

Article

Molecular Cloning, Tissue Distribution, and Pharmacological Characterization of GPR84 in Grass Carp (*Ctenopharyngodon Idella*)

Yang Li ^{1,*}, Wei-Jia Song ^{1,†}, Shao-Kui Yi ², Hui-Xia Yu ¹, Hao-Lin Mo ¹, Ming-Xing Yao ¹, Ya-Xiong Tao ³ and Li-Xin Wang ¹

¹ College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China; songvega@163.com (W.-J.S.); yuhuixia@nwsuaf.edu.cn (H.-X.Y.); 2017010832king@nwsuaf.edu.cn (H.-L.M.); ymx1114@nwsuaf.edu.cn (M.-X.Y.); fisherwanglx@nwsuaf.edu.cn (L.-X.W.)

² College of Life Sciences, Huzhou University, Huzhou 313000, China; 02844@zjhu.edu.cn

³ Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, USA; taoyaxi@auburn.edu

* Correspondence: yangli2001@nwsuaf.edu.cn

† These authors contributed equally to this work.

Simple Summary: GPR84 is a G protein-coupled receptor expressed in immune cells. In mammals and amphibians, it is activated by medium-chain fatty acids and plays critical roles in inflammation and eye development. However, our understanding of GPR84 in fish remains limited. In this study, we successfully cloned the coding sequence of grass carp GPR84, revealing its high expression in the liver and spleen. In cells transfected with ciGPR84, we observed its responsiveness to medium-chain fatty acids such as capric acid, undecanoic acid, and lauric acid. Surprisingly, ciGPR84 did not respond to a synthetic activator called diindolylmethane. Notably, we found that lauric acid and capric acid exhibited the strongest activation and inhibition of ERK and cAMP signaling, respectively, suggesting their potential as immune modulators. These findings provide valuable insights for mitigating chronic inflammation in farmed fish, especially grass carp.



Citation: Li, Y.; Song, W.-J.; Yi, S.-K.; Yu, H.-X.; Mo, H.-L.; Yao, M.-X.; Tao, Y.-X.; Wang, L.-X. Molecular Cloning, Tissue Distribution, and Pharmacological Characterization of GPR84 in Grass Carp (*Ctenopharyngodon Idella*). *Animals* **2023**, *13*, 3001. <https://doi.org/10.3390/ani13193001>

Academic Editors: Øivind Bergh, László Ardó and Janka Nagyné Biró

Received: 15 July 2023

Revised: 9 September 2023

Accepted: 20 September 2023

Published: 23 September 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: The G-protein-coupled receptor GPR84, activated by medium-chain fatty acids, primarily expressed in macrophages and microglia, is involved in inflammatory responses and retinal development in mammals and amphibians. However, our understanding of its structure, function, tissue expression, and signaling pathways in fish is limited. In this study, we cloned and characterized the coding sequence of GPR84 (ciGPR84) in grass carp. A phylogenetic analysis revealed its close relationship with bony fishes. High expression levels of GPR84 were observed in the liver and spleen. The transfection of HEK293T cells with ciGPR84 demonstrated its responsiveness to medium-chain fatty acids and diindolylmethane (DIM). Capric acid, undecanoic acid, and lauric acid activated ERK and inhibited cAMP signaling. Lauric acid showed the highest efficiency in activating the ERK pathway, while capric acid was the most effective in inhibiting cAMP signaling. Notably, DIM did not activate GPR84 in grass carp, unlike in mammals. These findings provide valuable insights for mitigating chronic inflammation in grass carp farming and warrant further exploration of the role of medium-chain fatty acids in inflammation regulation in this species.

Keywords: GPR84; medium-chain fatty acid; grass carp; expression; signaling

1. Introduction

Fatty acids were traditionally viewed as metabolic intermediaries that primarily serve as an energy source. However, mounting evidence in recent years has unveiled their pivotal role as endogenous signaling molecules, orchestrating a wide range of physiological processes. A growing body of literature demonstrates that fatty acids possess the ability

to regulate insulin secretion, impact cellular apoptosis, and modulate inflammatory and immune responses [1,2]. Free fatty acids include short-chain fatty acids (SCFAs) (C1–C8), medium-chain fatty acids (MCFAs) (C9–C14), and long-chain fatty acids (LCFAs) (more than C14). SCFAs in animals, primarily produced by intestinal microorganisms fermenting cellulose, improve inflammatory diseases and allergic asthma, and promote the maturation of T helper cells [3,4]. MCFAs, ingested directly from food [5], have been shown to stimulate the differentiation of Th1 and Th17 cells, thereby promoting an inflammatory response [6]. LCFAs, derived from milk and aquatic products, inhibit macrophage-induced tissue inflammation, with anti-inflammatory activity [3,7]. Fatty acids primarily exert the aforementioned physiological functions through a class of cell membrane-bound G protein-coupled receptors (GPCRs), known as free fatty acid receptors (FFARs). Upon sensing fatty acid signals, FFARs activate the second messenger system, thereby triggering physiological regulatory effects [1]. Nature has developed distinct FFARs to bind fatty acids with different carbon chain lengths. For example, GPR43 is a SCFA receptor that can affect T-helper cell function [8] and leukocyte chemotaxis [9]. GPR120 is a LCFA receptor that participates in the regulation of obesity-related inflammation after being activated by omega-3 fatty acids [10]. The GPR84 receptor is an MCFA receptor promoting inflammation [11]. A recent study showed that GPR84 is highly expressed in the inflamed colon tissues of active ulcerative colitis (UC) patients and dextran sulfate sodium (DSS)-induced colitis mice, and that the knockout of GPR84 could help mice resist the development of colitis induced by DSS [12]. In addition, under non-pathological conditions, GPR84 is highly expressed in activated mouse macrophages [13] and is significantly increased in response to lipopolysaccharide (LPS) stimulation [14]. Th2 cytokines, such as IL-4 with anti-inflammatory activity, are increased in T cells from GPR84 knockout mice [15].

The pharmacology of GPCRs is a widely researched topic due to their ubiquitous presence and importance as drug targets [16]. This field of study aims to investigate the interactions and effects of ligands or drugs with GPCRs, encompassing a comprehensive understanding of the activation and signal transduction mechanisms of GPCRs, as well as the binding mechanisms of ligands to GPCRs, and the modulation of their activities [17]. Pharmacological investigations of fish GPCRs were initiated by the pioneering work of Richard Peter, as reported in Cerdá-Reverter et al. [18]. Peter and his colleagues transfected the goldfish melanocortin-4 receptor (MC4R) into human embryonic kidney 293 (HEK293) cells, and used radiolabeled [Nle4, D-Phe7]- α -MSH (NDP-MSH), a synthetic potent agonist of MC4R, to probe the binding activity of goldfish MC4R and its downstream effect on cAMP concentration. Subsequently, other researchers employed HEK-293 cells and Chinese hamster ovary (CHO) cells to investigate the pharmacology of diverse fish GPCRs, including melanocortin receptors in species such as the channel catfish (*Ictalurus punctatus*) [19], spotted scat (*Scatophagus argus*) [20], grass carp (*Ctenopharyngodon idella*) [21], snakehead (*Channa argus*) [22], ricefield eel (*Monopterus albus*) [23], topmouth culter (*Culter alburnus*) [24], rainbow trout (*Oncorhynchus mykiss*) [25], and elephant shark (*Callorhynchus milii*) [26]. These studies, conducted in mammalian cells as a platform, not only elucidated the functional regulation of fish GPCRs on downstream signaling, but also revealed distinct characteristics of fish GPCRs, such as higher constitutive activity compared to their human orthologs [27].

In addition to MCFAs, diindolylmethane (DIM) [28], embelin [29], and 6-N-octylamino uracil (6-OAU), are all alternative agonists of GPR84, and 6-OAU is the most potent agonist [14]. 6-OAU rapidly induces microglia cell folding and increases microglia cell movement by stimulating mouse GPR84 [30]. The inflammatory mediator CXCL1 is increased in rats injected with 6-OAU [31]. These experiments further demonstrated that the function of GPR84 is associated with inflammation and macrophage activity. Human and mouse GPR84 is coupled to Gi/o protein, with receptor activation resulting in decreased intracellular cAMP accumulation, which is inhibited by pertussis toxin [11]. When stimulated by 6-OAU, GPR84 also increases the phosphorylation of Akt and ERK and the nuclear translocation of NF- κ B in macrophages and promotes inflammation by increasing the expression of key cytokines and chemokines [14].

