Two alternatively spliced GPR39 transcripts in seabream: molecular cloning, genomic organization, and regulation of gene expression by metabolic signals

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Abstract

Two GPR39 transcripts, designated as sbGPR39-1a and sbGPR39-1b, were identified in black seabream (*Acanthopagrus schlegelii*). The deduced amino acid (aa) sequence of sbGPR39-1a contains 423 residues with seven putative transmembrane (TM) domains. On the other hand, sbGPR39-1b contains 284 aa residues with only five putative TM domains. Northern blot analysis confirmed the presence of two GPR39 transcripts in the seabream intestine, stomach, and liver. Apart from seabream, the presence of two GPR39 transcripts was also found to exist in a number of teleosts (zebrafish and pufferfish) and mammals (human and mouse). Analysis of the *GPR39* gene structure in different species suggests that the two GPR39 transcripts are generated by alternative splicing. When the seabream receptors were expressed in cultured HEK293 cells, Zn²⁺ could trigger sbGPR39-1a signaling through the serum response element

pathway, but no such functionality could be detected for the sbGPR39-1b receptor. The two receptors were found to be differentially expressed in seabream tissues. sbGPR39-1a is predominantly expressed in the gastrointestinal tract. On the other hand, sbGPR39-1b is widely expressed in most central and peripheral tissues except muscle and ovary. The expression of sbGPR39-1a in the intestine and the expression of sbGPR39-1b in the hypothalamus were decreased significantly during food deprivation in seabream. On the contrary, the expression of the GH secretagogue receptors (sbGHSR-1a and sbGHSR-1b) was significantly increased in the hypothalamus of the food-deprived seabream. The reciprocal regulatory patterns of expression of these two genes suggest that both of them are involved in controlling the physiological response of the organism during starvation.

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Introduction

Subsequent to the identification of the GH secretagogue receptor (GHSR; Howard *et al.* 1996), other GHSR family members were discovered including GPR38 and GPR39, which belong to the class A 7 transmembrane (TM) domain G-protein-coupled receptors (GPCRs; McKee *et al.* 1997). GHSR was orphanized in 1999 by the discovery of a novel gut-derived peptide ghrelin (Kojima *et al.* 1999). The endogenous ligand for GPR38 was also identified as a gastric peptide motilin (Feighner *et al.* 1999). The putative natural ligand for GPR39 was suggested to be an amidated 23-amino acid (aa) peptide designated as obestatin isolated from rat

stomach (Zhang *et al.* 2005). However, recent studies failed to demonstrate that obestatin could bind and activate GPR39 (Lauwers *et al.* 2006, Chartrel *et al.* 2007, Gourcerol *et al.* 2007, Holst *et al.* 2007). It was suggested that the impure obestatin used in the original report might be responsible for the aberrant results (Zhang *et al.* 2007).

GPR39 was encoded by a single-copy gene in the human genome mapped to the chromosome location 2q21–q22 (McKee *et al.* 1997). Northern blot and reverse transcription PCR (RT-PCR) analyses indicated that GPR39 is predominantly expressed in the gastrointestinal tract (McKee *et al.* 1997, Zhang *et al.* 2005, Nogueiras *et al.* 2007, Yamamoto *et al.* 2007). GPR39 is also widely expressed in the brain regions

The sequences reported in this paper have been deposited in the GenBank database at NCBI and are assigned the following accession numbers: EU076425, EU076426, EU076427, EU076428, EU076429, EU076430, EU076431, and EU076432.

(McKee *et al.* 1997, Jackson *et al.* 2006). Interestingly, GPR39 was found to be a constitutively activated receptor acting mainly through the serum response element (SRE) pathway (Holst *et al.* 2004). Although the endogenous ligand for GPR39 still remains to be ascertained, Zn^{2+} can activate GPR39 signaling through various pathways at micromolar concentrations (Holst *et al.* 2004, 2007, Lauwers *et al.* 2006). Recently, GPR39 knockout mice were generated. Compared with the wild-type counterpart, GPR39-deficient mice exhibit accelerated gastric emptying and reduced fasting-induced hyperphagia, suggesting a role of the receptor in gastrointestinal tract regulation (Moechars *et al.* 2006).

To date, the full-length cDNA sequences of *GPR39* have been reported in some mammals and chicken (McKee *et al.* 1997, Zhang *et al.* 2005, Yamamoto *et al.* 2007). Little information is available about GPR39 in teleost fish. Thus, isolating GPR39 from distant phylogenetic groups may help to elucidate the structural and functional evolution of GPR39. In addition, there is little information on the presence of GPR39 subtypes and their regulation of expression. In the present study, we have identified two GPR39 transcripts from black seabream, namely sbGPR39-1a and sbGPR39-1b, derived from alternative splicing of the same gene. We have also demonstrated that this phenomenon is conserved in a variety of vertebrates from teleosts to mammals. The two seabream GPR39 receptors were studied by transfection in cultured HEK293 cells and their expression levels investigated in different seabream tissues. GPR39 is a member of GHSR receptor family, which plays important roles in food intake and energy homeostasis (Kojima & Kangawa 2005); thus regulation of the expression of GPR39 and GHSR during food deprivation (FD) in seabream was also investigated.

Materials and Methods

Animals

Black seabream (*Acanthopagrus schlegelii*) and pufferfish (*Takifugu rubripes*) were obtained from the Guangdong Daya Bay Fishery Development Center (Guangdong Province, China). Zebrafish (*Danio rerio*) were purchased from a local fish shop. Fish were anesthetized and killed by decapitation. Tissue samples were collected immediately and snap frozen in liquid nitrogen. All the animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees at Sun Yat-Sen University and The Chinese University of Hong Kong.

Molecular cloning of *GPR39* cDNAs in a number of teleosts and mammals

According to the reported mammalian *GPR39* cDNA sequences and the predicted *GPR39* cDNA sequences in the fish genomes, degenerate primers were designed for cloning the

GPR39 cDNA in seabream. Total RNA from seabream intestine was prepared using TRIzol reagent (Invitrogen). One microgram of total RNA was used to synthesize the first-strand cDNA following the manufacturer's instructions of the ReverTra Ace- α First-strand cDNA Synthesis kit (TOYOBO, Tokyo, Japan). Subsequently, a cDNA fragment of the putative seabream GPR39 (sbGPR39) was obtained.

