The presence of two distinct prolactin receptors in seabream with different tissue distribution patterns, signal transduction pathways and regulation of gene expression by steroid hormones

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Abstract

Two prolactin receptors (PRLRs) encoded by two different genes were identified in the fugu and zebrafish genomes but not in the genomes of other vertebrates. Subsequently, two cDNA sequences corresponding to two PRLRs were identified in black seabream and Nile tilapia. Phylogenetic analysis of PRLR sequences in various vertebrates indicated that the coexistence of two PRLRs in a single species is a unique phenomenon in teleosts. Both PRLRs in teleosts (the classical one named as PRLR1, the newly identified one as PRLR2) resemble the long-form mammalian PRLRs. However, despite their overall structural similarities, the two PRLR subtypes in fish share very low amino acid similarities (about 30%), mainly due to differences in the intracellular domain. In particular, the Box 2 region and some intracellular tyrosine residues are missing in PRLR2. Tissue distribution study by real-time PCR in black seabream (sb) revealed that both receptors (sbPRLR1 and sbPRLR2) are widely expressed in different tissues. In gill, the expression level of sbPRLR2 is much higher than that of sbPRLR1. In the intestine, the expression of sbPRLR1 is higher than that of sbPRLR2. The expression levels of both receptors are relatively low in most other tissues, with sbPRLR1 generally higher than sbPRLR2. The sbPRLR1 and sbPRLR2 were functionally expressed in

cultured human embryonic kidney 293 cells. Both receptors can activate the β -casein and c-fos promoters; however, only sbPRLR1 but not sbPRLR2 can activate the Spi promoter upon receptor stimulation in a ligand-specific manner. These results indicate that both receptors share some common functions but are distinctly different from each other in mobilizing post-receptor events. When challenged with different steroid hormones, the two PRLRs exhibited very different gene expression patterns in the seabream kidney. The sbPRLR1 expression was up-regulated by estradiol and cortisol, whereas testosterone had no significant effect. For sbPRLR2, its expression was down-regulated by estradiol and testosterone, while cortisol exerted no significant effect. The 5'-flanking regions of the *sbPRLR1* and *sbPRLR2* genes were cloned and the promoter activities were studied in transfected GAKS cells in the absence or presence of different steroid hormones. The results of the promoter studies were in general agreement with the *in vivo* hormonal regulation of gene expression results. The *sbPRLR1* gene promoter activity was activated by estradiol and cortisol, but not by testosterone. In contrast, the *sbPRLR2* gene promoter activity was inhibited by estradiol, cortisol, and testosterone.

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Introduction

Prolactin (PRL) is recognized as the most versatile pituitary hormone among diverse species (Manzon 2002). In fish, various physiological functions have been attributed to PRL, such as enhancement of immune functions (Harris & Bird 2000), pigment dispersion in the tegumentary chromatophores (Kitta *et al.* 1993), synergism with production of

steroid hormones in the gonads (de Ruiter *et al.* 1986), and reproduction (Cavaco *et al.* 2003).

PRL exerts its actions via specific PRL receptor (PRLR) which belongs to the cytokine class I receptor superfamily (Bole-Feysot *et al.* 1998). Besides mammals and other tetrapods, PRLRs have also been cloned and characterized in different fish species (Sandra *et al.* 1995, Tse *et al.* 2000, Le Rouzic *et al.* 2001, Santos *et al.* 2001, Lee *et al.* 2006).

In mammals, there are long, intermediate, and short PRLR forms that are generated by alternative splicing and promoter usage (Freeman *et al.* 2000). These isoforms have identical extracellular domains but differ in the length and composition of their intracellular domains as well as in their signal transduction mechanisms (Bole-Feysot *et al.* 1998). However, the presence of well-characterized multiple forms of PRLR in teleost has not been reported. The fact that there are two different PRLs in tilapia (PRL177 and PRL188) and their different physiological functions imply the possible existence of more than one PRLR in tilapia (Sandra & Prunet 1998). Yet no other forms of PRLR in Nile tilapia have been identified (Sandra *et al.* 1995). In other teleosts, PRLR transcripts of different sizes have been found by northern blot analysis in seabream (Santos *et al.* 2001) and goldfish (Tse *et al.* 2000), but their identities have not been ascertained.

In order to identify the presence of multiple genes for PRLR in a single species, *in silico* data mining was carried out in different fish and tetrapod genomes. Such search results indicated the presence of two putative PRLR genes in teleosts but not in other vertebrates. To further substantiate the coexistence of two PRLRs in a single fish species, we have cloned two types of PRLR cDNAs in black seabream (sbPRLR1 and sbPRLR2). We have also cloned a novel PRLR subtype (ntPRLR2) from Nile tilapia in which the other PRLR subtype (ntPRLR1) is already known. Subsequently, a phylogenetic tree was constructed from all the available PRLR sequences. The results clearly indicated that the PRLR sequences could be grouped into two different clades, namely PRLR1 and PRLR2. Using black seabream (*Acanthopagrus schlegelii*) as the model organism, we have investigated the tissue distribution and signal transduction mechanisms of the two PRLRs, as well as their *in vivo* regulation of gene expression by steroid hormones. We have also cloned the promoters of these two genes in black seabream and studied their responsiveness to steroid hormone challenge in transfected cultured cells.

Materials and Methods

Experimental animals

Black seabream (body length 22.5 ± 0.3 cm, body weight 235 ± 11 g) and Nile tilapia (body length 20 ± 4 cm, body weight 200 ± 20 g) of both sexes were purchased from a local fish farm. All experiments were conducted in accordance with guidelines as established by the University Committee on the use and care of laboratory animals at the Chinese University of Hong Kong.

Data mining and phylogenetic analysis

Genomic contigs corresponding to the putative PRLR genes were identified by searching the genome databases (Ensembl

genome browser <http://www.ensembl.org/index.html> and NCBI database <http://www.ncbi.nlm.nih.gov/> using TBlastN algorithm) of zebrafish (*Danio rerio*, fish), fugu (*Tetraodon nigroviridis*, fish), chicken (*Gallus gallus*, avian), Xenopus (*Xenopus tropicalis*, amphibian), and mouse (*Mus musculus*, mammal; Jiao *et al.* 2006). Nucleotide and amino acid (aa) sequence editing, comparison, and alignment were performed using Editseq and Megalign from the DNASTAR package (DNASTAR, Madison, WI, USA). The multiple alignment software ClustalX (Thompson *et al.* 1997) was employed to construct the phylogenetic tree by using the neighbor-joining method. Treeview was used to display the phylogenetic tree.