Compared with studies in mammals, there are only three reports on fish GPR84. Huang et al. [32] reported that zebrafish GPR84 is involved in the accumulation of lipid droplets and can up-regulate the expression of IL-12 p40 upon undecanoic acid treatment. The authors of [33] showed that the overexpression of zebrafish GPR84 in the RAW264.7 cell line can enhance phagocytic activity. Another study found that the brain of rainbow trout can sense MCFAs such as octanoate through GPR84, which can subsequently affect their food intake [34]. These findings suggest that, akin to mammals, GPR84 plays a role in regulating the immune system of fish and may also be partially involved in maintaining energy homeostasis.

GPR84 plays a crucial role in regulating the immune system of fish, making it an attractive target for drug development and physiological regulation to mitigate the incidence of metabolic diseases in farmed fish. Among the fish species affected by metabolic inflammation, the grass carp stands out as a significant example. The grass carp holds great economic importance in China, boasting the highest yield globally [35]. However, due to high-density culture practices and the widespread use of compound feed, cultured grass carp often experience chronic inflammation accompanied by various metabolic disorders. Consequently, investigating the potential involvement of MCFAs in regulating chronic inflammation in grass carp becomes an area of significant interest. In this study, we successfully cloned the full-length coding region of grass carp GPR84, determined its tissue expression pattern, and demonstrated its capacity to modulate downstream signaling pathways upon MCFA stimulation.

2. Materials and Methods

2.1. Chemicals, Reagents, and Plasmids

To elucidate the pharmacological properties of grass carp GPR84 (ciGPR84), we employed DIM as a representative agonist, known for its potent activation of GPR84 in mammalian systems. Additionally, considering their potential endogenous role, we investigated the activation potency of three commonly occurring MCFAs in gastrointestinal tracts: capric acid, undecanoic acid, and lauric acid. These chemicals were obtained commercially from Shanghai Macklin Biochemical Technology (Shanghai, China), and their purities are as follows: DIM (purity $\geq 98\%$), capric acid (purity $\geq 99.5\%$), undecanoic acid (purity $\geq 99\%$), and lauric acid (purity $\geq 99.5\%$).

Since the aforementioned compounds are insoluble in water, they were initially dissolved in 3% dimethyl sulfoxide (DMSO), then coated with 2% bovine serum albumin (BSA) to create a stock solution with a concentration of 10 mM. Prior to use, the stock solution was diluted to the working concentration using a serum-free DMEM medium. The concentration of fatty acids in the solution was detected using a Tecan F200 microplate reader (Tecan, Männedorf, Switzerland) through colorimetric analysis. The luciferase assay kits were obtained from Beyotime Biotechnology (Shanghai, China). The plasmids pGL4.29 and pGL4.33 used for examining signaling were obtained from Promega (Madison, WI, USA).

2.2. Total RNA Extraction and cDNA Synthesis

Grass carp specimens were obtained from Western Orchid Ecological Park (Yangling, China). A total of 9 grass carp individuals, with an average body length (SD) of 35.2 ± 4 cm and an average weight (SD) of 674.4 ± 57 g, were included in this study. Prior to sacrifice, the grass carp were temporarily reared for three days. Due to the immaturity of the grass carp used in this study, it was not feasible to differentiate their sexes based on their gonads. The fish were anesthetized using a 1:500 dilution of 2-phenoxyethanol, followed by prompt euthanasia and subsequent dissection. Tissues including kidney, brain, heart, gill, liver, spleen, intestines, and muscle were collected for RNA extraction. For the RNA extraction, tissues of the same type from different individuals were combined to create a pooled sample. Total RNA was extracted using the RNA pure Tissue & Cell Kit (CW BIO, Shanghai, China). The quality of the RNA extraction was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and a minimum

OD260/280 ratio of 1.9 was considered satisfactory for quality assurance. To remove any DNA contamination, a Turbo DNase kit (Ambion, Austin, TX, USA) was utilized, and the resulting RNA was employed for cDNA synthesis.

The RNA was reverse transcribed into cDNA using the Revert Aid RT Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). For the reverse transcription process, a premix containing 5 µg of total RNA and 1 µL of oligo-deoxythymidine was prepared in a total volume of 12 µL. The mixture was incubated at 70 °C for 4 min and immediately cooled on ice for 2 min. Subsequently, 5× reaction buffer, 20 mM deoxynucleotide triphosphate (dNTP), and 200 U of Moloney murine leukemia virus reverse transcriptase (M-MLV) were added to the mixture, bringing the final reaction volume to 20 µL. The reverse transcription reaction was carried out at 42 °C for 1 h, followed by heating at 70 °C for 5 min to inactivate the reaction. The resulting cDNA products were stored at −20 °C and used for subsequent molecular cloning and tissue-specific quantitative expression studies of *ciGPR84*.

2.3. Molecular Cloning of Grass Carp *GPR84*

The predicted nucleotide sequence of grass carp *gpr84* used for designing primers was acquired from the Grass Carp Genome Database [36]. The PCR procedure was performed at the following conditions: 95 °C for 5 min, followed by 32 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, as well as a final 10 min extension at 72 °C. The primer sequences are shown in Table 1 and were synthesized by AUGCT (Beijing, China). The PCR product containing the entire open reading frame (ORF) was subcloned into the pcDNA3.1(+) (*pciGPR84*) and used for transfection.

Table 1. Primers used for cloning and RT-PCR.

Primer Name	Primer Sequence (5'-3')	Product Size (bp)	Ta ¹ (°C)
<i>ciGPR84</i> -F	ATGTGGAACGAGACGGA	1224	53
<i>ciGPR84</i> -R	AGCTTTAGGTCATATTCGGA		
qPCR-F	CTGATAGTAAATCTTGCCCTTCGC	194	60
qPCR-R	ACAACCAGGTAACGGCTCATAG		
<i>ciβ-actin</i> F	CGTGACATCAAGGAGAAG	215	57
<i>ciβ-actin</i> R	GAGTTGAAGGTGGTCTCAT		

¹ Ta, annealing temperature.

2.4. Homology, Phylogenetic, and Chromosome Synteny Analysis of *ciGPR84*

Multiple alignments of amino acid sequences of *GPR84*s from different species were performed with the ClustalW multiple sequence alignment program by MEGA X software. The putative disulfide bridges of *ciGPR84* were predicted by PROSITE (<https://prosite.expasy.org/>, accessed on 22 April 2022). The putative transmembrane domains (TMDs) of *ciGPR84* were predicted by the Conserved Domain Search Service (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, accessed on 22 April 2022). A phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA X software (version 10.2), based on alignments of amino acid sequences of *GPR84*. Bootstrap values were estimated from 1000 replicates to assess the robustness of the tree. A chromosome synteny analysis was performed between several fish and mammalian species with the Genomicus (<http://www.genomicus.biologie.ens.fr/genomicus>, accessed on 22 April 2022) and National Center for Biotechnology Information (NCBI) genome browser (<https://www.ncbi.nlm.nih.gov>, accessed on 22 April 2022).

2.5. Real-Time PCR for Tissue Expression

The expression of grass carp *gpr84* mRNA in various tissues was assessed using a real-time PCR (RT-PCR) technique. The primers for the RT-PCR procedure, synthesized by AUGCT (Beijing, China), are listed in Table 1. The PCR cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for

10 s and annealing/extension at 60 °C for 30 s. A dissociation curve analysis was performed to confirm the specificity of the amplified products. To normalize the mRNA expression across different tissues, the housekeeping gene *actb* (encoding β -actin) was used as an internal control. The relative expression levels of grass carp GPR84 were calculated using the comparative threshold cycle method ($2^{-\Delta\Delta C_t}$) [37].

2.6. Cell Culture

Pharmacological studies of GPCRs generally utilize mammalian cells to avoid the presence of endogenous GPCRs and achieve higher expression efficiency on the cell membrane. HEK293 and its derivative cell lines, such as HEK293T, as well as CHO cells, are the most commonly used cell lines in this field of research [19,22,26,38]. In this study, we employed HEK293T cells as the research platform according to a method we established previously [39].

Human embryonic kidney cells HEK293T obtained from Beyotime Biotechnology (Shanghai, China) were cultured with Dulbecco's Modified Eagle Medium (DMEM) containing a 10% fetal bovine serum in an incubator (37 °C and 5% CO₂ humidified atmosphere). The cells were plated into 24-well plates and cultured for 24 h before assays. The Countess 3 cell counter (Invitrogen, Waltham, MA, USA) was used for cell counting.