Rapid amplifications of cDNA ends (RACE) were then performed to obtain the full-length *GPR39-1a* cDNA using the 5'- and 3'-RACE amplification kits (Invitrogen). For 5'-RACE, sbGPR39-1a RACE R1 and AAP were used in the first-round reaction. Then the amplified products were diluted and employed as a template for the second-round reaction using sbGPR39-1a RACE R2 and AUAP as primers. For 3'-RACE, sbGPR39-1a RACE F1 and AUAP were used in the first-round reaction and sbGPR39-1a RACE F2 and AUAP were used in the second-round reaction. To confirm that the full-length cDNA sequence was correctly assembled, two gene-specific primers sbGPR39-1a full-length F and sbGPR39-1a full-length R were designed to amplify the entire open reading frame (ORF) of sbGPR39-1a.

For sbGPR39-1b, primers sbGPR39-1b RACE F1 and sbGPR39-1b RACE F2 were used to obtain the 3'-end sequence. 3'-RACE was performed as described above using first-strand cDNA prepared from the seabream hypothalamus as the template. The full-length cDNA sequence of sbGPR39-1b was eventually confirmed by PCR with a pair of gene-specific primers sbGPR39-1a full-length F and sbGPR39-1b full-length R flanking the ORF of sbGPR39-1b.

Data mining was carried out for identifying the presence of the GPR39-1b transcript by searching the genome sequence information (Ensembl Genome Browser (<http://www.ensembl.org/>) and NCBI database (<http://www.ncbi.nlm.nih.gov/>) using TBLASTN algorithm) in human (*Homo sapiens*: Ensembl 48, NCBI 36), mouse (*Mus musculus*: Ensembl 48, NCBI m37), zebrafish (*D. rerio*: Ensembl 48, assembly Zv7), and pufferfish (*T. rubripes*: Ensembl 48, FUGU 4.0). Using the deduced sbGPR39-1a polypeptide sequence as query, putative ORFs encoding for GPR39-1a were identified in zebrafish and pufferfish using the TBLASTN algorithm. The GPR39-1b transcripts were predicted to be encoded by the first exon of GPR39-1a and part of the intron. To substantiate that the two GPR39 transcripts, especially GPR39-1b, really exist in these species, RT-PCR was performed to amplify the ORFs of the GPR39 transcripts in these species of vertebrates. Sequence information of all the primers used is listed in Table 1.

Sequence and phylogenetic analyses

Multiple alignment of aa sequences was performed using the CLUSTAL_X program. Hydrophobicity analyses were performed by the TMHMM 2.0 program (www.cbs.dtu.dk/services/TMHMM). Phylogenetic trees were generated using MEGA3.1 (Kumar *et al.* 2004). The data were resampled by 1000 bootstrap replicates to determine the confidence indices within the phylogenetic tree.

Table 1 The primer sequences used in the present study

Primer name	Sequence (5' to 3')	Purpose
Degenerate F Degenerate R	GGAGCTCTACAGYGCCATCTG GTACATCACCCA GTGCTNCC	cDNA fragment generation
AP AAP AUAP	GGCCACGCGTCGACTAGTAC(oligoT ₁₆) GGCCACGCGTCGACTAGTAGGGGGGGGGGGG GGCCACGCGTCGACTAGTAC CCTGCAAGA	RACE
sb GPR39-1a RACE F1 sb GPR39-1a RACE F2 sb GPR39-1a RACE R1 sb GPR39-1a RACE R2 sb GPR39-1b RACE F1 sb GPR39-1b RACE F2	TCTACAACCTCC GCCCATAATCTGACGTTCTGTAC GTAGATCTTGACAGAAACGTTGC ACGAGAACGGGAACCATGATGG CCTGCAAGATCTACAACCTCC GTTTCGCTTCAAAGCACTC	
sb GPR39 full-length F sb GPR39-1a full-length R sb GPR39-1b full-length R Human GPR39 full-length F Human GPR39-1a full-length R Human GPR39-1b full-length R Mouse GPR39 full-length F Mouse GPR39-1a full-length R Mouse GPR39-1b full-length R Pufferfish GPR39 full-length F Pufferfish GPR39-1a full-length R Pufferfish GPR39-1b full-length R Zebrafish GPR39 full-length F Zebrafish GPR39-1a full-length R Zebrafish GPR39-1b full-length R	TGACTCGTGA ACTCTCTGACAG CGCTCACCTCTGGATCTTGG GGTTCACCTAACAGCTGGTG GTGCTCTTCTCATGGCTTCAC CATTCAAACCTCATGCTCCTG TGGAAGCTGCTCAGTGTG ATGGCTTCATCCAGTGGCTC TCATACTTCTGCTCTGTAA TCAGTGAACCTCCGGTTTCAC GAACAGAGAAGAAGACCTGAG GACCTGAAGGATGAGACGATG CTGTTTAGTTACATGCAGG ATGGACCCGCAGCAGCCGG TCAGCTGCTTTGTTCATCAG CTAGTGTGACCACAGGATAAG	Full-length amplification
Probe F Probe R	TCGCTCACCTCTGGATCTTGG ACGAGAACGGGAACCATGATGG	Probe preparation
sb GPR39-1a real-time F sb GPR39-1a real-time R sb GPR39-1b real-time F sb GPR39-1b real-time R sb GHSR-1a real-time F sb GHSR-1a real-time R sb GHSR-1b real-time F sb GHSR-1b real-time R	CTACATGATCGTGTGGTCTG CAGCACCTGGACGAACACC CTACATGATCGTGTGGTCTG CTCTAACGAATGGACACGTAG CATCAAGATGTTGGTGGTGG CACTAAGCTGCAGTACTCGG CATTACGCAGTGGAGTCCG AGACACATATACTCTACATGG	Real-time PCR
sb GPR39-1a F sb GPR39-1a R sb GPR39-1b F sb GPR39-1b R	CCTGCAAGATCTACAACCTCC CTGGAGGAGAGGTTGTAGAG CTACATGATCGTGTGGTCTG GGTTCACCTAACAGCTGGTG	Tissue distribution studies
sb-actin-F sb-actin-R	ACCCAGATCATGTTCCGAGACC ATGAGGTAGTCTGTGAGGTCTG	β-actin amplification

Northern blot analysis of seabream tissues

A probe was prepared by labeling a fragment of the *sbGPR39* cDNA with a pair of primers (Table 1) using the PCR Dig Labeling System (Roche). Thirty micrograms of total RNA of each seabream tissue mixed with loading dye were denatured and separated on gel, transferred onto Hybond-N nylon paper (Northern Max-Gly kit, Ambion, Austin, TX, USA), and cross-linked by u.v. irradiation. Hybridization and detection were performed in accordance with the manufacturer's protocol. Briefly, after prehybridization at 42 °C for 4 h, 0.5 µl of the probe was added and hybridization

was performed overnight at 42 °C. The blots were washed twice with washing buffer and non-specific binding sites were blocked by 1:10 000 anti-Dig antibody. One milliliter of CDP-Star at 1:10 000 dilution was added to the membrane, and the chemiluminescent signal was then detected on a Syngene Bioimaging System (Synoptics, Cambridge, UK).

