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Cd36 is a candidate lipid sensor involved in the sensory detection of fatty acid in zebrafish

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ABSTRACT

Recently more and more evidences raise the possibility for the taste system in the role of the perception of lipids in mammals, and the fatty acid receptor CD36 has been proved to be as an important candidate receptor of fat taste. Fish has different taste modality with mammals. No information was known about the function of *cd36* in fish taste till now. Here, using in situ hybridization and immunofluorescence technologies, we showed that fish *cd36*/Cd36 localized in taste buds. Real-time PCR technology demonstrated that, in zebrafish *cd36* (*zcd36*)-transfected cells, linoleic acid (LA) increased the expression level of tryptophan hydroxylase-1 (*TPH-1*), which encodes the enzyme involved in the biosynthesis of monoamine neurotransmitter of 5-HT. Moreover, the LA-induced up-regulation expression of *TPH-1* was significantly curtailed by SSO, a specific inhibitor of LCFA binding to CD36, suggesting zCd36 is implicated in the LA-induced release of neurotransmitter. Importantly, we observed that *zcd36* gene knockout zebrafish reduced the preference for LA contrast to wild-type zebrafish. Together, our findings indicate that Cd36 is a candidate lipid sensor involved in the sensory detection of fatty acid in zebrafish.

1. Introduction

Taste acts as an important step for an animal's food acceptance or rejection behavior by evaluating the nutritious content and the overall pleasure of the food. It is generally accepted that five basic taste qualities are distinguished: sweet, umami, bitter, sour and salty. However, there is accumulating evidence for the existence of a fat “taste” mediated by a gustatory response to fatty acids in rodents [1–11]. Moreover, a fatty taste receptor function for CD36 (fatty acid translocase) is suggested by its localization in taste bud cells [1,2,12] and the lack of linoleic acid or attenuated triglycerides preference in CD36 knockout (KO) mice [12,13]. In addition, the role of CD36 as a taste-based lipid receptor is supported by the predicted structure of this protein. This plasma membrane protein has a hairpin structure, with a large extracellular hydrophobic pocket located between two short cytoplasmic tails [14].

CD36-mediated fat taste transduction pathway has also been uncovered [15,16]. Long-chain fatty acids (LCFA), by binding to CD36, will induce the phosphorylation of Src-PTK (protein tyrosine kinase), leading to a rise in intracellular free calcium concentration by calcium influx via the opening of store-operated calcium (SOC) channels. The interaction of LCFA and CD36 also increases IP3 concentration, which will recruit Ca^{2+} from the endoplasmic reticulum (ER) pool. An abundant increase in $[Ca^{2+}]i$ will induce exocytosis of 5-HT (5-hydroxytryptamine, serotonin) and NA (noradrenaline) into the extracellular medium, and the neurotransmitters in turn will activate afferent termination nerve fibers, which will transmit information on the quality and intensity of taste stimuli to the brain.

Taste perception varies enormously across different species of animals. For example, mice and humans perceive various sweet substances by only one T1R2/T1R3 heterodimer [17,18]. However, more than one T1R2 genes have been identified in fish, all of which act as receptors to amino acids but not to sugars [19,20]. In case of CD36, the identification and evolution have been reported in fish and amphioxus [21,22]. However, its role in fish taste is still unknown. In this study, the zebrafish was used as a model to explore whether the Cd36 function as a fat taste receptor. We revealed the localization of Cd36 in taste buds in fish and attenuated LA preference in *cd36* KO zebrafish, indicating the conservation of CD36 in taste signaling from fish to mammals.

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2. Materials and methods

2.1. Animals

Zebrafish (*Danio rerio*) and channel catfish (*Ictalurus punctatus*) purchased from local suppliers were used for in situ hybridization and immunohistochemistry. Embryos from the zebrafish AB line were used for the gene knockout. Artificial spawning and fertilization were performed as previously reported [23]. All animals used in the experiment followed the ethical guidelines established by the Institutional Animal Care and Use Committee of the Ocean University of China (permit number, SD2007695).

2.2. Three-dimensional structure prediction

The three-dimensional structure prediction of zebrafish Cd36 was performed by SWISS-MODEL online software at the Expert Protein Analysis System (http://www.expasy.org/) [24].

2.3. Total RNA extraction and reverse transcription

Total RNA extraction of tissues or embryo and reverse transcription were performed as described before [25]. Briefly, total RNA was extracted using RNAiso Plus (TaKaRa). The reverse transcription protocol is the following steps: DNaseI (RNase free) was incubated with the RNA sample at 37 °C for 30 min to digest any contamination of genomic DNA. Then EDTA was mixed into the tube in 65 °C for 10 min to inactivate the DNaseI. The oligo dT (2.5 mM, Promega) is used as the reverse transcription primer with the temperature 70 °C for 10 min. After 2 min in the ice, the rest of components (0.5 mM dNTPs, 5 × RT Buffer 2 μl, 12.5 U ribonuclease inhibitor, 12 U EasyScript RT RNase H-M-MLV in 10 μl volume) were mixed into the tube with the react condition 42 °C for 60 min, 70 °C for 15 min. Samples without reverse transcriptase were also added as control.