2.7. Functional Characterization of ciGPR84 in Cultured HEK293 Cells

The functionality of ciGPR84 was assessed using our established method [40]. Specifically, the coding sequence (CDS) of ciGPR84 was subcloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA), resulting in the construction of pcDNA3.1-ciGPR84 (pciGPR84). HEK293T cells were co-transfected with pciGPR84 (or pcDNA3.1 empty vector), two luciferase reporter vectors (pGL4.29 and pGL4.33) containing a cAMP response element (CRE) and serum response element (SRE), respectively, and pEGFP-N1 (as an internal control for transfection normalization) in a mixture with polyethyleneimine (PEI) transfection reagent (Fusheng Biotechnology, Shanghai, China). The cells were then incubated in the original medium for 24 h, transferred to a 48-well plate, and grown for an additional 24 h to reach a cell density of 2×10^5 cells per well. For cells transfected with pGL4.29 and pGL4.33, four ligands (DIM, capric acid, undecanoic acid, and lauric acid) were diluted to working concentrations in a serum-free DMEM medium and added to the 48-well plates to treat the cells for 6 h. It is important to note that due to the anticipated inhibitory effect of MCFA on the intracellular cAMP, 5 μ M forskolin was added to all wells transfected with pGL4.29 to pre-activate the cellular adenylyl cyclase.

After the treatment, cells were lysed with $1 \times$ passive lysis buffer (Beyotime Biotechnology, Shanghai, China), and a luciferase substrate was added to initiate a glow-generating reaction. The luminescence signals were measured using a Tecan F200 (Tecan, Männedorf, Switzerland) microplate reader to determine the relative luciferase activity in each well. Three independent experiments were performed for each agonist concentration ($n = 3$), and the data were presented as a mean \pm SEM.

2.8. Statistical Analysis

According to our previously established method [40], the luciferase activities were converted to fold change relative to the control group (DMEM serum-free medium) and fitted to a dose-response curve using a nonlinear regression analysis. A data analysis was performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Analysis of the Full Length of the Sequence

The ciGPR84 sequence contains an ORF of 1224 bp encoding 407 amino acids (GenBank Accession No. MW535309), and the molecular weight is 45.37 kDa. The structure of ciGPR84 has seven typical TMDs. The *N*-glycosylation site prediction shows that ciGPR84 has three Asn sites at 3, 10, and 359. A3 and A10 are outside the plasma membrane, which are highly

likely glycosylation sites (Figure 1). The results of the disulfide bond prediction show that a disulfide bond could be formed between C97 and C174. The TMD3 and TMD7 of ciGPR84 contain a conserved SRY motif and NPxxY motif, respectively. The results of the multi-sequence alignment showed that, compared with mammals, fish GPR84 was relatively conserved in the TMDs and the extracellular loops, but showed low similarity in the intracellular loops. We found that three amino acids essential for decanoic acid binding in human GPR84 (L100, F101, and N104) are also conserved in ciGPR84 (L104, F105, and N108). Similarly, other residues (Y69, Y81, Y186, H352) that have hydrogen bonding capability in human GPR84 and may participate in the formation of ligand-binding pockets can also be found in ciGPR84 (Y73, Y85, Y192, and H354) (Figure 2).

1	ATG	TGG	AAC	GAG	ACG	GAT	CCT	CTG	AGA	AAT	GAT	ACC	TTT	TCT	TGT	GCG	TCC	CCT	TCT	GTG	60
1	M	W	N	E	T	D	P	L	R	N	D	T	F	S	C	A	S	P	S	V	20
61	GAG	GGC	TAC	CGT	TAT	TTT	GGC	GTC	CTC	TTG	GGA	TCG	GCT	GTG	ACC	ATA	GTA	GGA	ACC	ATA	120
21	E	G	Y	R	Y	F	G	V	L	L	G	S	A	V	T	I	V	G	T	I	40
121	GGG	AAT	ATT	CTG	ACG	GTG	CTC	GCC	TTT	GCT	ACT	GAT	GTC	AAC	CTG	AGG	ACG	CGT	TTC	AAC	180
41	G	N	I	L	T	V	L	A	F	A	T	D	V	N	L	R	T	R	F	N	60
181	GTT	CTG	ATA	GTA	AAT	CTT	GCC	TTC	GCT	GAC	CTC	CTA	TAC	TGC	ACC	ATG	CTC	CAA	CCT	GTC	240
61	V	L	I	V	N	L	A	F	A	D	L	L	Y	C	T	M	L	Q	P	V	80
241	AGT	GTT	GAC	TCG	TAT	CTG	CAC	CTG	CAC	TGG	AGG	GGC	GGA	GCC	ACA	TGG	TGC	CAG	ATG	TTT	300
81	S	V	D	S	Y	L	H	L	H	W	R	G	G	A	T	W	C	Q	M	F	100
301	GGA	TTC	CTA	CTG	TTC	CTG	TCC	AAC	AGC	GTG	TCT	ATT	ATT	ACA	TTA	TGC	CTC	ATC	GCT	ATG	360
101	G	F	L	L	F	L	S	N	S	V	S	I	I	T	L	C	L	I	A	M	120
361	AGC	CGT	TAC	CTG	GTT	GTT	GCG	CAT	CGC	ACC	TCT	CGC	TGT	GCC	CGT	CTG	CTC	CTA	TCC	CAH	420
121	S	R	Y	L	V	V	A	H	R	T	S	R	C	A	R	L	L	S	H	140	
421	CGT	GGA	GTG	GCG	GTG	CTC	CTC	ATC	TCC	TCC	TGG	GTA	CTA	GGT	GTG	GCC	AGT	TTC	GGC	CCA	480
141	R	G	V	A	V	L	L	I	S	S	W	V	L	G	V	A	S	F	G	P	160
481	CTT	TGG	CCT	GTC	TAC	GTG	TTC	GCC	CCG	CAA	GTG	TGC	ACC	TGC	AGT	TTT	CAC	CGC	ACT	AAG	540
161	L	W	P	V	Y	V	F	A	P	Q	V	C	T	C	S	F	H	R	T	K	180
541	GGC	CGG	CCT	TAC	ACC	ACT	GTG	CTT	CTT	TTC	ATG	TAC	TTC	TTC	CTG	GGC	TTG	GGT	TGC	GTG	600
181	G	R	P	Y	T	T	V	L	L	F	M	Y	F	F	L	G	L	G	C	V	200
601	GGT	CTT	TTT	TAC	TTT	CTG	ATT	TAC	CGT	AAG	GTG	AGC	GTA	GCC	GCC	AAA	GCA	TTC	CTG	AAG	660
201	G	L	F	Y	F	L	I	Y	R	K	V	S	V	A	A	K	A	F	L	K	220
661	TAC	AGG	CCC	AGT	CGT	CGC	TCA	TCA	AAA	CGC	AAG	CAG	GCT	GAA	GCC	GAA	GGG	ACG	ACG	GAT	720
221	Y	R	P	S	R	R	S	S	K	R	K	Q	A	E	A	E	G	T	T	D	240
721	GAC	AGC	GGG	ATC	AGC	GCC	ACA	GCC	TCA	ACA	ACT	CAA	AGC	TGT	GAG	ATC	AGC	AGT	GAT	GGG	780
241	D	S	G	I	S	A	T	A	S	T	T	Q	S	C	E	I	S	S	D	G	260
781	GCG	GGA	GCA	GAG	CAG	CAT	AAA	AAC	AGA	GCG	CAA	GAA	GGC	CAC	ACA	ACC	ACA	CAA	GAG	TCA	840
261	A	G	A	E	Q	H	K	N	R	A	Q	E	G	H	T	T	T	Q	E	S	280
841	AAA	AAC	TCC	ATT	CAA	AGT	GCC	TCC	AAT	GAG	GTA	AAA	ACA	TCT	TCT	AAA	CCA	GCC	CGG	GTA	900
281	K	N	S	I	Q	S	A	S	N	E	V	K	T	S	S	K	P	A	R	V	300
901	GTA	ATC	CAG	ACC	ACT	CCA	GCT	GCC	AAC	TCA	GGA	GAG	GAC	AGT	GAA	TTT	AAA	CGC	GTG	ACT	960
301	V	I	Q	T	T	P	A	A	N	S	G	E	D	S	E	F	K	R	V	T	320
961	CGG	ATG	TGT	TTC	ACA	GTC	TTT	TTG	TGT	TTT	GTT	GGC	TGC	TTT	GCA	CCA	TTT	CTG	CTG	CTA	1020
321	R	M	C	F	T	V	F	L	C	F	V	G	C	F	A	P	F	L	L	L	340
1021	AGT	GTT	GCC	GAT	AAG	GCA	AAT	CGC	GCA	CCA	CAG	GTC	GTT	CAT	ATG	TTT	TGT	GCA	AAC	CTC	1080
341	S	V	A	D	K	A	N	R	A	P	Q	V	V	H	M	F	C	A	N	L	360
1081	ACT	TGG	TTA	AGT	AGT	TGC	ATT	AAC	CCT	TTG	TTG	TAT	GCC	GCC	ATG	AAC	CGG	CAG	TTT	AAC	1140
361	T	W	L	S	S	C	I	N	P	L	L	Y	A	A	M	N	R	Q	F	N	380
1141	CAG	GCC	TAC	AAA	GAC	CTA	CTA	AGC	AAA	GCT	GTA	CAT	CCA	CTA	ACC	TGG	ATA	TGG	AGA	ACC	1200
381	Q	A	Y	K	D	L	L	S	K	A	V	H	P	L	T	W	I	W	R	T	400
1201	CGC	CGT	TTC	ACA	ATA	AAG	CGA	TAA													1224
401	R	R	F	T	I	K	R	*													407

Figure 1. Nucleotide and deduced amino acid sequence of ciGPR84. Positions of nucleotide and amino acid sequences are marked on both sides. The seven TMDs are shaded in grey. The conserved motifs, SRY and NPxxY, are highlighted in black. Open boxes frame the presumptive *N*-glycosylation sites, and the boxes outlined by dash lines indicate the cysteines that possibly form disulfide bonds. Asterisk (*) denotes stop codon.