Genomic organization of the *GPR39* gene

Seabream genomic DNA was isolated from the liver. Two pairs of primers used to confirm the ORFs of the two *sbGPR39* transcripts were used in the genomic PCR. The

genomic PCR band was isolated and sequenced to compare with the cDNA sequences.

For species in which the genome sequence information is already available, a comparison of the cloned cDNA sequences with the genome sequences by BLAST would yield information regarding the genomic organization of the *GPR39* genes.

Tissue distribution of GPR39 in seabream

The expression profiles of two *GPR39* transcripts in seabream were analyzed by RT-PCR using gene-specific primers for the two receptors. The cDNA templates of different tissues from seabream were prepared as described above. Mock RT reactions without reverse transcriptase were used as negative controls for the reactions.

Two pairs of gene-specific primers differentiating sbGPR39-1a and sbGPR39-1b (Table 1) were employed to investigate the expression patterns of the two *GPR39* transcripts. β -Actin was amplified in parallel to verify the integrity of all the cDNA samples. Five microliters of each reaction product were resolved on a 1.5% agarose gel containing ethidium bromide and visualized on a Gel Doc System (Bio-Rad). No contamination was found since all the negative controls gave no amplified products. The integrity of all the cDNA templates was verified by the successful and consistent amplification of the β -actin transcript.

Cell culture, transfection, and functional assay

Cell culture materials were purchased from Invitrogen. The HEK293 cell line was purchased from ATCC (Manassas, VA, USA). Cells were maintained at 37 °C in DMEM containing 10% fetal bovine serum. All media were supplemented with antibiotics (10 U/ml penicillin and 100 μ g/ml streptomycin).

In the transfection experiments, Lipofectamine reagent (Invitrogen) was used. Twenty hours before transfection, 1.5×10^5 cells/well were seeded onto 24-well tissue culture plates. Five hundred nanograms of the pSRE-Luc (Stratagene, La Jolla, CA, USA), 20 ng of pcDNA 4.0-sbGPR39-1a or pcDNA 4.0-sbGPR39-1b, and 50 ng of pRL-CMV (for normalization of transfection efficiency) containing the *Renilla* luciferase reporter gene were co-transfected into the cells in 250 μ l serum-free medium. Six hours after transfection, the cells were incubated with 10 μ M ZnCl₂ or 0.1 μ M human obestatin (ALEXIS Biochemicals, San Diego, CA, USA) for a further 20 h. Luminescence was measured on a Lumat LB 9501 luminometer (EG & G, Berthold, Bad Wildbad, Germany) and the activities of both luciferases were measured sequentially on the same sample.

Real-time PCR analysis of sbGPR39 and sbGHSR expression in seabream during food deprivation

Experimental design and tissue sampling Experimental fish were obtained from the Guangdong Daya Bay Fishery Development Center (Guangdong Province, China).

Forty black seabream of mixed sexes with body weight of 240 ± 15 g were divided into two groups (20 fish per tank) and kept separately in 0.5 m³ indoor tanks supplied with constant seawater flow. Fish were fed once daily at a scheduled time (0900 h) with $\sim 3\%$ body weight of commercial diet. Fish were allowed to acclimatize to the feeding routine for 2 weeks. Feeding was abstained in one tank (food deprivation; FD), and fish in the other tank were fed as usual to serve as the control. Eight fish from each tank (control and FD groups) were anesthetized by immersing in 0.05% MS222 solution and killed at 1100 h, 2 days, and 7 days after food withdrawal respectively. Tissues (hypothalamus and mid-intestine) were collected and immediately frozen in liquid nitrogen for later use.

Real-time PCR To monitor the expression levels of sbGPR39 and sbGHSR in the tissues, real-time PCR was performed on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). One microgram of total RNA from each sample digested with DNase I was used as the template in the reverse transcription reaction. Real-time PCR was performed using the SYBR Green I kit (TOYOBO), according to the manufacturer's protocol. Standard curves for sbGPR39-1a, sbGPR39-1b, sbGHSR-1a, sbGHSR-1b, and β -actin were generated by serial dilutions of plasmid constructs of the target transcripts. Expression of β -actin was used as the internal control to correct for variations in template amount and RT efficiency. The primers used for each transcript are listed in Table 1. Real-time PCR was performed as described previously (Li *et al.* 2007). Fluorescent data were converted to threshold cycle (C_T) values (Livak & Schmittgen 2001). The concentration of the template in the sample was determined by relating the C_T value to the standard curve. Transcripts levels were normalized against β -actin. Other studies have also reported on the use of β -actin as the internal control in gene expression studies in fish (Celius *et al.* 2000, Vong *et al.* 2003, Yada *et al.* 2005).

Statistical analysis

All data were expressed as the mean $\pm$ S.E.M. from three or more independent measurements as indicated in the figure legends. Data were considered statistically significant at $P < 0.05$ using either one-way ANOVA or unpaired *t*-test using the software Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Results

Isolation of two sbGPR39 transcripts from black seabream

A fragment of sbGPR39 with a size of 343 bp was isolated from seabream intestine using degenerate primers designed from the conserved regions of *GPR39* in other species. A BLAST search indicates that the sequence of the isolated

fragment is a putative GPR39. According to the sequence information of the fragment, gene-specific primers were designed and employed in 5'- and 3'-RACE reactions. Amplicons with a size of 1200 and 550 bp from the 5'- and 3'-RACE reactions respectively were cloned and sequenced. The RT-PCR and RACE fragments were assembled. Two primers designed at the 5'- and 3'-untranslated regions (UTR) were used to confirm that the full-length cDNA sequence was correctly assembled. The full-length cDNA amplified from the seabream tissue was sequence confirmed and designated sbGPR39-1a. The sbGPR39-1a contains an ORF of 1272 bp with a 5'-UTR of 254 bp and a 3'-UTR of 449 bp. When translated, sbGPR39-1a encodes for a polypeptide of 423 aa, which shares 49.9%, 47.3%, and 49.6% in overall aa identity with the human, mouse, and

chicken GPR39 respectively. Hydrophobicity analysis using the TMHMM program indicates sbGPR39-1a contains seven putative TM motifs (Fig. 3).