Molecular cloning of sbPRLR1 and sbPRLR2 from black seabream and ntPRLR2 from Nile tilapia

According to the known fish PRLR1 cDNA sequences and the predicted PRLR2 cDNA sequences in fugu and zebrafish, degenerate primers were designed for cloning the PRLRs in black seabream and Nile tilapia. A plasmid cDNA library of average insert size larger than 2 kb that was constructed from poly(A)⁺ mRNA prepared from seabream gill using the pSPORT1 vector (Invitrogen) was used as the PCR template to obtain a fragment each of sbPRLR1 and sbPRLR2. Methods of rapid amplification of cDNA ends (RACE) were applied to obtain the full-length sequences of sbPRLR1 and sbPRLR2. Seabream gill total RNA was extracted according to the manufacturer's protocol (Roche Applied Science). 5' and 3' RACE were performed using the 5' and 3' RACE amplification kits (Invitrogen) respectively. The full-length cDNA clones were confirmed by DNA sequencing in at least three different individual clones. For the Nile tilapia PRLR2, the whole cloning procedure was similar to that of sbPRLRs, except that the tilapia gill tissues were used to prepare the first strand cDNA as the template for PCR.

For all the PCRs in the present study, reactions were carried out with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52–58 °C for 30 s, and 72 °C for 1–1.5 min. The reaction was ended by a further 10 min at 72 °C. The amplicons were resolved on a 1% agarose gel and the target DNA fragment was purified by the QIAquick Gel Extraction Kit (Qiagen). The fragment was then cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced by an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). Sequence information of all the primers used in the present study is listed in Table 1.

Functional expression of sbPRLR1 and sbPRLR2 in cultured eukaryotic cells

Functional expression is a prerequisite for demonstrating the biological activities of cloned sequences (Chan & Cheng

Table 1 The primer sequences used in the present study

Primer	Sequences (5' to 3')	Purpose
sbPRLR1-F1	TCT CCW GAG AAR GAG ACC TTC	cDNA fragment PCR
sbPRLR1-F2	TGG GTC AAC TAC AAC ATC ACT GT	
sbPRLR1-R6	ACY YCT TTG ACC TTG CTG TAG T	RACE
sbPRLR1-R2	TCS TCW GAC TTK CCR CTC TTG AG	
sbPRLR1-F8	TCA ATG AGG AGG GTA TGG TGC	Full-length PCR amplification
sbPRLR-UTR-R1	ACA TAK CCA CTY ACT GCC AG	
sbPRLR-UTR-R2	TCC ASA MCT GCC WGT TYG G	cDNA fragment PCR
sbPRLR1-Race-R1	TGC ATG CGT GAA GAC TAG CAG CA	
sbPRLR1-Race-R2	GAT AAC TTC AAG GAC TTT CC CAT	Full-length PCR amplification
sbPRLR1-UTR-F	GGA GAG ACT GAA AGC TGC TGT CA	
sbPRLR1-UTR-R	CCC ACC TGC CAG TTT GGG CAC CTA	cDNA fragment PCR
sbPRLR2-F4	ATT GGA GGG AGT GCA CGA GTGT	
sbPRLR2-R4	GTG CAG GGA TGG TAT CTA TAT AT	RACE
sbPRLR2-F6	CTC CTG TTC CTG GTC CAA AGAT	
sbPRLR2-Race-R1	TTC CCT GGT GGA GAC GAG CTG	Full-length PCR amplification
sbPRLR2-Race-R2	CTG CAC ACA GCA CAA GCA C	
sbPRLR2-UTR-F1	CAG CTG GAC ACA CTG CGG GAC A	cDNA fragment PCR
sbPRLR2-UTR-R	ACT ACA CAA TAC CAT CAG CCT	
ntPRLR2-F1	GKT CCC CHG AVA ARG AGA C	RACE
ntPRLR2-R1	GGA CCA GGR ACW GGD GGC AG	
ntPRLR2-F2	CAG CCT CTC TCT GAT TCC GTT GC	Real-time PCR
ntPRLR2-F3	TAT TCT GCC CCC TGT TCC TGG TC	
ntPRLR2-R2	CAA GTC AAA AGT AAG GGG GTC TG	c-myc tag PCR
ntPRLR2-R3	GGA GTG TGC TGT GTG GTA GTC TG	
sbPRLR1-sense	CGG TCT TCT GTG CCT TCA TCT	PRLR1 genome walking
sbPRLR1-antisense	CAC CAG CAA GTC CTC GTA GTT AG	
sbPRLR2-sense	TCT GCC TCC TGT TCC TGG TC	PRLR2 genome walking
sbPRLR2-antisense	GAA GCC AGC AGG AAT AAT CAA G	
sbPRLR1-ORF-R	TAT AGC GGC CGC GTA GGT AGG CAG TGT GAA A	sbPRLR1 promoter cloning
sbPRLR2-ORF-R	TAT AGC GGC CGC CAT TAA AGG TGA TGC AGG GA	
c-myc-F	TAT AGC GGC CGC TGA GCA GAA GCT GAT CTCA	sbPRLR2 promoter cloning
c-myc-R	TAT ATC TAG ACT ACA GAT CCT CCT CTG AGA T	
sbPRLR1-PR1	CGG CTC CCA GAA GAA CGA ACT GAA CT	RACE
sbPRLR1-PR2	CTC CCA GAA GAA CGA ACT GAA CTC ACC	
sbPRLR1-PR3	GTC AGT AGT TAC TTT GCA TCA GAG C	Library PCR
sbPRLR2-PR1	ACT CCA CTG AGT CAC TCA ACT CCA TGT	
sbPRLR2-PR2	TCA CTC AAC TCC ATG TTG CTG CTG TC	Genome walking
sbPRLR2-PR3	GCA CAG CAG CAA AGA TTT GTA CAC AG	
PR1F1	TCG TGA GAT GTT TAG GAG AAT GC	Actin PCR amplification
PR1F2	TAT AAC GCG TGG AGA ATG CTG CAG CTC TGT (Mlu I)	
PR1R1	TAT AAG ATC TTC CAC CGC CTC TCA GTC CGC (Bgl II)	
PR2F1	GGG TGA GCT CGA ATC TTA AGG GAT CTC TCT	
PR2R1	TGG GAA GCT TCA CCT GAG GTG GAG TAT GTA	
AP	GGC CAC GCG TCG ACT AGT AC(T)16	
AAP	GGC CAC GCG TCG ACT AGT AC GGG GGG GGG G	
AUAP	GGC CAC GCG TCG ACT AGT AC	
T7	TAA TAC GAC TCA CTA TAG GG	
SP6	ATT TAG GTG ACA CTA TAG AA	
AP-1	GTA ATA CGA CTC ACT ATA GGG C	
AP-2	ACT ATA GGG CAC GCG TGG T	
sb-actin-F	ACC CAG ATC ATG TTC GAG ACC	
sb-actin-R	ATG AGG TAG TCT GTG AGG TCG	

