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The orphan G protein-coupled receptor 25 (GPR25) is activated by Apelin and Apela in non-mammalian vertebrates

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ABSTRACT

G protein-coupled receptor 25 (GPR25) is an orphan G protein-coupled receptor in vertebrates, that has been implicated to be associated with autoimmune diseases and regulate blood pressure in humans. However, the endogenous ligand of GPR25 remains unknown in vertebrates. Here, we reported that in non-mammalian vertebrates (zebrafish, spotted gars, and pigeons), GPR25 could be activated by Apelin and Apela peptides, which are also the two endogenous ligands of vertebrate Apelin receptor (APLNR). Using the pGL3-CRE-luciferase reporter assay and confocal microscopy, we first demonstrated that like APLNR, zebrafish GPR25 expressing in HEK293 cells could be effectively activated by zebrafish Apelin and Apela peptides, leading to the inhibition of forskolin-stimulated cAMP production and receptor internalization. Like zebrafish GPR25, pigeon and spotted gar GPR25 could also be activated by Apelin and Apela, and their activation could inhibit forskolin-induced cAMP accumulation. Interestingly, unlike zebrafish (/spotted gar/pigeon) GPR25, human GPR25 could not be activated by Apelin and Apela under the same experimental conditions. RNA-seq analysis further revealed that *GPR25* is expressed in a variety of tissues, including the testes and intestine of zebrafish/spotted gars/humans, implying the potential roles of GPR25 signaling in many physiological processes in vertebrates. Taken together, our data not only provides the first proof that the orphan receptor GPR25 possesses two potential ligands 'Apelin and Apela' and its activation decreases intracellular cAMP levels in non-mammalian vertebrates, but also facilitates to unravel the physiological roles of GPR25 signaling in vertebrates.

1. Introduction

Up to date, nearly 87 class A G protein-coupled receptors (GPCRs) are still classified as orphan GPCRs and their ligands have not yet been identified [1]. The G protein–coupled receptor 25 (GPR25) is an example of orphan receptors in the class A GPCR family. GPR25 was first cloned in 1997 and mapped on human chromosome 1 [2]. GPR25 is expressed in human memory T-cells and NK-cells and identified as a primary causal gene associated with autoimmune diseases revealed by *cis*-eQTL mapping based on a genome-wide association study (GWAS) [3]. Moreover, GPR25 is reported to be associated with arterial stiffness, and presumably, it may be capable of binding an unknown ligand to regulate blood pressure (BP) [4].

Despite the progress on understanding the potential roles of GPR25, the identity of its endogenous ligand remains unknown. Within the class A GPCR family, GPR25 shares a relatively high degree of amino acid sequence identity (29–34%) with vertebrate Apelin receptor (APLNR) [5]. It is clear that APLNR is activated by two distinct peptides, named Apelin and Apela (also known as Elabela and Toddler), which are encoded by two separate genes (*APELIN* and *APELA*), in humans [6], rats [7] and zebrafish [8,9].

Apelin is a peptide of 36 or 13 amino acids (designated as Apelin-36 and Apelin-13 respectively) and identified as the first endogenous ligand of APLNR [10]. Both Apelin and APLNR are reported to be widely expressed in the central nervous system (CNS) and peripheral tissues and involved in the regulation of many physiological/pathological processes, including the cardiovascular functions, angiogenesis, water balance and stress-induced disorders in vertebrates [11–13]. Apela is an alternative endogenous ligand of APLNR which was identified in zebrafish and mammals

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just recently. Apela is a peptide of 36 (Apela-36) or 22 (Apela-22) amino acids. Despite the low degree of amino acid sequence identity (25%) shared between Apelin and Apela peptides [14], both peptides can equipotently activate APLNR, which is functionally coupled to Gi protein and its activation decreases intracellular cAMP levels. In zebrafish, Apela is reported to be essential for cell movement during gastrulation and heart development [8,9]. In mammals, Apela is highly expressed in the kidneys of humans [6] and rats [7] and plays important roles in angiogenesis [6], fluid homeostasis [7], placental development [15] and cardiovascular development [16].

The structural similarity shared between APLNR and GPR25 led us to hypothesize that like APLNR, GPR25 could be activated by Apelin and/or Apela in vertebrates. To test this hypothesis, in this study, we cloned GPR25 from several representative vertebrate species, including zebrafish, pigeons, spotted gars and humans, and tested whether this receptor could be activated by Apelin and Apela *in vitro*. Our data, for the first time, demonstrated that the orphan receptor GPR25 can be activated by Apelin and Apela in non-mammalian vertebrates, and its activation can decrease intracellular cAMP levels. Our findings will help to unravel the physiological roles of GPR25 signaling in vertebrates.