Another GPR39 transcript, designated sbGPR39-1b, was isolated from the seabream hypothalamus. 5'-RACE performed in the hypothalamus gave a band of identical size and sequence when compared with the 5'-RACE product from the intestine. A band of larger size (700 bp) was obtained from the 3'-RACE reaction in the hypothalamus. The RT-PCR and RACE fragments were assembled to yield a complete cDNA sequence designated as sbGPR39-1b consisting of an ORF of 852 bp, a 5'-UTR of 254 bp, and a 3'-UTR of 229 bp. Again gene-specific primers at the 5'- and 3'-UTRs were used to confirm that the full-length *sbGPR39-1b* cDNA sequence was correctly assembled. As shown in Fig. 1,

		AGAAGAAGCTGCTGAAGAAACCTCATGTTTAACTTCTGTGCTGGGTCTGAAATGA	56
		TTCACCTCTTGATTGGAGCTTCTCACCTGCTGGATTACTCCCATTTGAAGGATCAGGAGTTATTAGAGACCAAGAGAAGTTTCAGAGTGAGCTGTT	155
		ATCTCGCTCACCTCTGGATCTTGGCTGCGCCCGGCAGAGCTGATAAGCTAAAAGCAAGAATCAATAGCAGGAAAATCAGAGCTCAGCGCTCCGAGCC	254
255		ATGAGTGAGGAAGTGGAGATATCAGAAGAGAAGGACTGGAGGAGCTGGAGCCCACTACCCCTCAAAATCTTCCTGACCGCTTTTACAGCTTCATC	353
1a	M S E E V E I S E E K D W R E L E P N Y P L K I F L T V F Y S F I		33
1b	- -		33
		CTGTGTACGGGCATTGTGGGTAAATTCGTCCTATCAGAGTGACGAGGTGTTGAGGCGCAACGGCTACCTGCAGAAGAAGCTGACGGACCATGTGTC	452
1a	L V T G I V G N S V T I R V T Q V L R R N G Y L Q K N V T D H M V		66
1b	- -		66
		AGCCTGGCGTGTCTGGACCTGCTGGTGCTCCTCATCGGCATGCCTGTGAGCTCTACAGCGCCATCTGGTTCCCGTCTCTGTCACCTCGGGCAACGTT	551
1a	S L A C S D L L V L L I G M P V E L Y S A I W F P F S S T S G N V		99
1b	- -		99
		TCCTGCAAGATCTACAACCTCTGTTCGAAGCCTGCAGTACGCCACCATCTCAATGTGGCCACGCTCAGTTTCGAGCGCTACGTGGCGATCTGCCAC	650
1a	S C K I Y N F L F E A C S Y A T I L N V A T L S F E R Y V A I C H		132
1b	- -		132
		CCGTTTCGCTTCAAAGCACTCGGTGGGAAACGACCTCCGCCCTCATACCTTCGCTGCTGGTGTGCTGGCGCTGCCGCTGCTGATTGCG	749
1a	P F R F K A L G G K R T S A L I T F A W L V S V L V A L P L L I A		165
1b	- -		165
		ACGGGAACCCAGGGGCACATTCCCGTCCGCTCAGACATACCCGCCAGAATCTGACGTTCTGTACAACTGCGGGAGCGCTGGGTGATGTACCGCAGC	848
1a	T G T Q G H I P V R S D I P A Q N L T F C T N L R E R W V M Y R S		198
1b	- -		198
		AGCATCTTCATCGCCTTCATCATCTACATGATCGTGTGGTCTGCGTGGCCGTCATGTGCCGCGCCATGATCCTCGTGCTCCGGAGGCGTTTGGGTGGC	947
1a	S I F I A F I I Y M I V L V C V A V M C R A M I L V L R R P L G G		231
1b	- -		231
		CCGGACAGCGACGCCAAGGAGCTCCAGGAGCCTCCAAGCATGAAAGTGCAAAGGTCAAGACGCTCCAGGAAGCAGACCATCATTTTTCTTGGTACGTTG	1046
1a	P D S D A N G A P G A S K H E S A K V K T S R K Q T I I F L G		262
1b	- -		262
		CTGTTTTATTTATCTCAAAATGTTCTCTCTCTGCTCCGTTTGTGTGCTGAGCTTTAATACCTCTCGGGATCTACGTGTCCATTCTGTAGAGAG	1145
1b	L F Y L S Q K C S S S L S V C L L S E L *		284
		GAAACCTCCGAAAAGTGTGCTCGGAAAAGCTACTGAGTGTGTTATTTTTGGCCAACCTTTGTATGGAGGTGTGTGAGTGTGTGTGTGTGTGAGTGA	1244
		TGCTGCTCGGGATGTTTGGACATTTTTCAGCCACTTCATCTGTTTACACCACTGTTAAGTGAACCACTTTGACTGTCTGCCACAAGTGAAGTAAAAA	1343
		AAAAAAAAAA	1354
		CTCATCTCGGATCCTTGATGGTCTGCTGGCTTCCCAATCAGATCCGTCGCCTCATGATGGCCGCGTGCACCAAGTCCCGCTGGACCACTTCTCTCTTC	1139
1a	L I V G S L M V C W L P N Q I R R L M M A A V P K S R W T T S F F		295
		CGGAGCTACGTCACTTCTGCACCCCGTGGCCGACACCTTCTTCTACCTGAGCTCCGTCATCAACCCGTTCTCTACAACCTCTCTCCAGACAGTCCGG	1238
1a	R S Y V I L H P V A D T F F Y L S S V I N P F L Y N L S S R Q F R		328
		GAGGTGTTGCTCCAGGTGCTGCGGTGCGCGCTCACCATCGAACCATCGAACAGCAACGACCTCCCGCGCGTCTCCGCGCGTCTCCCTGCGAG	1337
1a	E V F V Q V L R C R L T I E H V N K R N V R T S R A V S A R S L Q		361
		CCGCTGCTGATGAAATCTCTGCGGCGCAGCAAAGCTAACCGCTCCACCATCGCAGAGGAGAAACAAGCTCCAACCTTCATCACCTTCCGGCAGGAGAGC	1436
1a	P L L M K S L R R S K A N R S T I A E E K Q A P T F I T F R Q E S		394
		GACTCTGAGGCTTCAAACTCTGACTGAAGAGTCCGAGTGCAGCTGCACGACAGACACATCAACCATCAGAGTCTGAAATATAATACCTTTAAT	1535
1a	D S G V A S N T L T E E S E S T R T T D T H Q P S E S E I *		
		GCAACACGGTTAAAAAACAAGATGCAGAAAAAACTCAGATATCTCTCGTACACAGGAAACACGGTACAAAGTGTGCTGCTCAGTAGATCACGCT	1634
		CAGAAACAAGCTGCAGACTTTAACTCAAGGAAAATGATAAAACGCGAGCTGAAATATTCAGTAAAGCATGAAACTCTGCGAGCTGGAGTGAAAGTGGAG	1733
		TTTTTTTGACCACTAGAGGCGCTAAAGCAGCTCAGTCGATCCACGAGAGCTCAGCTGGAGTGTGATGTTTGTGTTTAAATATGTTAACTGACAGAAGAAA	1832
		TAAAGTCGTGTTGTATCTGTCATGTTTCTGTGAGAGAGTTCAGAGTTCGACGCTTAAATATATATATACAGCATTTTATATCTACAGATTACACGTC	1931
		TACAGAGCTCGTAGGAAGGTGCAGAATAAAATCTTTTACCATACAAAAA	1975