2004, Chan *et al.* 2004). The full-length sbPRLR1 and sbPRLR2 cDNA clones were functionally expressed in cultured human embryonic kidney (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) and their biological activities studied. HEK293 cells were transiently transfected with 50 ng pcDNA3.1 vector (Invitrogen) carrying the entire coding region of either sbPRLR1 or

sbPRLR2 between the *EcoR* V and *Not* I sites. In order to compare the transfection efficiency and protein expression level of the two receptor constructs, a c-myc tag was attached to the C terminus of both receptors by taking advantage of the *Not* I and *Xba* I sites of the pcDNA3.1 vector. This would allow western analysis of the expression levels of the two receptor proteins in the transfected cells using specific

antibodies against the c-myc tag. The functionality of the expressed receptors in the transfected cells was evaluated by the ability of the receptors to transactivate certain gene promoters in a reporter gene assay format. Briefly, 500 ng luciferase reporter plasmid containing either the rat β -casein promoter in pLucDSS or the human c-fos promoter in pFL711 or the rat serine protease inhibitor (Spi 2.1) promoter in pGL2, were co-transfected together with either one of the two PRLR expression constructs. After 6 h of transfection, purified salmon growth hormone (sGH), PRL (sPRL), or somatolactin (sSL) was added. The cells were then incubated with the respective hormones for a further 20 h. Afterwards, luminescence was measured using a Lumat LB 9501 luminometer (EG&G, Berthold, Germany) and activities of both the firefly luciferase and the *Renilla* luciferase were measured sequentially from a single sample using the Dual Luciferase Kit (Promega).

Western blot

Western blot analysis was carried out according to a standard protocol (Sambrook *et al.* 1989). Briefly, HEK293 cells were lysed at 4 °C. The BCA protein assay kit (Pierce, Rockford, IL, USA) was used to determine the protein concentrations of the cell lysates. Equal amounts of protein were loaded onto polyacrylamide gels, electrophoresed, and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) using a semi-dry electroblotter (Owl Scientific, Woburn, MA, USA). After transfer, the membranes were blocked overnight in 5% dry milk and probed for protein expression using the anti-c-myc antibody (Promega) and then with goat anti-mouse IgG peroxidase-conjugated secondary antibodies (Promega). The bands were stained and viewed on an alpha imager (Alpha Innotech Co., San Leandro, CA, USA).

Expression studies of sbPRLR1 and sbPRLR2 in seabream tissues by real-time PCR

The sbPRLR1 and sbPRLR2 transcript levels in different seabream tissues were measured by real-time PCR using β -actin as the internal control. Gene-specific primers for sbPRLR1, sbPRLR2, and β -actin are listed in Table 1. The primers were designed on different exons so that any genomic contamination could be detected. The primers for sbPRLR1 and sbPRLR2 amplified two specific amplicons of 215 and 235 bp, respectively.

First strand cDNA was synthesized from 3 μ g total RNA prepared from seabream tissues using oligo-dT₁₈ as primer and MMLV-H⁻ reverse transcriptase as enzyme (GeneSys, Borehamwood, Herts, England). PCR amplification and analysis were performed on an MJ Real-time Detection System (MJ, Hercules, CA, USA). The final volume of the reaction was 25 μ l, using the ABI SYBR Green Supermix (ABI, Warrington, UK). The specificity of the SYBR Green PCR signal was confirmed by melting curve analysis and agarose gel electrophoresis. For each particular gene, the efficiency of the PCR

(91–98%) was the same for serial dilutions of the standards and samples. Agarose gel electrophoresis exhibited a single band of the expected size, and all amplified products showed a single melting peak on real-time PCR. The threshold cycle (Ct) was defined as the fractional cycle number by the method of global minimum. The ratio change in the target gene relative to β -actin control gene was determined by the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001, Vong *et al.* 2003). There was some minor but insignificant variation in the Ct values of β -actin in the different tissues as well as in the samples of the hormonal treatment study described in the next section, indicating the validity of using β -actin as the internal control in our study. 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, Yada *et al.* 2005).

Hormonal regulation of sbPRLR1 and sbPRLR2 gene expression in seabream kidney in vivo by real-time PCR

Black seabreams (sexually matured, gonad regressed, body length 22.5 ± 0.3 cm, body weight 235 ± 11 g) were acclimatized for at least 1 month before the experiment. Throughout the study, fish were kept in fully aerated seawater and were exposed to natural photoperiod at ambient temperature (24–29 °C) in May, 2004. Fish were fed with commercially available fish pellet diet until satiety during the experimental period. Fish were injected with either cortisol (5 μ g/g; Sigma), 17 β -estradiol (E₂; 2 μ g/g; Sigma), or testosterone (2 μ g/g; Sigma), intraperitoneally once daily for 4 days. Cortisol, E₂, and testosterone were dissolved in olive oil. The injections were performed at 1000 h every day and the tissues were collected from the fish at 1500 h. on the day of killing. Olive oil was used as the vehicle in the control groups. The fish were then killed by decapitation and tissues were kept at -70 °C after freezing in liquid nitrogen. The transcript levels of sbPRLR1 and sbPRLR2 were measured by real-time PCR as described in the section on tissue distribution studies.