2. Materials and methods

2.1. Chemicals and peptides

Chicken Apelin-36, Apela-32 (without a disulfide bond) and zebrafish Apelin-36, Apela-36 and Apela-22 (without a disulfide bond) peptides were synthesized (>95% purity) by GL Biochem (Shanghai, China) and their structures were verified by mass spectrometry. Dual-luciferase reporter assay system was purchased from Promega (Madison, Wisconsin). Primers used in this study were listed in Supplementary Table 1. All other experimental reagents were purchased from Sigma (Sigma-Aldrich, China).

2.2. Animal experiments

Zebrafish, pigeons and spotted gars purchased from local suppliers were sacrificed and various tissues were collected. All these animal experiments were conducted in accordance with the Guidelines for Experimental Animals issued by the Ministry of Science and Technology of the People's Republic of China. The experimental protocol used in this study was approved by the Animal Ethics Committee of College of Life Sciences, Sichuan University.

2.3. Cloning of zebrafish, spotted gar, pigeon and human GPR25 and construction of expression plasmids

Genomic DNA was extracted from human embryonic kidney 293 (HEK293) cells or from heart tissues of zebrafish, pigeon and spotted gar using a genome DNA extraction kit (Tiangen Biotech, China) following the manufacturer's instructions. According to the predicted (or reported) cDNA sequences of zebrafish (XM_021479994), pigeon (XP_005510493), spotted gar (XM_015342099), and human (NM_005298) *GPR25* deposited in the GenBank, gene-specific primers were designed to obtain the complete open reading frame (ORF) of GPR25 from these species. The ORF of GPR25 was amplified with the high-fidelity KOD DNA polymerase (Toyobo, Japan) using genomic DNA as the template (note: the coding regions of *GPR25* in these species are intronless). Then, PCR products were cloned into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA) and sequenced by Beijing Genome Institute (BGI).

Using the same approach, the expression plasmids encoding zebrafish APLNR (also named APLNRa, NM_001075105) and human APLNR (NM_005161) were also constructed in this study. For internalization assays, three pcDNA3.1 (+) expression plasmids encoding zebrafish GPR25-EGFP, human GPR25-EGFP and APLNR-EGFP were constructed by tagging EGFP to the C-terminus of GPR25 or APLNR.

2.4. Functional characterization of GPR25 in cultured HEK293 cells

According to the method established in our previous studies [17,18], the functionality of various cloned receptors was examined in HEK293 cells by a system of co-transfected pGL3-CRE-luciferase reporter construct and receptor expression plasmids. In brief, HEK293 cells transiently expressing GPR25/APLNR were treated with Apelin/Apela peptide for 6 h in the presence of forskolin (5 μM). Then, receptor-inhibited cAMP levels were monitored by a pGL3-CRE-Luciferase reporter assay [17,18].

2.5. Internalization assay by confocal microscopy

HEK293 cells cultured on six-well plates were transfected with 1 μg/well expression plasmid encoding zebrafish/human GPR25-EGFP (or human APLNR-EGFP). Then, cells were subcultured on poly-D-lysine coated glass coverslips in 24-well plates for 24 h. Cells were treated with serum-free DMEM medium or ligands (10 μM Apelin or Apela) for 30 min, and then fixed with 4% paraformaldehyde for 10 min. After fixation, cells were washed 3 times in cold PBS, and a subsequent 1-min incubation with 2 μg/ml DAPI in PBS was used to stain the nuclei. EGFP signals were examined by a Leica TCS SP5 II confocal microscope (Leica, Germany).

2.6. Statistical analysis

The luciferase activities in each treatment group were expressed as percentage compared with the control group. The data were analyzed by nonlinear regression followed by one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA). To validate the results, all these experiments were repeated at least 3 times.

3. Results

3.1. Cloning of GPR25 from zebrafish, pigeons and spotted gars

To identify the potential ligands for the orphan receptor GPR25 in vertebrates, we first cloned the coding region of *GPR25* from several representative vertebrate species, including zebrafish (MG732937), spotted gars (MG732939) and pigeons (MG732938) (Supplementary Figs. 1–3). The coding region of zebrafish, spotted gar and pigeon *GPR25* are predicted to encode a receptor of 377, 376 and 371 amino acids respectively, which are similar in length to human GPR25 (361 amino acids, NM_005298). Amino acid sequence alignment revealed that zebrafish GPR25 shows high amino acid identity with that of spotted gars (52%), pigeons (38%), coelacanth (45%) and humans (39%), and the highest identity was noted in the seven transmembrane domains (TM1-7). In addition, the conserved D/ERY motif known for G protein-coupling and two cysteine residues for a disulfide bond formation are also present in GPR25 of all vertebrate species examined (Fig. 1).

Interestingly, we also found that vertebrate GPR25 shares some degree of amino acid sequence identity with zebrafish/human APLNR (34%/29%) (Fig. 1).

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