Figure 1 Nucleotide and deduced amino acid sequences of the sbGPR39-1a and sbGPR39-1b. Nucleotides (upper sequence) and amino acid (lower sequence) are numbered on the right-hand side. Identical sequences are shown as dashes. The stop codon (TAA) is indicated by asterisk, while the different nucleotide sequence of sbGPR39-1b is underlined.

comparison of sbGPR39-1b with sbGPR39-1a revealed that the first 1040 bp, which encodes for the first 262 aa of the translated polypeptide, were the same. The sbGPR39-1b ORF encodes for a polypeptide of 284 aa with five putative TM domains in the hydrophobicity analysis.

To further confirm that the two sbGPR39 transcripts indeed exist in seabream tissues, northern blot analysis was performed. Two bands of ~2.0 and 1.4 kb corresponding to the sizes of the cloned sbGPR39 transcripts were detected in the intestine, stomach, and liver RNA samples (Fig. 5).

Isolation of two GPR39 transcripts from other teleosts and GPR39-1b from mammals

The presence of GPR39-1a and GPR39-1b transcripts in zebrafish and pufferfish was predicted *in silico* from the genome information, followed by experimental cloning of their complete ORFs using the primers described in Table 1. The presence of GPR39-1b transcripts in human and mouse was also predicted *in silico*, followed by cloning of their complete ORFs. Hydrophobicity analyses indicate that all the GPR39-1a sequences contain seven putative TM domains and all the GPR39-1b sequences contain five putative TM domains.

Phylogenetic analysis was performed on the GPR39 sequences reported in the literature and those obtained in the present study, together with other members of the GHSR family. As shown in Fig. 4, GPR39 and GHSR were found to form separate related clades. The teleost GPR39 sequences obtained in the present study, although sharing less than 50% aa identity with the mammalian counterparts, do cluster together with the mammalian GPR39 sequences to form a phylogenetically distinct clade separate from the GHSR and GPR38 sequences. The GHSR and GPR38 sequences appear to be more related to each other phylogenetically than to GPR39.

GPR39 gene structure analysis

The structures of the *GPR39* genes in human, mouse, zebrafish, and pufferfish were analyzed by comparison of the *GPR39-1a* cDNA sequence with the respective genome sequence. In all of these species, the *GPR39* gene contains two exons and one intron, and the intron is located between TM5 and TM6. Comparison of the *GPR39-1b* cDNA sequence with the respective genome sequence in these species revealed that the *GPR39-1b* cDNA sequence is identical to the respective genomic sequence corresponding to the first exon of GPR39-1a and part of the 5'-end of the intron. Therefore, as shown in Fig. 2, two GPR39 transcripts could be generated by alternatively splicing the same gene. The GPR39-1a transcript is generated by splicing away the intron and the GPR39-1b transcript is generated by retaining part of the intron.

Using two primers flanking the ORF of sbGPR39-1b, a portion of the *sbGPR39* gene was amplified from the genomic DNA prepared from seabream tissue and sequenced. The nucleotide sequence of the amplified GPR39 genomic sequence was found to be identical to the *sbGPR39-1b* cDNA

Intron* refers to part of intron

Figure 2 Schematic representation of the vertebrate *GPR39* gene and alternative splicing mechanism. The *GPR39* gene contains two exons and one intron. The two GPR39 transcripts are generated by removing the intron or retaining part of it.

sequence. It appears therefore that the scheme proposed in Fig. 2 is conserved in all the species that we have studied in the present study from mammals to teleosts (Figs 3–5).

Tissue expression patterns of the two sbGPR39 transcripts in seabream

The expression profiles of the two GPR39 transcripts in different seabream tissues were investigated by RT-PCR. As shown in Fig. 6, *sbGPR39-1a* mRNA was found to be abundantly expressed in the gastrointestinal tract, liver, adipose tissue, and kidney. Some expression of the *sbGPR39-1a* transcript was also detected in the midbrain and testis. However, *sbGPR39-1a* expression was very low or virtually undetectable in most brain regions.

Different from the expression pattern of *sbGPR39-1a*, which is confined to a relatively small number of tissues, the *sbGPR39-1b* mRNA was found to be expressed in all the tissues examined except muscle and ovary. In the brain regions, high *sbGPR39-1b* expression was found in the midbrain, hindbrain, and medulla. Appreciable expression was also detected in the other brain regions such as the forebrain, pituitary, and hypothalamus. In the peripheral organs, *sbGPR39-1b* was found to be expressed predominantly in the gastrointestinal tract, liver, and kidney where *sbGPR39-1a* is also expressed abundantly. Therefore, co-expression of *sbGPR39-1a* and *sbGPR39-1b* is evident in these peripheral tissues.

Expression of the two cloned sbGPR39 receptors in cultured cells

As shown in Fig. 7, when the *sbGPR39-1a* receptor was transfected into HEK293 cells in culture, Zn^{2+} at 10 μM could trigger the SRE signaling pathway, as indicated by the induction

Figure 3 Multiple alignment of GPR39-1a amino acid sequences. Sequences were aligned by the CLUSTAL_X program. Identical sequences are indicated by asterisks. The TM domains are indicated. Gaps (indicated by hyphens) are introduced in some sequences to maximize alignment.

of luciferase activity of the reporter gene. On the other hand, however, transfected HEK293 cells bearing the sbGPR39-1b receptor could not be stimulated by Zn^{2+} . Also, in line with the findings that obestatin is unable to activate the GPR39 receptor, our attempts to stimulate the sbGPR39-1a and sbGPR39-1b receptors with this peptide failed to detect any bioactivity.