Isolation of sbPRLR1 and sbPRLR2 5'-flanking regions and preparation of promoter reporter constructs

The sbPRLR1 and sbPRLR2 transcription start sites were determined by 5' RACE. Gene-specific primers were designed at the most 5' ends of both cDNAs. Isolation of the 5'-flanking regions of the sbPRLR genes was achieved using the Universal Genome Walker Kit (BD Biosciences Clontech). Five Genome Walker libraries (*Dra* I, *Eco*R V, *Pvu* II, *Stu* I and *Sca* I) were constructed according to the manufacturer's instructions (Yeung *et al.* 2004, 2006).

The sbPRLR1 gene fragment was obtained from two primers containing two restriction enzyme cutting sites, namely *Mlu* I and *Bgl* II, and then the product was cloned into the pCR2.1-TOPO vector (Invitrogen). The TOPO vector with the cloned gene fragment was digested with *Mlu* I and *Bgl* II and cloned into the *Mlu* I/*Bgl* II sites of the pGL3-basic vector. The sbPRLR2 gene fragment was directly inserted into the *Sac* I/*Hind* III sites of the pGL3-basic vector. All the plasmid DNA used in the present study for transfection experiments was prepared using

the QIAfilter Plasmid Maxi Kit (Qiagen). The primer sequences and enzyme cutting sites are listed in Table 1.

Gene promoter assays

The culture media used for cell culture experiments were obtained from Invitrogen. GAKS cells are goldfish scale fibroblast cells from the RIKEN Bioresource Center (Ibaraki, Japan). This fish cell line expresses glucocorticoid receptor (GR) and androgen receptor (AR), but not estrogen receptor (ER) as indicated by RT-PCR using gene-specific primers (data not shown). Transient transfection of the GAKS cells was carried out using the Lipofectamine Reagent (Invitrogen). The cells were seeded onto 24-well culture plates (Iwaki) at a density of 1.5×10^5 cells/well 24 h prior to transfection. The GAKS cells were transfected with 0.5 µg of either the *sbPRLR1* or *sbPRLR2* gene promoter construct in pGL3-basic, and 0.05 µg pRL-CMV vector containing the *Renilla* luciferase reporter gene in 250 µl serum-free medium. After 6 h of transfection, the medium was aspirated away and replaced with complete growth medium. The transfected cells were treated with different steroid hormones, namely E_2 , cortisol, and testosterone in serum-free medium for 24 h. For the cells treated with E_2 , 50 ng goldfish ER α was also co-transfected and the medium was phenol-red free. Then the cells were washed and lysed for determination of luciferase activities. The promoter activity was normalized against the *Renilla* luciferase activity and expressed as the percentage increase relative to the activity of the promoterless pGL3-basic vector.

Data analysis

All data were expressed as mean values $\pm$ S.E.M. Data were considered statistically significant at $P < 0.05$ using either one-way analysis of variance (ANOVA) or unpaired *t*-test.

Results

Data mining and molecular cloning of PRLR1 and PRLR2 in different fish species

Recently, several vertebrate genome databases such as zebrafish, fugu, African clawed frog, chicken, mouse, and human have become available. Using these genome information, we conducted bioinformatics analysis to look for PRLR genes in different organisms. Briefly, the available PRLR protein sequences were used to blast the different genomes using the TblastN method. Then the blast hits with the highest scores and lowest expect value were singled out for identifying the coding regions of the respective PRLR. Such search results indicated the presence of two PRLR genes in the fish genome, a phenomenon unique for teleosts.

By RT-PCR and RACE techniques, two PRLR cDNAs were obtained from black seabream. The full-length *sbPRLR1* cDNA contains 2069 bp encompassing a

5'-untranslated region (UTR) of 182 bp, a coding region of 1866 bp and a partial 3'-UTR. The coding region encodes a protein of 621 aa with a putative single transmembrane domain (Fig. 1). The *sbPRLR2* contains 2751 bp encompassing a 5'-UTR of 158 bp, a coding region of 1614 bp, and a 3'-UTR of 979 bp together with a poly (A) tail. The coding region encodes a protein of 537 aa (Fig. 2). Several characteristic landmarks of PRLR including the WS motif, two pairs of cysteine residues in the extracellular region, and a conserved intracellular Box 1 motif were found in both *sbPRLR1* and *sbPRLR2*. However, unlike *sbPRLR1*, the absence of a typical Box 2 in *sbPRLR2* was noted. In addition, we have also obtained a PRLR2 cDNA in Nile tilapia containing 2011 bp, encompassing a 5'-UTR of 217 bp, a coding region of 1590 bp encoding a protein of 529 aa, and a 3'-UTR of 204 bp (Fig. 3). Again, the Box 2 region was missing in this sequence. Despite the overall similarities between PRLR1 and PRLR2, distinct differences were found in their intracellular regions where several deletions were identified in PRLR2 (Fig. 4). The overall sequence similarity between PRLR1 and PRLR2 is rather low, being 28–40% at the aa level (Table 2). The sequence similarity among the PRLR2s themselves is also low, being 30–46% at the aa level (Table 3).

PRLR1 and PRLR2 alignment and phylogenetic analysis

A multiple alignment of PRLR1 and PRLR2 sequences was performed in the four fish species in which both PRLR1 and PRLR2 sequences are known to date, viz. fugu, zebrafish, Nile tilapia, and black seabream. As shown in Fig. 4, PRLR1 and PRLR2 are very similar in the extracellular region, including the four conserved cysteine residues and the WS motif. On the other hand, remarkable differences were found in the intracellular region. Notably, PRLR2 does not possess the typical Box 2 motif and several other missing regions in the intracellular domain are also found. Using the available PRLR sequences to construct a phylogenetic tree, the results clearly showed that there are two clades of PRLR representing two different subtypes of PRLR in fish (Fig. 5). The PRLR1 clade encompasses the sequences previously reported in a number of fish species including the goldfish (Tse *et al.* 2000), Nile tilapia (Sandra *et al.* 1995), halibut (Higashimoto *et al.* 2001), rainbow trout (Prunet *et al.* 2000), carp (San Martin *et al.* 2006) as well as the black seabream PRLR1 and fugu PRLR1. The PRLR2 clade, unique to teleosts, encompasses the previously reported gilthead seabream PRLR (Santos *et al.* 2001), fugu PRLR2 (Lee *et al.* 2006), as well as the black seabream PRLR2, Nile tilapia PRLR2, and zebrafish PRLR2.