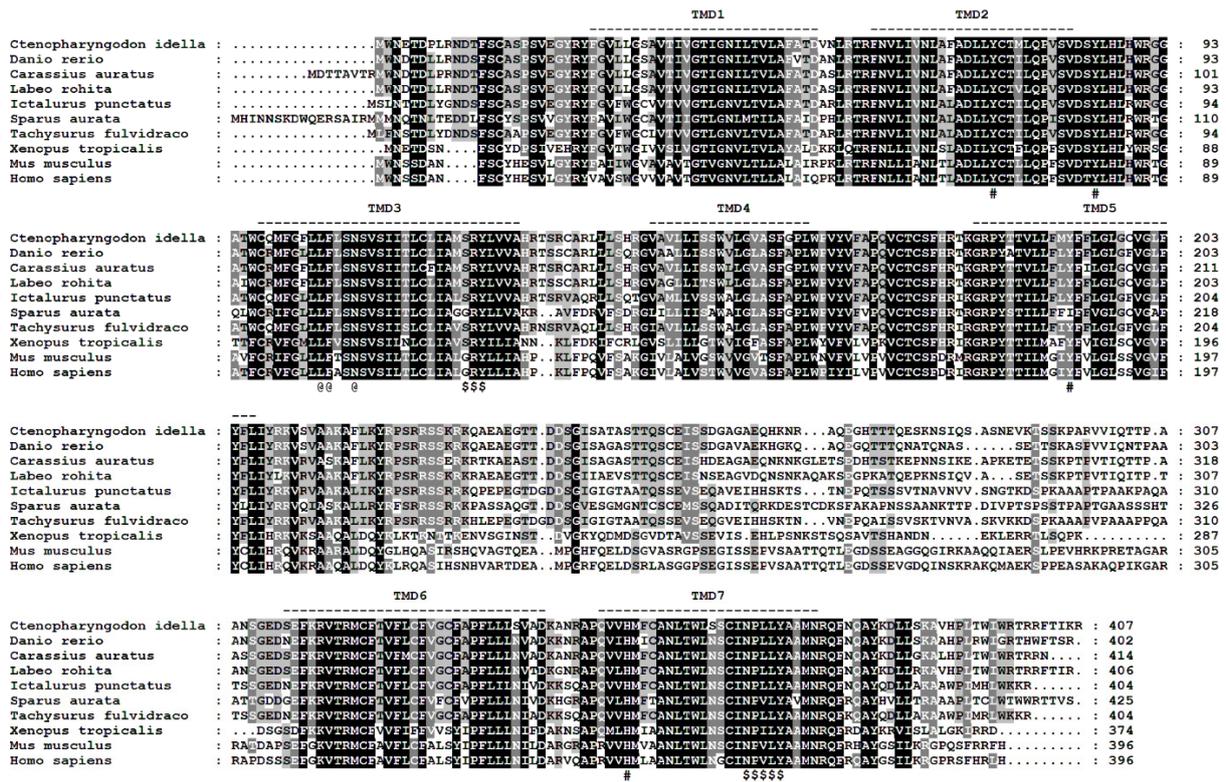


Figure 2. Multiple amino acid sequence alignments of GPR84s. Dashed lines represent the seven TMDs. The black shading represents amino acid sequences with 100% identity, while the grey shading indicates sequences with identity greater than 60% in the amino acid sequence alignment. @ indicates the amino acid residues essential for decanoic acid binding. # denotes amino acid residues that are capable of hydrogen bonding and may participate in the formation of the ligand-binding pockets. \$ represents the conserved SRY and NPxxY motifs. GenBank accession numbers of the GPR84s: *Ctenopharyngodon idella* (MW535309), *Danio rerio* (NP_001099167.1), *Carassius auratus* (XP_026115535.1), *Labeo rohita* (RXN07797.1), *Ictalurus punctatus* (XP_017336000.1), *Sparus aurata* (XP_030274675.1), *Tachysurus fulvidraco* (XP_027029919.1), *Xenopus tropicalis* (NP_001072335.1), *Mus musculus* (NP_109645.1), *Homo sapiens* (NP_065103.1).

3.2. Phylogenetic and Synteny Analysis of ciGPR84

We performed a phylogenetic analysis of ciGPR84 and other GPR84s. Grass carp GPR84 was more closely related to those of rohu, crucian carp, and zebrafish, than to those of amphibians and mammals (Figure 3). A synteny analysis showed that the adjacent genes, *os9* and *apof*, in grass carp were also found in rainbow trout. However, no conserved adjacent genes were observed between grass carp and human and rat GPR84s (Figure 4).

3.3. The Expression of Grass Carp GPR84 in Grass Carp

The tissue expression of grass carp *GPR84* was determined by a real-time PCR procedure. Grass carp *gpr84* mRNA had the highest expression in the liver, followed by the spleen. The expression in other tissues, including the brain, gill, heart, intestine, kidney, and muscle, was lower than that of the liver and spleen (Figure 5).

3.4. Functional Characterization of ciGPR84 in Cultured HEK293 Cells

We co-transfected HEK293 cells with pciGPR84 and pGL4.29 or pGL4.33, and treated the cells with capric acid, undecanoic acid, lauric acid, or DIM to investigate whether the different agonists could activate ciGPR84. As shown in Figure 6, the treatment of MCFAs could inhibit forskolin-induced cAMP levels in a dose-dependent manner in cells co-transfected with pciGPR84 and pGL4.29. The inhibitory potency of MCFAs on cAMP

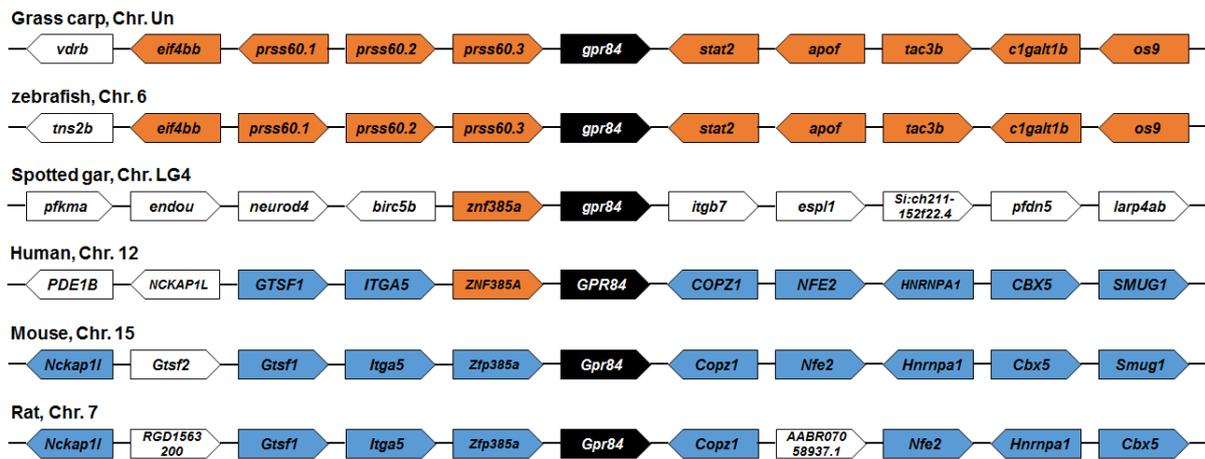


Figure 4. Synteny analyses of GPR84s. The syntenic genes are displayed as boxes with directions and linked by lines. GPR84 genes are shown in black boxes. The genes exhibiting conserved synteny in fishes are indicated in orange boxes, and those in mammals are shown in blue boxes.