Effect of FD on sbGPR39 and sbGHSR expression in seabream

The expression patterns of sbGPR39 and sbGHSR during FD in seabream were investigated by real-time PCR. As shown in Fig. 8, after 2 days of FD, the expression level of sbGPR39-1a was somewhat lowered in the mid-intestine when compared with the control. This decrease became significant after 7 days of FD, with the sbGPR39-1a level decreased to less than 50% of the control level (Fig. 8A). However, the expression level of sbGPR39-1b was not significantly changed in the mid-intestine after FD (Fig. 8B). On the contrary, the expression level of sbGPR39-1b was significantly decreased in the hypothalamus after 7 days of FD (Fig. 8C). The sbGHSR-1a expression level was increased sharply in the hypothalamus after 2 days of FD. After 7 days of FD, this increase was enhanced even more (Fig. 8D). Similar to that of sbGHSR-1a, the expression level of sbGHSR-1b was also significantly increased in the hypothalamus after FD (Fig. 8E).

Discussion

Sequence and phylogenetic analysis of GPR39

GPR39 is a GPCR belonging to the ghrelin receptor subfamily (McKee *et al.* 1997). We have cloned and characterized ghrelin and ghrelin receptor in black seabream previously (Chan & Cheng 2004, Yeung *et al.* 2006). In the present study, we report the identification and characterization of another ghrelin receptor family member, namely sbGPR39-1a and sbGPR39-1b, from the same organism.

Several characteristics of the cloned sbGPR39-1a sequence revealed that it is an ortholog of the GPR39 identified in avian and mammalian species. Apart from the characteristic feature of possessing seven putative TM domains, the SXXNPXXY motif in TM7 and the D(E)-R-Y(F) motif at the end of TM3, both of which are landmarks of the rhodopsin family members, are also found in sbGPR39-1a. The aa sequences of the two cytoplasmic loops between TM1 and TM2, and between TM3 and TM4, as well as an extracellular loop between TM6 and TM7, are highly conserved (Fig. 2). In addition, phylogenetic analysis revealed that GPR39 formed one separate clade distinct from the other GHSR receptor family members. These findings suggest that GPR39 is structurally conserved among different species.

Identification of an additional *GPR39-1b* transcript among various vertebrates

A careful examination of the mRNA expression of *GPR39* in the seabream hypothalamus has led us to the identification of another *GPR39* transcript in seabream. Two pairs of primers flanking different regions of sbGPR39-1a were initially employed in preliminary experiments to determine whether *GPR39* is expressed in the hypothalamus. Interestingly, expression of sbGPR39-1a was clearly detected in the hypothalamus using one pair of primer flanking the 5'-UTR and TM2. However, another pair of primer (sbGPR39-1a F and sbGPR39-1a R) flanking TM4 and TM7 failed to detect *GPR39* expression in the hypothalamus. Based on this observation, we hypothesized that another transcript of *GPR39*, which shares the same region with the isolated sbGPR39-1a at the 5'-end, is exclusively expressed in the hypothalamus. Therefore, 3'-RACE were performed using the seabream hypothalamus as the template. The sbGPR39-1b transcript was subsequently isolated from the hypothalamus. By northern blot analysis, we confirmed that two *GPR39* transcripts indeed exist in seabream tissues.

The cDNA sequences of *GPR39* have been reported in human, rat, mouse, and, more recently, in chicken (McKee *et al.* 1997, Zhang *et al.* 2005, Yamamoto *et al.* 2007). In the present study, we have clearly demonstrated the presence of two alternatively spliced forms of *GPR39* in teleosts as well as in mammals, and that these two receptors in seabream are distinct in biological function and are differentially expressed. During the preparation of this manuscript, Egerod *et al.* (2007) also reported that *GPR39-1b* transcripts are found in human, mouse, rat, and chicken, further supporting the notion that the presence of two *GPR39* transcripts is a general phenomenon among vertebrates.

The structure of the *GPR39* gene

The structures of the mammalian and teleostean *GPR39* genes containing two exons and one intron were shown to be similar to that in chicken. The position of the intron, located between TM5 and TM6, is also conserved among these species. This observation suggests that the gene structure of *GPR39* is evolutionarily conserved from teleost to human. Despite our repeated trials, attempts to isolate the whole intron of the sbGPR39 gene in seabream were unsuccessful. This was probably due to the large size of the intron in

seabream. In this context, it should be noted that the intron is about 61 kb in the chicken gene, and even in the more compact genome of pufferfish, this intron is more than 4.5 kb in size.

Comparison of the two *GPR39* transcripts with the genomic sequence revealed that alternative splicing of the same gene may give rise to the two *GPR39* transcripts in human, mouse, zebrafish, and pufferfish. In one situation, if the intron is removed, it will generate a long-form receptor with seven TM motifs. In another situation, if the intron is not spliced away completely, it will generate a truncated receptor with only five TM domains (Fig. 2). The presence of an inadvertent stop codon in the intron sequence retained in the mRNA is responsible for the generation of the truncated receptor form. A very similar situation was observed for the generation of the two GHSR transcripts (Howard *et al.* 1996, Chan & Cheng 2004) and the two motilin receptor transcripts (Feighner *et al.* 1999). The occurrence of two alternatively spliced forms of the same receptor gene appears to be a common phenomenon in the ghrelin receptor family. Although the whole intron of the sbGPR39 gene has not been isolated, we have demonstrated that the sbGPR39-1b sequence, the same as that seen in human and mouse, was identical to the genomic sequence. This observation suggests that sbGPR39-1b is probably generated in a fashion similar to that in humans and mice. The conserved gene structure, together with a partial sequence of the sbGPR39 gene determined, indicates that two sbGPR39 transcripts are probably generated by alternative splicing of the *GPR39* gene in seabream.

Tissue distribution of *GPR39* transcripts in seabream

Study on the expression pattern of *GPR39* may shed light on the physiological roles of the receptor. In some aspects, the expression pattern of *GPR39-1a* in seabream is similar to that reported in human and chicken where the gastrointestinal tract is the primary site of expression (Zhang *et al.* 2005, Nogueiras *et al.* 2007, Yamamoto *et al.* 2007). The high expression in the gastrointestinal tract suggests that *GPR39* might be involved in regulating gut functions. Consistent with this notion, the volume of gastric secretion was increased and gastric emptying accelerated in *GPR39* knockout mice (Moechars *et al.* 2006). A previous report has also demonstrated that *GPR39-1a* is expressed in the hypothalamus and pituitary of mammals (Zhang *et al.* 2005). On