Functional expression of *sbPRLR1* and *sbPRLR2* in transfected HEK293 cells

As shown in Fig. 6, when *sbPRLR1* and *sbPRLR2* were individually expressed in cultured HEK293 cells, receptor

Figure 1 Nucleotide and deduced aa sequences of the sbPRLR1. Nucleotides (upper sequence) and aa (lower sequence) are numbered on the right. The following features on the sequence are highlighted: conserved cysteine residues in the extracellular domain (circled), WS motif (underlined with a solid line), transmembrane domain (underlined with a dashed line), Box 1 and Box 2 regions (boxed in shaded rectangles), intracellular tyrosine residues (circled with polygons), and the stop codon (marked with an asterisk).

activation by sPRL could stimulate both the β -casein and c-fos promoters (Fig. 6A and B) but only the sbPRLR1 can stimulate the Spi 2.1 promoter (Fig. 6C). For the β -casein promoter, sbPRLR1 could trigger a bigger stimulation than sbPRLR2. But for the c-fos promoter, the two receptors exhibited similar activities. This could not be attributed to the

difference in the expression levels of the two receptors in the transfected cells since determination of receptor levels in the cultured cells by western blot (Fig. 6D) indicated that the two receptors had very similar expression levels in the transfected cells. Ligand specificity of receptor interaction was ascertained by stimulating the receptor-transfected cells with sGH and

ACAGATGCGAGGGTTACCAGGTGTGAGAAGGTCGTCGACGACAATGTTGCGGCCGCGAGG
 AGGATTCTGTTGCTCTGTCGACAGCAGCAACATGGAGTTTGAAGTACTGAGTGGTGTAGGTGCGACACTCAGCTGGACACACTGCGGGACAGTTGGTGCC
 ATG ATG TGG AGA GAT CTT CGA TTG GCA GTG CTT GTG CTG CTG TGT GCA GCT GTA AAG TCC AAC AGC TCG TCT CCA 75
 M M W R D L R L A V L V L L C A A V K S N S S S P 25
 CCA GGG AAA CCA GTC CTG CTC AGC TGC AGG TCC CCT GAG AAG GAG ACG TTT ACG TGC TGG TGG CAG CCA GGC TCT 150
 P G K P V L L S C R S P E K E T F T C W W Q P G S 50
 GAC GGA GGG CTG CCC ACC ACC TAC AAA CTC TAT TAT GAG AGA GAA AGA TTG GAG GGA GTG CAC GAG TGT CCG GAC 225
 D G G L P T T Y K L Y Y E R E R L E G V H E C P D 75
 TAT CGG TCA GCA GGT AGC AAC TCC TGT TTC TTC AGC AAG AGC CAC ACC TCC ATC TGG GTT GAG TAC TAC CTG ACT 300
 Y R S A G S N S C F F S K S H T S I W V E Y Y L T 100
 GTG GTG GCC TCC AAT GCC CTC GGG AAT GCC ACC TCA GAT GTC TTT AAA ATG GAT GTG ATG CAA ATA ATA AAG CCC 375
 V V A S N A L G C N A T S D V F K M D V M Q I I K P 125
 AAT GTT CCT GAA AAT GTA ACG CTG CTG GTG GAG ACG GGG GAC AGC CCA TAT CTC AAT ATC AGA TGG GAA CAT 450
 N V P E N V T L L V V E T G D S P Y L N I R W E H 150
 CCA CGT AAC ACA GAC ACC GGG TCC GGC TGG GTC ACC ATT AAA TAT GAA CTA AGA GTC AAA CAA GAA AAC AAT AAC 525
 P R N T D T G S G W V T I K Y E L R V K Q E N N N 175
 AAG TGG AAG GCG TAC ATG TCA GGT ACA CAA ACC CAT TTC AGC CTG TAC AGC ATC ACT CCT GGA GTG GTG CAC ACA 600
 K W K A Y M S G T Q T H F S L Y S I T P G V V H T 200
 GTG CAG GTG CGC TGT AGA CTG GAC CAC GGC TCC TGG AGT GAC TGG ACC AAC AGT ACC CAC GTG AAG GTC CCA AAC 675
 V Q V R C R L D H G S W S D W T N S T H V K V P N 225
 TAT GTT CAG AAT GAA AAA TCA TTT TGG ATC TTG GTC GCC ACC CTG TCT GCA ATT CCA TTT ATG GCA GCA ATG TGC 750
 Y V Q N E K S F W I L V A T L S A I P F M A A M C 250
 ATC TTG GTT GTA AAG AGG GAA AAT GTG AAG CAG TGT GTT CTG CCT CCT GTT CCT GGT CCA AAG ATC AGA GGA GTT 825
 I L V V K R E N V K Q C V L P P V P G P K I R C V 275
 GAT TTC CAC CTT CTC ATG AGT GGA CAA TAT GAA GAC ACC ACC AAT GCC CTG ATC ATC AAC CAG AAC TTT CCT CTC 900
 D F H L L M S G Q Y E D T T N A L I I N Q N F P L 300
 ATG ATG GGC TGG AAG AAC CAG ATC GAG GAG TAC CTC ATA GTG ACC GAG AAT AGC ATC GGG CCT CCG TTG GAT GCC 975
 M M G G W K N Q I E E Y L I V T E N S I G P P L D A 325
 TCG AAT TG CAA AAA AGG AAA AAG E C TTG ATT CCT GCT GGC TTC TGC TCA GAT TGG GAA ATT GAA TGC AAC 1050
 S N C Q K R K K S L I I P A G G F C S D W E I E C K 350
 TCA ACC CAC ATT CAG AAT GAC TGT GCA GGG GGA ACA AAG AAT GAA ACA GGT AAC TTT CCC AAG AAC AAT AAG TCT 1125
 S T H I Q N D C A G G T K N E T G N F P K N N K S 375
 CTC TCA GGG GAG AGT TTA TCT AAC GCG GAG CCA TCC AAA CTG CAG AAA CAG CAG AGT TTG GGT CAA AAC TTA ATG 1200
 L S G E S L S N A E P S K L Q K Q Q S L G Q N L M 400
 AAC ACA GAA GCG ACA GAA CCC AGT CCA CTG AAC CAG GAG AAC GAC GTC AAA CTC TTC ACA AAC AGC GAC TAC GTG 1275
 N T E A T E P S P L N Q E N D V K L F T N S D V V 425
 GAT ATT CGG AAA CAT GTG GAG AAG ACG CAG GAG GTG AAT GTG AAA CAG TTG GAC TAC AGC AGA ATG GAG GAA GTG 1350
 D I R K H V E K T Q E V N V K Q L D V S R M E E V 450
 AAT GGT GAC AAT GTG CTC ATC CTC AAC AAC AAA AAT CTC CCC TTT AAC AGC TAC ATG GAT TTT CAG AGG CAA GAG 1425
 N G D N V L I L N N K N L P F N S Y M D F Q R Q E 475
 GAG AAA CTA TCA GCG GAT TAC AGC AGG GTG AAA GAG GTG GAC AGT AAC AAT GTA GTG GTC CTG CAG AGG CAA GAC 1500
 E K L S A D V S R V K E V D S N N V V V L Q R Q D 500
 GTG TCA GCT GAC ACT AAA ACT CTT CAT GCG ACT GGA GCC CAC AAA ATG GGG GTG TGC ACA GAA CTC ATC AAC AGC 1575
 V S A D T K T L H A T G A H K M G V C T E L I N S 525
 GGA TAT GTA GAT ACC ATC CCT GCA TCA CCT TTA ATG TAA 1613
 G Y V D T I P A S P L M * 550