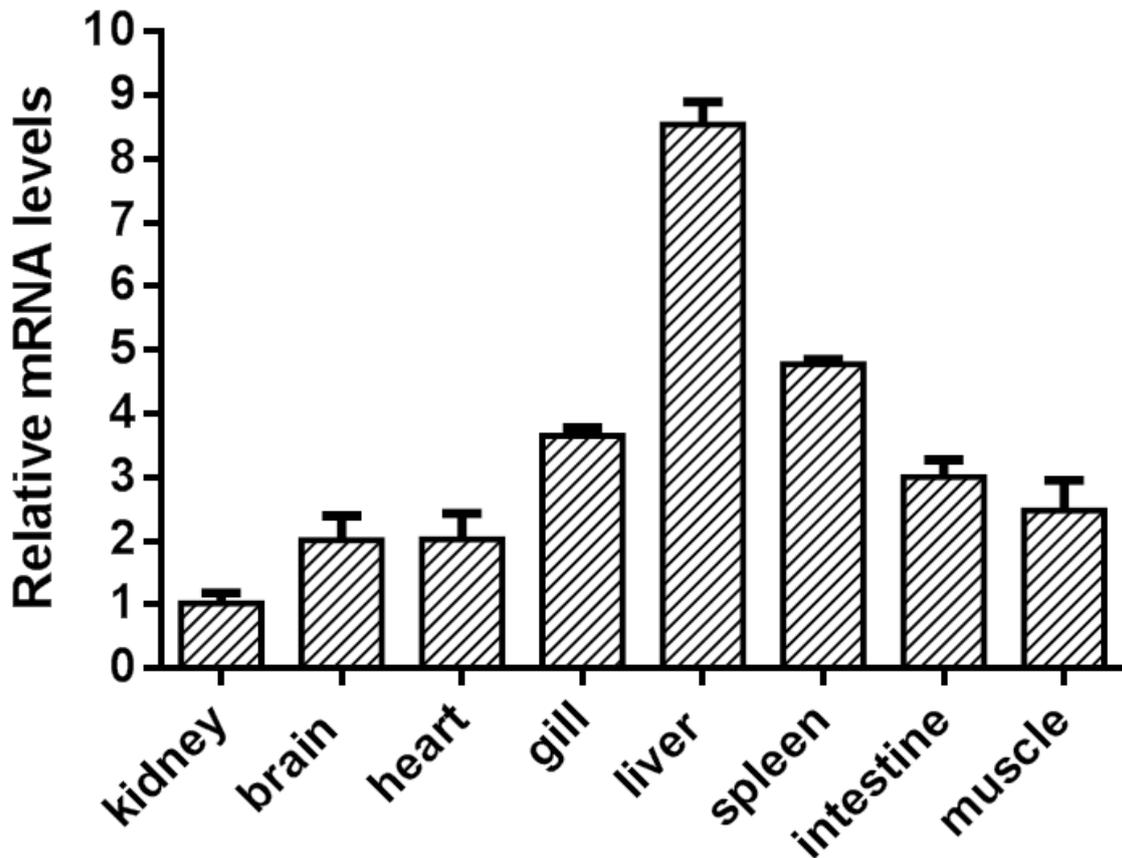


Figure 5. Tissue expression of grass carp GPR84 in different tissues. Quantitative real-time PCR analysis of GPR84 and *actb* mRNA levels in grass carp was performed. *actb* served as an internal reference gene to normalize the mRNA expression in different tissues. Fold difference was calculated by the $2^{-\Delta\Delta Ct}$ method. All the other tissues were compared with the kidney. Vertical bars represent the mean \pm SD ($n = 3$).

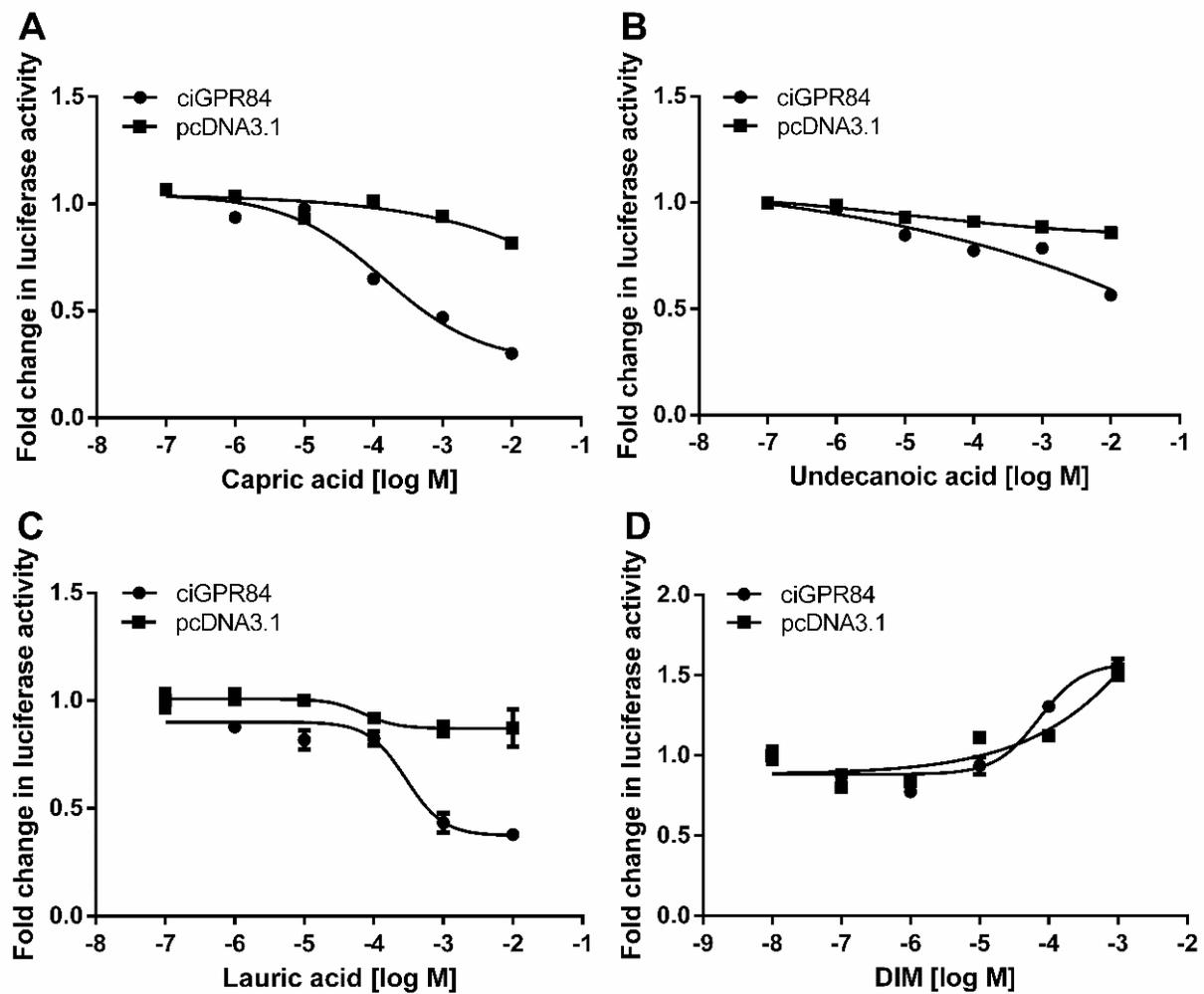


Figure 6. Activation of cAMP signaling mediated by ciGPR84. Induction of the luciferase activities of HEK293T cells expressing ciGPR84 in responses to different concentrations of MCFAs and DIM treatment. The cells were co-transfected with pGL4.29 and p ciGPR84 for 24 h and treated with agonists for 6 h. (A) capric acid (10^{-7} to 10^{-2}), (B) undecanoic acid (10^{-7} to 10^{-2}), (C) lauric acid (10^{-7} to 10^{-2}), (D) DIM (10^{-8} to 10^{-3}) treatments are shown. The co-transfected empty pcDNA3.1 and pGL4.29 were used as a control group. Each data point represents the mean \pm SEM of 3 independent experiments.

We used the pGL4.33 (pGL4-SRE-luciferase) reporter system to detect the activation of three MCFAs and DIM on the ERK signaling of cells transfected with ciGPR84. The results showed that all three MCFAs could activate this signaling pathway in a dose-dependent manner. The potency order of the three fatty acids was lauric acid (EC_{50} : 4250 μ M) > capric acid (EC_{50} : 13,000 μ M) > undecanoic acid (EC_{50} : 13,800 μ M). DIM did not activate the ERK signaling (Figure 7).

