttttt PCR anchor primer gaccacgactatcgatgtcgac	3sites Adaptor Primer ctgatctagaggtaccggatcc
Gapdh	medaka gapdh-F acctccactccacctaagca	medaka gapdh-R gcttcatgcactggaagaca

2.5. 3′ RACE analysis for Lpar1 and Lpar6

3′ RACE was performed using a 3′ RACE kit (Takara Bio, Kyoto, Japan). Brain-derived mRNAs were purified from total RNA using oligoTex (Takara Bio), and reverse-transcribed to generate cDNA using oligo dT-3sites adaptor primers. The resultant cDNA was then amplified using Accuprime DNA polymerase HiFi or GoTaq, and Lpar1 75014-s14 or Lpar6 75288-s1 and 3sites adaptor primers. When using the Accuprime DNA polymerase HiFi, the cycling protocol was 60 s at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 90 s at 68 °C; and finally 7 min at 68 °C to complete extension. When using GoTaq polymerase, the cycling protocol was 60 s at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 90 s at 72 °C; and finally 7 min at 72 °C to complete extension. The PCR products were further subjected to TA cloning using the pGEM-T vector and the DNA sequences were determined.

2.6. Genomic DNA isolation and determination of the new Lpar1 Exon

A small piece of medaka fin was incubated in lysis buffer (10 mM Tris–Cl, 100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecylsulfate, and 100 μg/ml proteinase K, pH 8) at 37 °C overnight and genomic DNAs were then extracted using phenol/chloroform, followed by ethanol precipitation with ammonium acetate (Strauss, 1998). To determine the