TGTTTAAACATGCAATGTCCATATACAAATGGTAGATGTCCCTTCTGATTACTGTTCAGAGTTTCAGAGAAAAAGGCTGATGGTATTGTGTAGTTTGTCAATAT
 AATCTGAGGAGTTTCCATGACATTTGGCACAGACATTATGGTCTCCAGAGATAAATCTTACCAACTTCAGTGACACCTGAACTTCCCTATAGCATCATGAC
 GTTCTGCTTTTGTTCAGAAATGAAATATCTTGACACTTTGGAATTTATTTGTGTGAATTTGATACATATATTCAGGTCACAGAGATAAAGCTTAAATCTTT
 TGGTGATCCCGACTCTCTATCTAGCCCCATAATCATCCCAAAGTTTAAATATATCACCTGACTTACTGTATGTAAACAGTAGTTGTAGTTGTATTAATATACATGAT
 AGGTATAAGGATATACGTTTATGGCTCAGGCTAAGGTAACCTTTTGAAGATCTGAAAAGAAAAATACTCAGCCACTCCTTCTTAGATGCTTGCCACTACTGGGA
 CACCTGGTGCAATTGTACAGTGTATTGATGCAATTGAAGCACAGGTCATGTCTAGTCTCCAAGCTATACTACCTTGTGGTGAAGTTACAGTTGCCCTTAAAGAA
 AATCTGCACTGATTTTACCAGTCTGACTGAAGTTTCTCCAGGTGGTGTGAAGTAAACCATATGATAACATGATTACTCTCAGACAGGACTATTTTACT
 CCTCTGAAACTGTCGATTAATCTGAATTTGAGTACATCATTTCTTAATTCGAATCCGATATATTTTGGAGGAAAAATACCTTTTACTCTACTGACTCTTT
 TCAGTTGTTGAGGAAATACTCACCTTTGGTAACAAAGTGATACCAGTGTAAAAATACATGTATGCATAAACGTCCTATTCATCATAATATTATTTGTATTAAAC
 GATAAGTTTACCCCAATTTGAAAAAATAAATAA

Figure 2 Nucleotide and deduced aa sequences of the sbPRLR2. Nucleotides (upper sequence) and aa (lower sequence) are numbered on the right. The conserved cysteine residues in the extracellular domain, WS motif, transmembrane domain, Box 1 region, intracellular tyrosine residues, and the stop codon are highlighted in the same way as in Fig. 1.

Figure 3 Nucleotide and deduced aa sequences of the ntPRLR2. Nucleotides (upper sequence) and aa (lower sequence) are numbered on the right. The conserved cysteine residues in the extracellular domain, WS motif, transmembrane domain, Box 1 region, intracellular tyrosine residues, and the stop codon are highlighted in the same way as in Fig. 1.

sSL in parallel. It was found that sGH and sSL could not stimulate the β -casein, c-fos, or Spi 2.1 promoters at a concentration where stimulation by sPRL could be readily demonstrated (Fig. 6). It is worth noting that for most of the

positive response curves in Fig. 6A–C, a characteristic bell-shaped dose–response relationship was found, indicating the requirement of a certain ratio between the ligand and the receptor in order to generate a maximal productive biological

Figure 4 Multiple alignment of PRLR1 and PRLR2 in fish where both sequences are known to date. These polypeptide sequences were aligned by the Clustal X program. The conserved cysteine residues in the extracellular domain are marked with white triangles at the bottom of the aligned sequences. The WS motifs are boxed. The transmembrane (TM) domains are underlined with two solid lines. The intracellular Box 1 and Box 2 regions are also boxed. The intracellular conserved tyrosine residues are marked with black triangles at the bottom of the aligned sequences. The major defined regions in the intracellular domain of PRLR2 are numbered at the bottom of the aligned sequences. The abbreviations fg, zf, nt, and sb stand for fugu, zebrafish, Nile tilapia, and black seabream respectively.

Table 2 Amino acid similarity between fish prolactin receptors 1 (PRLR1s) and PRLR2s

	PRLR1			
	B_seabream	N_tilapia	Fugu	Zebrafish
PRLR2				
G_seabream	32.9	32.9	32.0	34.4
B_seabream	32.5	32.1	31.3	32.1
N_tilapia	31.0	29.2	27.9	30.6
Fugu	34.9	32.5	32.0	32.0
Zebrafish	39.5	36.5	36.3	35.8

The Clustal method of the DNASTAR software was employed to compare PRLR1 and PRLR2 polypeptide sequences among the fish. Data are expressed as percentage aa identity. G_seabream and B_seabream stand for gilthead seabream and black seabream respectively. N_tilapia stands for Nile tilapia.

response (Tse *et al.* 2000) due to the one ligand–two receptors proportionality. It is apparent that this proportionality would give rise to different dose–response curves for different receptors and for different signaling events.