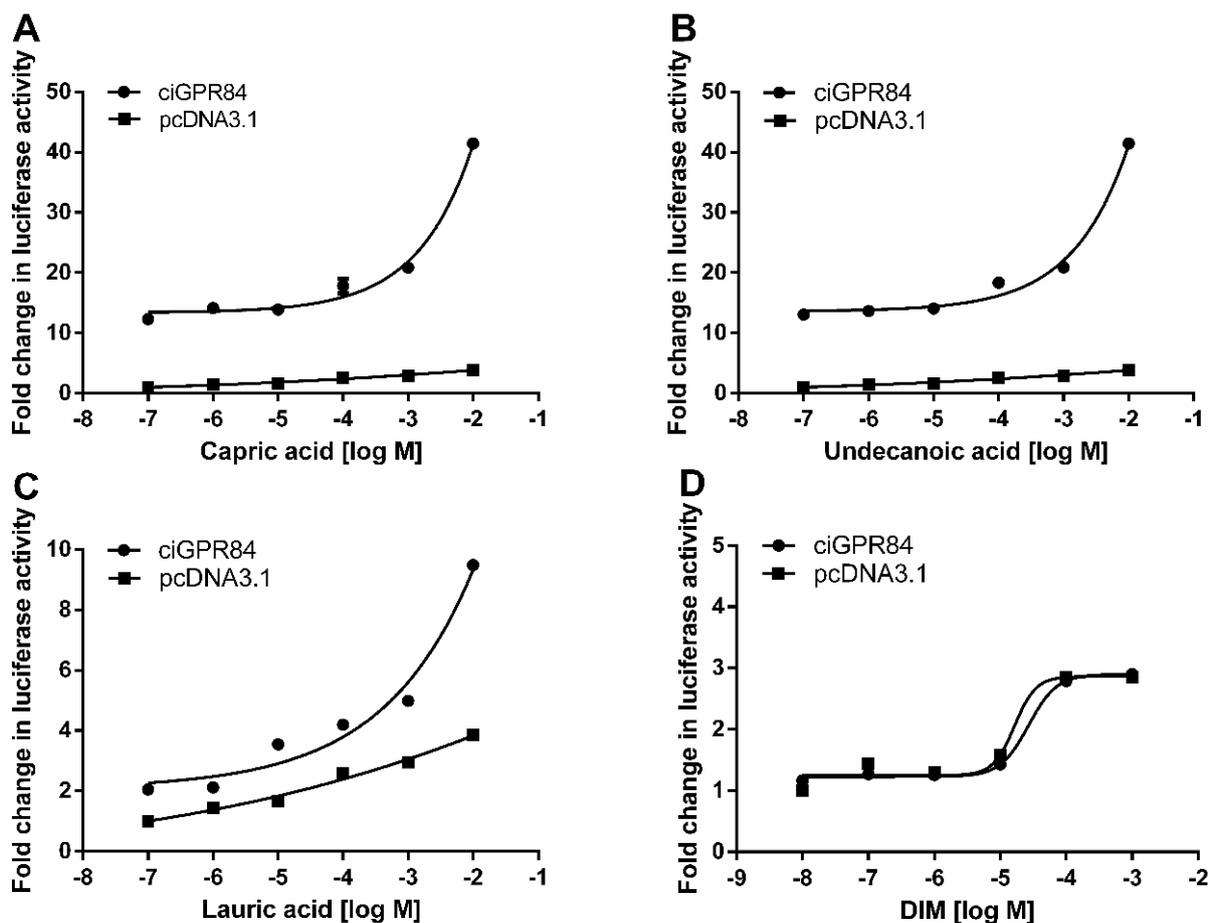


Figure 7. Activation of ERK signaling mediated by ciGPR84. Induction of the luciferase activities of HEK293T cells expressing ciGPR84 in responses to different concentrations of MCFAs and DIM treatment. HEK293T cells were co-transfected with pGL4.33 and ciGPR84 vectors for 24 h and treated with agonists for 6 h. Shown are (A) capric acid (10^{-7} to 10^{-2}), (B) undecanoic acid (10^{-7} to 10^{-2}), (C) lauric acid (10^{-7} to 10^{-2}), (D) DIM (10^{-8} to 10^{-3}). Cells co-transfected with empty vectors, pcDNA3.1 and pGL4.33, were used as the control group. Each data point represents the mean \pm SEM of 3 independent experiments.

4. Discussion

In this study, we cloned the full-length CDS region of grass carp GPR84, analyzed the nucleotide and amino acid sequences, determined its tissue expression pattern, and confirmed downstream signaling pathways regulated by ciGPR84 using the luciferase reporter system.

Grass carp GPR84 cDNA was 1224 bp long and encoded a protein of 407 amino acids, similar to that of fish such as rohu (406 amino acids) and zebrafish (402 amino acids), and different from those of mammals (396 amino acids for human and mouse GPR84s). The multi-sequence alignment of GPR84s of different species showed that ciGPR84 was highly conserved in the seven TMDs and three extracellular loops. Grass carp GPR84 had a higher homology with those of crucian carp, South Asian scaly mackerel, and zebrafish, but a lower homology with those of mammals. The same conclusion was reached in the evolutionary tree constructed by the NJ method. These results collectively demonstrate the evolutionary conservation of GPR84 in bony fish.

Despite the relatively lower homology between ciGPR84 and human GPR84, key motifs and amino acids identified in human GPR84 are also present in ciGPR84. For instance, the highly conserved NPxxY motif in TMD7 is found in ciGPR84, and the tyrosine within this motif potentially interacts with the tyrosine at position 7.53 of the activated receptor,

suggesting a typical activation mechanism observed in rhodopsin-family receptors [28]. Moreover, certain crucial amino acids (such as L104, F105, and N108) in ciGPR84, known to impact decanoic acid binding and hydrogen bond formation in human GPR84 [28], indicate the evolutionary conservation of the receptor's role in fatty acid binding and receptor function. Additionally, the SRY motif, which appears as SRY in fish and amphibian GPR84 but as DRY in mammals, is observed in ciGPR84, exhibiting consistency with other fish species and suggesting motif conservation in lower vertebrates.

Studies have shown that GPR84 has the highest expression in macrophages and peripheral white blood cells and is also highly expressed in the mouse spleen [11]. In zebrafish, GPR84 is highly expressed in the intestinal tract and liver, which are also involved in immune defense in zebrafish [32]. In grass carp, the expression of GPR84 in the liver and spleen was significantly higher than that in other tissues, similar to the distribution of GPR84 in mice. Both the liver and spleen are essential immune organs in fish and mice, suggesting that ciGPR84 may be involved in the regulation of inflammation, similar to mammals.

GPR84 can enhance phagocytic activity and promote an inflammatory response through G α i protein [41]. We showed that MCFAs could activate the G α i-cAMP and ERK signaling pathways through ciGPR84. The results showed that capric acid activated the G α i-cAMP signaling pathway through ciGPR84 better than the other ligands, whereas lauric acid activated the ERK signaling pathway better than the other ligands. When compared to mouse GPR84, which exhibited EC₅₀ values of 52, 4, 8, and 9 μ M for nonanoic acid, capric acid, undecanoic acid, and lauric acid, respectively [11], ciGPR84 demanded higher ligand concentrations for activation. Based on these findings, although the precise levels of various MCFAs in the intestines of grass carp under high-density culture conditions remain unclear, reducing the content of capric acid from dietary sources may serve as an effective strategy to mitigate inflammatory responses in grass carp farming.

In mammals, the small molecule DIM acts as an agonist for GPR84 [42], but our results showed that DIM could not activate ciGPR84, likely due to the structural differences between ciGPR84 and mammalian GPR84s. We observed a similar phenomenon with fish melanocortin-4 receptors (MC4Rs), where peptide ligands act similarly but small molecules act differently in fish and mammalian MC4Rs. For example, THIQ (*N*-[*(3R)*-1,2,3,4-tetrahydroisoquinolinium3-ylcarbonyl]-*(1R)*-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(*1H*-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine), is an orthosteric agonist of human MC4R but allosteric agonist of spotted scat MC4R [20], ricefield eel MC4R [43], and spotted sea bass MC4R [44]. By comparing the effects of four human MC4R antagonists, including endogenous peptide antagonists, agouti-related peptides, and synthetic small molecule antagonists, on the cAMP and ERK1/2 signaling of two fish MC4Rs, spotted scat MC4R and grass carp MC4R, we showed that AgRP acts as an inverse agonist in cAMP signaling pathway in both fish MC4Rs, similar to human MC4R; but the small molecule ligands have effects that are different from human MC4R, with some even different between the two fish MC4Rs [27]. These results suggest that either in research or production application, ligands developed in mammalian systems need to be tested in the aquaculture species of interest. Extrapolation from mammalian system to fish system is not feasible.