Figure 4 Phylogenetic analysis of the amino acid sequences of *GPR39*, *GPR38*, and *GHSR* from different vertebrates. Phylogenetic analyses were performed using MEGA3.1 by the maximum parsimony method with 1000 bootstrap replicates. The sbGPR39-1a and sbGPR39-1b are highlighted by asterisks. The *GPR39*, *GPR38*, and *GHSR* sequences used in the analysis and their GenBank accession numbers are: black seabream (*Acanthopagrus schlegelii*) sbGHSR-1a AAN77875 and sbGHSR-1b AAN77876; chicken (*Gallus gallus*) chGHSR-1a BAC76445, chGHSR-1b BAC76446, and chGPR39-1a ABG75918; human (*Homo sapiens*) hGHSR-1a NP_940799, hGHSR-1b NP_004113, hGPR38 O43193, and hGPR39-1a AAI25047; mouse (*Mus musculus*) mGHSR-1a Q99P50 and mGPR39-1a AAH85285; and monkey (*Macaca mulatta*) monGPR38 XP_001101857, together with the sequences obtained in the present study: black seabream (*Acanthopagrus schlegelii*) sbGPR39-1a EU076425 and sbGPR39-1b EU076426; zebrafish (*Danio rerio*) zfGPR39-1a EU076427 and zfGPR39-1b EU076428; pufferfish (*Takifugu rubripes*) sbGPR39-1a EU076429 and sbGPR39-1b EU076430; human (*Homo sapiens*) hGPR39-1b EU076432; and mouse (*Mus musculus*) mGPR39-1b EU076431.

Figure 5 Northern blot analysis of the two sbGPR39 transcripts in seabream. Thirty micrograms of total RNA were isolated from the intestine (lane 1), stomach (lane 2), and liver (lane 3). Two bands of ~2.0 and 1.4 kb in sizes were detected in the mRNA samples.

the contrary, we did not detect any expression of GPR39-1a in the seabream hypothalamus and pituitary even using 40 cycles of PCR amplification. In fact, several other groups also failed to detect GPR39-1a expression in the hypothalamus using RT-PCR or *in situ* hybridization (Jackson *et al.* 2006, Holst *et al.* 2007, Nogueiras *et al.* 2007). The absence of sbGPR39-1a in the hypothalamus and pituitary probably suggests a relatively unimportant role of this receptor in modulating pituitary hormone release. However, the presence of sbGPR39-1a in other brain regions may imply that this receptor might be involved in some other yet unidentified neural functions. Interestingly, both sbGPR39-1a and sbGPR39-1b are abundantly expressed in many tissues such as the gastrointestinal tract, liver, and kidney. The

Figure 6 Analysis of *GPR39-1a* and *GPR39-1b* mRNA expression in seabream tissues by RT-PCR. The different tissues are: 1, forebrain; 2, midbrain; 3, hindbrain; 4, medulla; 5, pituitary; 6, hypothalamus; 7, intestine; 8, stomach; 9, muscle; 10, liver; 11, testis; 12, ovary; 13, gill filament; 14, heart; 15, adipose tissue; 16, kidney; 17, spleen; 18, negative control.

co-expression of the two GPR39 transcripts in peripheral regions might imply functional interaction between each other. However, sbGPR39-1b is also expressed in other tissues where there is little sbGPR39-1a, particularly in the brain regions including the hypothalamus and pituitary. A similar tissue expression pattern of GPR39-1b in mouse was reported by Egerod *et al.* (2007). This conserved GPR39-1b expression pattern in mammals and fish implies a possible physiological role of the truncated receptor in these tissues.

What factors, transcriptional or otherwise, that control the differential expression of the two alternatively spliced receptors in seabream tissues are not known at the moment. Whether there is alternative promoter usage of the 5'-flanking region of the single GPR39 gene is an interesting and worthwhile follow-up study.

Functional study of the two sbGPR39 transcripts

Despite the fact that the endogenous ligand for GPR39 is still unknown, previous studies have indicated that Zn²⁺ could activate human GPR39 in HEK293 cells (Holst *et al.* 2004, 2007, Lauwers *et al.* 2006). In the present study, we have also observed that Zn²⁺ is capable of triggering sbGPR39-1a signaling via the SRE pathway. In line with the findings of Zhang *et al.* (2007), the obestatin peptide was totally inactive in this regard. This observation suggests that GPR39 is functionally conserved during evolution. Therefore, GPR39 may serve important physiological functions in phylogenetically diversified species. However, the physiological roles and signaling pathways cannot be fully unraveled until the natural ligand for GPR39 becomes known.

It appears from our results that sbGPR39-1b is a truncated receptor devoid of signaling ability. However, the unavailability of the natural ligand for GPR39 has hampered us to test this assumption. At this juncture, it is interesting to note the occurrence of a similar situation in another receptor of the same family in seabream. We have previously reported that sbGHSR-1b, a truncated receptor form of the functional ghrelin receptor in seabream, is incapable of signal transduction (Chan & Cheng 2004). A comparison of the truncated receptor sbGPR39-1b with the functional sbGPR39-1a revealed that the main discrepancy between them lies on the topology architecture: the truncated receptor contains five TM domains and the functional one has seven TM domains. Therefore, the lacking of TM6 and TM7 in the truncated receptor may be responsible for its dysfunction. According to the global toggle switch model, activation of seven TM receptors requires an inward movement of TM6 and TM7 toward the inner face of TM3 (Schwartz *et al.* 2006). Consistent with this view, it is interesting to note that truncated chemokine receptors with five TM motifs lacking TM1 and TM2 act as fully functional GPCRs (Ling *et al.* 1999). Moreover, receptor mutation analysis of GHSR and GPR39 indicates that TM6 and TM7 are essential for the constitutive receptor activity, further supporting the hypothesis that lacking in TM6 and TM7

Figure 7 Effects of Zn^{2+} and human obestatin on SRE reporter activity in HEK293 cells transfected with sbGPR39-1a and sbGPR39-1b. In each experiment, cells were incubated with $10\ \mu\text{M}$ Zn^{2+} or $0.1\ \mu\text{M}$ human obestatin in triplicate wells for 20 h. Values are the means $\pm$ S.E.M. from three independent experiments, expressed as the percentage increase in luciferase activity above the control (without any stimulating agent).

has led to the inability of the truncated receptors in activating the signaling events (Holst *et al.* 2004).