Tissue distribution

Real-time PCR methods were developed to detect the transcript levels of sbPRLR1 and sbPRLR2 in different seabream tissues. The receptors appeared to be highly expressed in the osmoregulatory organs, particularly the gill and the intestine. In intestine and kidney, sbPRLR1 has a higher expression than sbPRLR2. But in the gill, the expression of sbPRLR2 is much higher than that of sbPRLR1. The expression level is similar for both receptors in the gonad. The expression levels of both receptors are relatively low in most other tissues, with sbPRLR1 generally higher than sbPRLR2. The expression of sbPRLR2 in the heart and spleen could not be detected even using this sensitive real-time PCR method (Fig. 7).

Differential regulation of sbPRLR1 and sbPRLR2 expression by steroid hormones in seabream kidney in vivo

The expression of sbPRLR1 and sbPRLR2 in the kidney of seabream administered with different steroid hormones *in vivo*

was investigated (Fig. 8). The expression of sbPRLR1 was significantly increased after treatment with E₂ and cortisol, but treatment with testosterone did not elicit any significant change. In contrast, expression of sbPRLR2 was significantly decreased after treatment with E₂ and testosterone, while treatment with cortisol did not bring about any significant change (Fig. 8).

Isolation of the 5'-flanking regions of the sbPRLR1 and sbPRLR2 genes

As a first step towards understanding the transcriptional regulation of the seabream PRLR1 and PRLR2 genes, the 5'-flanking regions of both genes were isolated in the present study. After several rounds of genome walking in a stepwise manner, two genomic fragments of 2366 and 2113 bp corresponding to the sbPRLR1 and sbPRLR2 genes respectively were isolated. Sequence analysis using TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=SEA-FR-QueryS>) revealed a number of putative transcription factor-binding sites on them (Figs 9 and 10). The promoter activities of these 5'-flanking regions were evaluated in cultured GAKS cells by measuring the promoter-driven luciferase activities. A 25-fold increase in luciferase activity over and above the promoter-less control (pGL3-Basic) was recorded for the sbPRLR1 gene promoter, and a 10-fold increase for the sbPRLR2 gene promoter (Fig. 11A). These strong promoter activities indicated that the cloned 5'-flanking regions did represent the functional promoters of the seabream PRLR genes.

Regulation of promoter activities by steroid hormones

As shown in Fig. 11, after treatment with E₂ and cortisol, the luciferase activity was significantly increased for the sbPRLR1 gene promoter, but decreased for the sbPRLR2 gene promoter. In the case of testosterone treatment, the luciferase activity was significantly decreased for the sbPRLR2 gene promoter, but remained unchanged for the sbPRLR1 gene promoter. The overall promoter study results in response to steroid hormone challenge were in general agreement with the hormonal regulation of gene expression studies *in vivo* shown in Fig. 8.

Table 3 Amino acid sequence similarity among the fish prolactin receptors 2 (PRLR2s)

	PRLR2				
	G_seabream	B_seabream	N_tilapia	Fugu	Zebrafish
PRLR2					
G_seabream	100				
B_seabream	86.9	100			
N_tilapia	41.0	41.0	100		
Fugu	46.3	45.9	37.6	100	
Zebrafish	35.6	34.0	29.7	34.5	100

The Clustal method of the DNASTAR software was employed to compare PRLR2 polypeptide sequences among the fish. Data are expressed as percentage aa identity. G_seabream and B_seabream stand for gilthead seabream and black seabream respectively. N_tilapia stands for Nile tilapia.

Figure 5 Phylogenetic tree of PRLRs from different vertebrates. The default settings of the ClustalX protein alignment program was employed using the black seabream GHR1 (NCBI accession no. AF502071) as the outgroup, and visualized by the treeview program. The values represent bootstrap scores out of 1000 trials, indicating the credibility of each branch. The two fish PRLR clades are named as PRLR1 and PRLR2. The PRLR sequences used in this phylogenetic analysis are: Bull frog (*Rana catesbeiana*) BAD14941; African clawed frog (*Xenopus laevis*) PRLR form a AAF05776 and form b AAF05777; Japanese firebelly newt (*Cynops pyrrhogaster*) BAB61107; silver-gray brushtail possum (*Trichosurus vulpecula*) AAD27039; brown capuchin (*Cebus apella*) AAO73437; dog (*Canis familiaris*) XP_536502; domestic pigeon (*Columba livia*) Q90374; rabbit (*Oryctolagus cuniculus*) P14787; chicken (*Gallus gallus*) AAS93635; pig (*Sus scrofa*) AAQ76842; human (*Homo sapiens*) AAH59392; turkey (*Meleagris gallopavo*) AAB01544; house mouse (*Mus musculus*) Q08501; Norway rat (*Rattus norvegicus*) P05710; red deer (*Cervus elaphus*) CAA64419; white-tufted-ear marmoset (*Callithrix jacchus*) CAB75847; cattle (*Bos taurus*) Q28172; sheep (*Ovis aries*) O46561; leopard gecko (*Eublepharis macularius*) BAD24103; zebrafish (*Danio rerio*) PRLR1 AAQ84555 and PRLR2 XP_690339; goldfish (*Carassius auratus*) AAF23268; common carp (*Cyprinus carpio*) PRLR form a AAK95833 and form b AAV71059; rainbow trout (*Oncorhynchus mykiss*) AAG44267; the Nile tilapia (*Oreochromis niloticus*) PRLR1 Q91513; bastard halibut (*Paralichthys olivaceus*) BAB62756; gilthead seabream (*Sparus aurata*) AAG17629; fugu (*Takifugu rubripes*) PRLR1 BAE94535; and PRLR2 NM_001078625; together with the three sequences obtained in the present study: black seabream (*Acanthopagrus schlegelii*) PRLR1 EF429092 and PRLR2 EF429093; and the Nile tilapia (*Oreochromis niloticus*) PRLR2 EF429094.