In conclusion, we cloned ciGPR84, and analyzed its tissue expression pattern and signaling properties. Grass carp GPR84 was highly expressed in the liver and spleen. Functional studies demonstrated that decanoic acid, undecanoic acid, and lauric acid could inhibit cAMP generation and activate ERK signaling. However, DIM, the highly potent agonist of mammalian GPR84, could not activate ciGPR84. The findings of this study provide insights into the regulation of inflammatory responses in farmed grass carp through nutrient modulation and lay the foundation for further research on the role of MCFAs in chronic inflammation in fish.

Author Contributions: Y.L.: Conceptualization. W.-J.S.: Writing – original draft preparation. S.-K.Y.: Data curation. H.-X.Y.: Methodology. H.-L.M.: Resources. M.-X.Y.: Formal analysis. Y.-X.T.: writing – review and editing. L.-X.W.: Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (grant number 31502180), the Natural Science Foundation of Shaanxi Province (grant number 2023-JC-YB-155), the Key Research and Development Program of Shaanxi Province (grant number 2023-YBSF-565), and the Teaching Reform Project of Northwest A&F University (grant number JY2103041).

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Northwest A&F University (protocol code: NWAUFU-314020038; date of approval: 12 January 2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available on request.

Acknowledgments: We sincerely appreciate the support of Animal Genetics and Breeding Laboratory, College of Animal Science and Technology, Northwest A&F University.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Alvarez-Curto, E.; Milligan, G. Metabolism Meets Immunity: The Role of Free Fatty Acid Receptors in the Immune System. *Biochem. Pharmacol.* **2016**, *114*, 3–13. [[CrossRef](#)] [[PubMed](#)]
2. Falomir-Lockhart, L.J.; Cavazzutti, G.F.; Giménez, E.; Toscani, A.M. Fatty Acid Signaling Mechanisms in Neural Cells: Fatty Acid Receptors. *Front. Cell. Neurosci.* **2019**, *13*, 162. [[CrossRef](#)] [[PubMed](#)]
3. Smith, P.M.; Howitt, M.R.; Panikov, N.; Michaud, M.; Gallini, C.A.; Bohlooly-Y, M.; Glickman, J.N.; Garrett, W.S. The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic Treg Cell Homeostasis. *Science* **2013**, *341*, 569–573. [[CrossRef](#)] [[PubMed](#)]
4. Trompette, A.; Gollwitzer, E.S.; Yadava, K.; Sichelstiel, A.K.; Sprenger, N.; Ngom-Bru, C.; Blanchard, C.; Junt, T.; Nicod, L.P.; Harris, N.L. Gut Microbiota Metabolism of Dietary Fiber Influences Allergic Airway Disease and Hematopoiesis. *Nat. Med.* **2014**, *20*, 159–166. [[CrossRef](#)]
5. Schönfeld, P.; Wojtczak, L. Short-and Medium-Chain Fatty Acids in Energy Metabolism: The Cellular Perspective. *J. Lipid Res.* **2016**, *57*, 943–954. [[CrossRef](#)]
6. Saresella, M.; Marventano, I.; Barone, M.; La Rosa, F.; Piancone, F.; Mendozzi, L.; D’Arma, A.; Rossi, V.; Pugnetti, L.; Roda, G. Alterations in Circulating Fatty Acid Are Associated with Gut Microbiota Dysbiosis and Inflammation in Multiple Sclerosis. *Front. Immunol.* **2020**, *11*, 1390. [[CrossRef](#)]
7. Haghikia, A.; Jörg, S.; Duscha, A.; Berg, J.; Manzel, A.; Waschbisch, A.; Hammer, A.; Lee, D.-H.; May, C.; Wilck, N. Dietary Fatty Acids Directly Impact Central Nervous System Autoimmunity via the Small Intestine. *Immunity* **2015**, *43*, 817–829. [[CrossRef](#)]
8. Stilling, R.M.; van de Wouw, M.; Clarke, G.; Stanton, C.; Dinan, T.G.; Cryan, J.F. The Neuropharmacology of Butyrate: The Bread and Butter of the Microbiota-Gut-Brain Axis? *Neurochem. Int.* **2016**, *99*, 110–132. [[CrossRef](#)]
9. Vinolo, M.A.R.; Ferguson, G.J.; Kulkarni, S.; Damoulakis, G.; Anderson, K.; Bohlooly-Y, M.; Stephens, L.; Hawkins, P.T.; Curi, R. SCFAs Induce Mouse Neutrophil Chemotaxis through the GPR43 Receptor. *PLoS ONE* **2011**, *6*, e21205. [[CrossRef](#)]
10. Talukdar, S.; Bae, E.J.; Imamura, T.; Morinaga, H.; Fan, W.; Li, P.; Lu, W.J.; Watkins, S.M.; Olefsky, J.M. GPR120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-Inflammatory and Insulin-Sensitizing Effects. *Cell* **2010**, *142*, 687–698.
11. Wang, J.; Wu, X.; Simonavicius, N.; Tian, H.; Ling, L. Medium-Chain Fatty Acids as Ligands for Orphan G Protein-Coupled Receptor GPR84. *J. Biol. Chem.* **2006**, *281*, 34457–34464. [[CrossRef](#)] [[PubMed](#)]
12. Zhang, Q.; Chen, L.; Yang, H.; Fang, Y.; Wang, S.; Wang, M.; Yuan, Q.; Wu, W.; Zhang, Y.; Liu, Z.; et al. GPR84 Signaling Promotes Intestinal Mucosal Inflammation via Enhancing NLRP3 Inflammasome Activation in Macrophages. *Acta Pharmacol. Sin.* **2021**, *43*, 2042–2054. [[CrossRef](#)] [[PubMed](#)]
13. Lattin, J.E.; Schroder, K.; Su, A.I.; Walker, J.R.; Zhang, J.; Wiltshire, T.; Saijo, K.; Glass, C.K.; Hume, D.A.; Kellie, S. Expression Analysis of G Protein-Coupled Receptors in Mouse Macrophages. *Immunome Res.* **2008**, *4*, 5. [[CrossRef](#)] [[PubMed](#)]
14. Recio, C.; Lucy, D.; Purvis, G.S.D.; Iveson, P.; Zeboudj, L.; Iqbal, A.J.; Lin, D.; O’Callaghan, C.; Davison, L.; Griesbach, E. Activation of the Immune-Metabolic Receptor GPR84 Enhances Inflammation and Phagocytosis in Macrophages. *Front. Immunol.* **2018**, *9*, 1419. [[CrossRef](#)]
15. Venkataraman, C.; Kuo, F. The G-Protein Coupled Receptor, GPR84 Regulates IL-4 Production by T Lymphocytes in Response to CD3 Crosslinking. *Immunol. Lett.* **2005**, *101*, 144–153. [[CrossRef](#)]
16. Langmead, C.J.; Summers, R.J. Molecular Pharmacology of GPCRs. *Br. J. Pharmacol.* **2018**, *175*, 4005–4008. [[CrossRef](#)]
17. Mafi, A.; Kim, S.-K.; Goddard, W.A., III. The Mechanism for Ligand Activation of the GPCR–G Protein Complex. *Proc. Natl. Acad. Sci. USA* **2022**, *119*, e2110085119. [[CrossRef](#)]