2.4. RT-PCR and real-time PCR analysis

According to the gene sequence, gene-specific primers for RT-PCR and real-time PCR were designed to amplify the specific cDNAs fragment. PCR amplification was performed as described by Liu et al. [22]. Real-time PCR reactions were performed using an ABI 7500 real-time PCR system (Applied Biosystems) as described by Huang et al. [25]. Triplicate reactions of each sample were performed. Relative gene expression levels were quantified using the comparative Ct method ($2^{-\Delta\Delta Ct}$) based on CT values [26]. All values were given as means and standard deviations (mean ± SD). One-way analysis of variance (ANOVA) followed by a Dunnett's two-tailed post-hoc test was used to determine differences among different groups. The primers sequences used are listed in Table 1.

2.5. zcd36-eGFP eukaryotic expression vector construction

The gene encoding the fluorescent protein eGFP was cloned into the plasmid expression vector *pcDNA3.1/V5-His* (Invitrogen) using the primers eGFP-S with the *XbaI* site and eGFP-AS with *ApaI* sites (Table 1), and the plasmid was designated *pcDNA3.1/eGFP*. A pair of specific primers zcd36-ORF-S with *EcoRI* site and zcd36-ORF-AS with *XhoI* site (Table 1) was used to clone the *cd36* ORF from zebrafish. Subsequently, the cloned zebrafish *cd36* ORF, which the stop codon was already removed, was sub-cloned into the plasmid expression vector *pcDNA3.1/eGFP*. The recombinant eukaryotic expression vector was designated *pcDNA3.1/zcd36/eGFP*.

2.6. Cell transfection and fluorescent microscopy

HEK 293T cells were the gifts of Jianfeng Zhou, Laboratory of Molecular Medicine, School of Medicine and Pharmacy, Ocean University of China. The cells were maintained with DMEM medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen). To examine the subcellular localization of zCd36, HEK 293T cells were seeded in 6-well plates and transfected with the different plasmids (*pcDNA3.1/eGFP* or *pcDNA3.1/zcd36/eGFP*) using Lipofectamine 2000 Reagent (Invitrogen) according to the instructions of the manufacturer. Twenty-four hours after transfection, the cells were observed under confocal microscopy (Leica TCS-SP8).

Table 1
Sequences of the primers used in this study (*EcoR I*, *Xho I*, *Xba I* and *Apa I* sites are underlined).

Primer	Primer sequence(5′-3′)	Sequence information
cd36-P1-S	TGAACGCTGGCAAGGTGAAACAT	zcd36 probe primer
zcd36-P1-AS	TCAAACCGATCATACAGCCCAGA	zcd36 probe primer
zcd36-ORF-S	CGGAATTCATGACCTGCTGCGATC	ORF primer
zcd36-ORF-AS	CCGCTCGAGAGAAAGATTCGTTTT	ORF primer
eGFP-S	GCTCTAGAATGGTGAGCAAGGGCG	Recombinant primer
eGFP-AS	AGAGGGCCCGATTACTTGTACAGCT	Recombinant primer
zcd36-actin-S	CCGTGACATCAAGGAGAAGC	Real-time PCR primer
zcd36-actin-AS	TACCGCAAGATTCCATACCC	Real-time PCR primer
HEK-actin-S	TGACCCTGAAGTACCCCATCGA	Real-time PCR primer
HEK-actin-AS	CCTGTTGGCTTTGGGGTTCAG	Real-time PCR primer
TPH-1-S	GTTGTCAGTGATGCCCTTGCTA	Real-time PCR primer
TPH-1-AS	TGTCCCTCCAGGATCAGGTGTT	Real-time PCR primer
zcd36-S	CCAAACCTCTTCGAGGCACAAGG	Real-time PCR primer
zcd36-AS	CACAACAACTGACGCGGCCTG	Real-time PCR primer
zcd36-SX1-S	GCTCACAGTGTCAAGGTCAAT	Nested PCR primer of TALEN
zcd36-SX1-AS	TTCCTCTAGGTGCTTCGGT	Nested PCR primer of TALEN
zcd36-SX2-S	TGTTAAATGAGCTGGGTGTT	Nested PCR primer of TALEN
zcd36-SX2-AS	AAAAGGGACTAATCCAAAGG	Nested PCR primer of TALEN

S and AS represent sense and antisense primers, respectively.

2.7. In situ hybridization histochemistry

Designed according to zebrafish cd36 sequences, the specific primers (Table 1) produced 710 bp of the fragments. RNA-probes were prepared using DIG-RNA Labelling Kit (SP6/T7) (Roche). The in situ hybridization on zebrafish jaw tissue was performed according to the method as described previously [22]. Briefly, zebrafish jaw tissue was embedded in paraffin, and sectioned at 10 μm. They were hybridized in the hybridization buffer with 1 μg/ml DIG-labeled *zcd36* riboprobes at 55 °C for 16 h in a moist chamber. The sections were incubated with anti-Dig alkaline phosphatase conjugated antibody (Roche) diluted 1:1000 for 2 h at room temperature. After coloring reaction, the sections were mounted in Canada balsam, and photographed under a BX51 Olympus microscope.

2.8. Immunohistochemistry

Rabbit anti-rat CD36 antibody (sc9154) was purchased from Santa Cruz Biotechnology (USA). Immunohistochemical staining was performed as described by Li et al. [27]. Briefly, channel catfish barbels were embedded in paraffin, sectioned at 10 μm. The sections were pre-incubated with 5% BSA in 10 mM PBS (pH 7.4) at room temperature for 30 min, and then incubated overnight in a humidified chamber at 4 °C with Rabbit anti-rat CD36 antibody diluted 1:400. For the channel catfish barbel sections, TRITC-conjugated goat anti-rabbit IgG (1:100