Effect of FD on sbGPR39 and sbGHSR expression in seabream

Since GPR39 is expressed mainly in the gastrointestinal tract and GPR39 is a member of GHSR receptor family, which plays important roles in regulating food intake and energy homeostasis (Kojima & Kangawa 2005), we have therefore investigated the gene expression of *GPR39* and *GHSR* during FD in seabream. Our observation that there is a drastic elevation in the gene expression of the *GHSR-1a* receptor in the hypothalamus of the seabream during FD agrees with the results of a previous study conducted in rats (Kim *et al.* 2003). In addition, we have also observed a concomitant decrease in the expression of the sbGPR39-1a receptor in the seabream intestine during food derivation in the present study. Interestingly, during the course of preparing this manuscript, Egerod *et al.* (2007) reported that *GPR39-1a* mRNA level was upregulated in adipose tissue upon fasting and down-regulated during adipocyte differentiation in rats. Taken together, these data suggest that *GPR39* and *GHSR* gene expression is highly responsive to metabolic signals related to the fed-fast states of the animals, and that the reciprocal regulatory nature of the two receptors appear to be conserved from teleosts to mammals.

The remarkable changes in the expression levels of GPR39 and GHSR may have important physiological bearings. Holst

et al. (2003) have shown that the constitutive signaling activity of GHSR can reach 50% of the maximal signaling efficiency evoked by its natural ligand ghrelin. Similar to GHSR, GPR39 is also a constitutively active receptor signaling mainly through the SRE pathway (Holst *et al.* 2004). We have also observed that the sbGHSR-1a receptor in seabream is a constitutively active receptor (Chan *et al.* 2004). The high ligand-independent constitutive signaling activity implies that changes in receptor expression are directly connected with the signaling and therefore physiological events. Thus, a change in receptor gene expression under different energy status would directly influence the constitutive signaling activities of the receptors. It has been suggested that the high basal signaling activity of GHSR-1a may provide a set point to act against inhibitory signals from the anorexigenic peptides such as leptin, insulin, and PYY3–36 (Holst & Schwartz 2004). Therefore, the significant increase in GHSR expression after FD could be interpreted as a demand for more stimulatory signals for food intake and less energy expenditure. Interestingly, natural mutations of the GHSR-1a receptor in human, Ala204Glu and Phe279Leu, leading to the loss of the constitutive signaling activity have been associated with short stature and obese phenotype (Wang *et al.* 2004, Pantel *et al.* 2006). These findings suggest that the constitutive signaling activity of the GHSR-1a is normally needed and dramatic changes in the mRNA levels of *GHSR-1a* have significant physiological consequence. On the other hand, however, the significance of the constitutive signaling activity of GPR39 is not clear. The concomitant and reciprocal expression patterns of GHSR and GPR39 after FD strongly suggest that these two receptors possess opposing roles in controlling food intake and energy homeostasis.

FD influences sbGPR39 and sbGHSR expression

It has been recognized that truncated GPCRs, generated by alternative splicing of GPCR transcripts, may modulate the function of the full-length 7 TM version of the receptor by influencing its expression, ligand binding properties, and cellular location (Karpa *et al.* 2000, Nakamura *et al.* 2004, McElvaine & Mayo 2006, Beaumont *et al.* 2007). We have previously reported that the truncated sbGHSR-1b receptor can modulate the sbGHSR-1a receptor signal transduction activities (Chan & Cheng 2004). Further investigations have demonstrated that the GHSR-1b receptor can attenuate the constitutive signaling activity of the GHSR-1a receptor by forming a heterodimer between GHSR-1a and GHSR-1b (Chu *et al.* 2007, Leung *et al.* 2007). Therefore, GHSR-1b acts as a dominant-negative mutant of the full-length receptor. How physiological status influences the expression of these receptor variants, and thus their dimerization, is largely unknown. Our results revealed that the expression of both sbGHSR-1a and sbGHSR-1b is increased after FD. Thus, the overall effect would represent a change in receptor signaling events responding to the negative energy balance. The concomitant increase of the 1b form in this case might

Figure 8 Real-time PCR analysis of relative mRNA levels of *sbGPR39-1a*, *sbGPR39-1b*, *sbGHSR-1a*, and *sbGHSR-1b* of food-deprived (FD) seabreams. Seabreams were food deprived for 2 and 7 days. The relative mRNA levels of (A) *sbGPR39-1a* in the mid-intestine, (B and C) *sbGPR39-1b* in the mid-intestine and hypothalamus, and (D and E) *sbGHSR-1a* and *sbGHSR-1b* in the hypothalamus were measured by real-time PCR. Results are expressed as mean $\pm$ S.E.M. ($n=8$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus the respective controls (CTR).

act as a self-limiting process to mitigate the action of the increased 1a form.

GPR39-1b is also a truncated receptor form, which shares much in common with GHSR-1b: a wide distribution pattern, 5 TM topology architecture and the apparent lack of

signaling activity. Given such similarities between GPR39-1b and GHSR-1b and a natural tendency to form oligomers among the GPCR family members (Woolf & Linderman 2003), a putative role for GPR39-1b could also be to act as a modulator of GPR39-1a constitutive signaling activity by

forming heterodimers, particularly in view of the fact that the 1a and 1b receptors are co-expressed in some of the central and peripheral tissues in seabream. Our results revealed that the expression of both GPR39 transcripts in the mid-intestine appeared to be decreased after FD, although the decrease in the 1b form did not reach statistical significant level within the period of study. If heterodimerization between the two GPR39 variants does occur, the overall effect would be envisaged to represent a composite change in receptor signaling events in response to the negative energy balance. Interestingly, the expression of sbGPR39-1b was also decreased in the hypothalamus after FD, suggesting some other functional role of GPR39-1b in some brain regions that do not normally express GPR39-1a. Another possible role for GPR39-1b could be to act as a scavenger receptor that influences ligand transportation or internalization, which was previously reported in a subfamily of GPCRs that interact with chemokines (Bonecchi *et al.* 2004).

Evolution of the ghrelin receptor family

GPR39 is an orphan receptor of the ghrelin receptor family. GPR39, GHSR and GPR38 share many common characteristics: from their gene structure, generation of receptor subtypes, to their responsiveness toward metabolic signals. However, each receptor has its own characteristic properties. For example, GPR39 and GPR38 are most abundantly expressed in the gastrointestinal tract while GHSR is most abundantly expressed in the brain. On the other hand, GPR39 and GHSR exhibit substantial ligand-independent constitutive signaling activities while GPR38 is devoid of such activity. These observations suggested that GPR39, GHSR and GPR38 might be derived from a common ancestral gene but have been functionally differentiated during evolution. Although differences have been accumulated during receptor evolution, they are all involved in some kind of metabolic regulation process, which might represent the ancient function of the common ancestral gene. If this hypothesis is valid, this differentiation must have occurred a long time ago before the divergence of vertebrates, as many of the characteristics and functionalities in these receptors are respectively conserved during the course of vertebrate evolution over a period of ≈ 400 million years from teleost fish to modern mammals.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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