18. Cerdá-Reverter, J.M.; Ringholm, A.; Schiöth, H.B.; Peter, R.E. Molecular Cloning, Pharmacological Characterization, and Brain Mapping of the Melanocortin 4 Receptor in the Goldfish: Involvement in the Control of Food Intake. *Endocrinology* **2003**, *144*, 2336–2349. [[CrossRef](#)]
19. Yang, L.; Zhang, Z.; Wen, H.; Tao, Y. Characterization of Channel Catfish (*Ictalurus punctatus*) Melanocortin-3 Receptor Reveals a Potential Network in Regulation of Energy Homeostasis. *Gen. Comp. Endocrinol.* **2019**, *277*, 90–103. [[CrossRef](#)]
20. Li, J.T.; Yang, Z.; Chen, H.P.; Zhu, C.H.; Deng, S.P.; Li, G.L.; Tao, Y.X. Molecular Cloning, Tissue Distribution, and Pharmacological Characterization of Melanocortin-4 Receptor in Spotted Scat, *Scatophagus argus*. *Gen. Comp. Endocrinol.* **2016**, *230–231*, 143–152. [[CrossRef](#)]
21. Li, L.; Yang, Z.; Zhang, Y.P.; He, S.; Liang, X.F.; Tao, Y.X. Molecular Cloning, Tissue Distribution, and Pharmacological Characterization of Melanocortin-4 Receptor in Grass Carp (*Ctenopharyngodon idella*). *Domest. Anim. Endocrinol.* **2017**, *59*, 140–151. [[CrossRef](#)] [[PubMed](#)]
22. Wen, Z.-Y.; Liu, T.; Qin, C.-J.; Zou, Y.-C.; Wang, J.; Li, R.; Tao, Y.-X. MRAP2 Interaction with Melanocortin-4 Receptor in Snakehead (*Channa argus*). *Biomolecules* **2021**, *11*, 481. [[CrossRef](#)] [[PubMed](#)]
23. Liu, T.; Yi, T.-L.; Yang, D.-Q.; Tao, Y.-X. Regulation of Melanocortin-5 Receptor Pharmacology by Two Isoforms of MRAP2 in Ricefield Eel (*Monopterus albus*). *Gen. Comp. Endocrinol.* **2021**, *314*, 113928. [[CrossRef](#)]
24. Tao, M.; Ji, R.L.; Huang, L.; Fan, S.Y.; Liu, T.; Liu, S.J.; Tao, Y.X. Regulation of Melanocortin-4 Receptor Pharmacology by Two Isoforms of Melanocortin Receptor Accessory Protein 2 in Topmouth Culter (*Culter alburnus*). *Front. Endocrinol.* **2020**, *11*, 1489–1501. [[CrossRef](#)]
25. Yu, H.; Li, Y.; Song, W.; Wang, H.; Mo, H.; Liu, Q.; Zhang, X.; Jiang, Z.; Wang, L. Functional Characterization of Melanocortin-3 Receptor in Rainbow Trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.* **2022**, *48*, 241–252. [[CrossRef](#)]
26. Barney, E.; Dores, M.R.; McAvoy, D.; Davis, P.; Racareanu, R.C.; Iki, A.; Hyodo, S.; Dores, R.M. Elephant Shark Melanocortin Receptors: Novel Interactions with MRAP1 and Implication for the HPI Axis. *Gen. Comp. Endocrinol.* **2019**, *272*, 42–51. [[CrossRef](#)] [[PubMed](#)]
27. Yang, Z.; Liang, X.-F.; Li, G.-L.; Tao, Y.-X. Biased Signaling in Fish Melanocortin-4 Receptors (MC4Rs): Divergent Pharmacology of Four Ligands on Spotted Scat (*Scatophagus argus*) and Grass Carp (*Ctenopharyngodon idella*) MC4Rs. *Mol. Cell. Endocrinol.* **2020**, *515*, 110929. [[CrossRef](#)] [[PubMed](#)]
28. Al Mahmud, Z.; Jenkins, L.; Ulven, T.; Labéguère, F.; Gosmini, R.; De Vos, S.; Hudson, B.D.; Tikhonova, I.G.; Milligan, G. Three Classes of Ligands Each Bind to Distinct Sites on the Orphan G Protein-Coupled Receptor GPR84. *Sci. Rep.* **2017**, *7*, 17953. [[CrossRef](#)]
29. Gaidarov, I.; Anthony, T.; Gatlin, J.; Chen, X.; Mills, D.; Solomon, M.; Han, S.; Semple, G.; Unett, D.J. Embelin and Its Derivatives Unravel the Signaling, Proinflammatory and Antiatherogenic Properties of GPR84 Receptor. *Pharmacol. Res.* **2018**, *131*, 185–198. [[CrossRef](#)]
30. Wei, L.; Tokizane, K.; Konishi, H.; Yu, H.-R.; Kiyama, H. Agonists for G-Protein-Coupled Receptor 84 (GPR84) Alter Cellular Morphology and Motility But Do Not Induce Pro-Inflammatory Responses in Microglia. *J. Neuroinflamm.* **2017**, *14*, 198. [[CrossRef](#)]
31. Kuwabara, T.; Ishikawa, F.; Kondo, M.; Kakiuchi, T. The Role of IL-17 and Related Cytokines in Inflammatory Autoimmune Diseases. *Mediat. Inflamm.* **2017**, *2017*, 3908061. [[CrossRef](#)] [[PubMed](#)]
32. Huang, Q.; Feng, D.; Liu, K.; Wang, P.; Xiao, H.; Wang, Y.; Zhang, S.; Liu, Z. A Medium-Chain Fatty Acid Receptor Gpr84 in Zebrafish: Expression Pattern and Roles in Immune Regulation. *Dev. Comp. Immunol.* **2014**, *45*, 252–258. [[CrossRef](#)]
33. Wang, M.; Zhang, X.; Zhang, S.; Liu, Z. Zebrafish Fatty Acids Receptor Gpr84 Enhances Macrophage Phagocytosis. *Fish Shellfish Immunol.* **2019**, *84*, 1098–1099. [[CrossRef](#)] [[PubMed](#)]
34. Velasco, C.; Conde-Sieira, M.; Comesaña, S.; Chivite, M.; Míguez, J.M.; Soengas, J.L. Role of the G Protein-Coupled Receptors GPR84 and GPR119 in the Central Regulation of Food Intake in Rainbow Trout. *J. Exp. Biol.* **2021**, *224*, jeb242360. [[CrossRef](#)] [[PubMed](#)]
35. Rao, Y.; Su, J. Insights into the Antiviral Immunity against Grass Carp (*Ctenopharyngodon idella*) Reovirus (GCRV) in Grass Carp. *J. Immunol. Res.* **2015**, *2015*, 670437. [[CrossRef](#)] [[PubMed](#)]
36. Chen, Y.; Shi, M.; Zhang, W.; Cheng, Y.; Wang, Y.; Xia, X.-Q. The Grass Carp Genome Database (GCGD): An Online Platform for Genome Features and Annotations. *Database* **2017**, *2017*, bax051. [[CrossRef](#)] [[PubMed](#)]
37. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2⁻ $\Delta\Delta$ CT Method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]
38. Davis, P.; Franquemont, S.; Liang, L.; Angleson, J.K.; Dores, R.M. Evolution of the Melanocortin-2 Receptor in Tetrapods: Studies on *Xenopus Tropicalis* MC2R and *Anolis Carolinensis* MC2R. *Gen. Comp. Endocrinol.* **2013**, *188*, 75–84. [[CrossRef](#)]
39. Yu, H.-X.; Li, Y.; Ezeorba, T.; Mo, H.-L.; Zhang, Z.-H.; Yang, Q.-Y.; Wang, L.-X. Molecular Characterization and Functional Exploration of GPR84 in Chinese Giant Salamander (*Andrias davidianus*). *Dev. Comp. Immunol.* **2022**, *137*, 104526. [[CrossRef](#)]
40. Wu, L.; Yu, H.; Mo, H.; Lan, X.; Pan, C.; Wang, L.; Zhao, H.; Zhou, J.; Li, Y. Functional Characterization of Melanocortin-3 Receptor in a Hibernating Cavefish *Onychostoma macrolepis*. *Animals* **2021**, *12*, 38. [[CrossRef](#)]
41. Lucy, D.; Purvis, G.S.D.; Zeboudj, L.; Chatzopoulou, M.; Recio, C.; Bataille, C.J.R.; Wynne, G.M.; Greaves, D.R.; Russell, A.J. A Biased Agonist at Immunometabolic Receptor GPR84 Causes Distinct Functional Effects in Macrophages. *ACS Chem. Biol.* **2019**, *14*, 2055–2064. [[CrossRef](#)]

42. Takeda, S.; Yamamoto, A.; Okada, T.; Matsumura, E.; Nose, E.; Kogure, K.; Kojima, S.; Haga, T. Identification of Surrogate Ligands for Orphan G Protein-Coupled Receptors. *Life Sci.* **2003**, *74*, 367–377. [[CrossRef](#)] [[PubMed](#)]
43. Yi, T.-L.; Yang, L.-K.; Ruan, G.-L.; Yang, D.-Q.; Tao, Y.-X. Melanocortin-4 Receptor in Swamp Eel (*Monopterus albus*): Cloning, Tissue Distribution, and Pharmacology. *Gene* **2018**, *678*, 79–89. [[CrossRef](#)] [[PubMed](#)]
44. Zhang, K.-Q.; Hou, Z.-S.; Wen, H.-S.; Li, Y.; Qi, X.; Li, W.-J.; Tao, Y.-X. Melanocortin-4 Receptor in Spotted Sea Bass, *Lateolabrax Maculatus*: Cloning, Tissue Distribution, Physiology, and Pharmacology. *Front. Endocrinol.* **2019**, *10*, 705. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.