Figure 6 Transactivation of the different promoters in transfected HEK293 cells expressing sbPRLR1 and sbPRLR2. HEK293 cells were co-transfected with a pcDNA3.1 vector containing the entire coding region of either sbPRLR1 or sbPRLR2 (both containing a c-myc tag attached to the C terminus of the receptor) together with a luciferase reporter plasmid driven by the β -casein promoter (A), the c-fos promoter (B), or the Spi 2.1 promoter (C). The control for the receptor constructs was the empty pcDNA3.1 vector. The transfected cells were subsequently stimulated by salmon PRL (sPRL) at concentrations indicated on the x-axis, salmon GH (sGH) and salmon SL (sSL) at the concentration of 300 ng/ml. Results are mean values $\pm$ S.E.M. ($n=6$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ as compared with the respective control by one-way ANOVA). The western blot result by the anti-c-myc antibody is shown in (D). I and II represent the western blot results of sbPRLR1-myc and sbPRLR2-myc, at the appropriate sizes of the about 68 and 56 kDa respectively. No other major bands could be seen. M represents the molecular weight standards. The results show that the two constructs had similar transfection efficiency and the expression levels of the two receptors were similar in the transfected cells. Cells transfected with the empty vector did not exhibit any of the receptor bands when western blotted with the anti c-myc antibody.

Figure 7 Distribution of PRLR1 and PRLR2 in seabream tissues. Results are normalized against β -actin expression and are expressed relative to the gonad PRLR1 level. Results are mean values $\pm$ S.E.M. from six fishes. Significant difference, as revealed by unpaired *t*-test between sbPRLR1 and sbPRLR2 expression in each tissue type, is marked by asterisks (* $P < 0.05$).

Discussion

Genome analysis predicts the presence of two *PRLR* genes in fugu and zebrafish. This *in silico* prediction was experimentally substantiated by demonstrating the presence of two distinctly different PRLR cDNA sequences in both black seabream and

Nile tilapia. Subsequent analysis of all the available PRLR sequences known to date revealed that there are indeed two clades of fish PRLR, which we call PRLR1 and PRLR2. In fact most of the PRLRs discovered so far in fish belong to the PRLR1 clade including those from goldfish (Tse *et al.* 2000), Nile tilapia (Sandra *et al.* 1995), halibut (Higashimoto *et al.* 2001), rainbow trout (Prunet *et al.* 2000), and carp (San Martin *et al.* 2006). The PRLR1 clade in fish is more akin in structure to the PRLRs found in other non-fish vertebrates. Nonetheless, there are also examples of previously discovered fish PRLRs which belong to the PRLR2 clade and these include those from gilthead seabream (Santos *et al.* 2001) and fugu (Lee *et al.* 2006). Without realizing that there are two types of PRLR in fish, the differences found in different fish PRLRs have been attributed to the diverse phylogeny of different fish species. Our data therefore represent the first report demonstrating the concomitant existence of two PRLR cDNA subtypes in a single fish species in different teleost lineages. This phenomenon is entirely different from the demonstration of two PRLRs found in carp (*Cyprinus carpio*; San Martin *et al.* 2006) in which both sequences belong to the PRLR1 clade, as shown in the phylogenetic analysis (Fig. 5). In view of the tetraploid nature of this fish, it is highly likely that other PRLR sequences belonging to the PRLR2 clade might also exist in carp, pending experimental proof. The same applies to other fish species in Fig. 5 in which only one subtype of the two PRLR clades has been discovered so far. Liu *et al.* (2006) did point out the presence of two genes for PRLR in zebrafish. However, they have neither reported the PRLR2 sequence nor demonstrated its functional significance. Moreover, they did not realize that it is a common phenomenon in teleosts.

Using black seabream as the animal model, we succeeded in obtaining the entire coding regions of the two PRLRs in this fish species. This renders parallel studies on these two receptors in a single fish species possible. Thus, these two receptors were functionally expressed in cultured eukaryotic cells in order to study their signaling events upon ligand stimulation. Real-time PCR methods were developed to enable simultaneous quantitative assessment of their expression levels. Moreover, the promoters of the respective genes have been cloned and studied in transfected cells by means of reporter gene assays. The two PRLR subtypes in seabream were shown to exhibit different patterns of tissue distribution, post-receptor signaling, and differential regulation by steroid hormones at the gene expression level as well as at the promoter level.

In the present study, the PRLR1 and PRLR2 sequences obtained feature the long form of mammalian PRLRs which contain an extracellular domain, a transmembrane domain, and an intracellular domain. Two pairs of conserved cysteine residues were found in fish PRLR1 and PRLR2, suggesting a similar hormone-binding site for the two receptor subtypes (Rozakis-Adcock & Kelly 1992). In the extracellular domain, the conserved WS motif was found in both PRLR subtypes, indicating the possible significance of this motif in the two fish

Figure 8 *In vivo* regulation of PRLR1 and PRLR2 expression in seabream kidney by hormone treatment. The hormone concentrations used were: cortisol (5 μ g/g), E₂ (2 μ g/g), and testosterone (2 μ g/g). Results are mean values $\pm$ S.E.M. from six independent fishes (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared with the respective control by one-way ANOVA).

AAAAAGGGCGTAAATGAAGCTAAAAAGGTTTATTGGGATCTCAGGGTTTTTTTCGATTGTTTGTCCCATCTTCGTGAGATGTTTAG	-2461
GAGAATGCTGCAGCTCTGTTTTGCTGTGAAGCTCCAGGAATGTTTTGAAACTTAACCTGACTTTCCCATAGCATGGGGTTAAGTAGA	-2373
HSF	STATx
TAATGACTGAATTTTTCTTTTTGGGTGAACGTATCCTTTAATGCATTAATCATATGATAATTGTATGATAGCTGATACAATCTCCTC	-2285
CATTTGAGTGCTTTTATTGTGATGATTTTCAGCACATGTTGCTATATTTTAGCAATTCATGCTTGTATGCGTGCTGATGCCCTTTT	-2197
TTTTTTGCCAGCCGTGCCCTGAAACATCCGGAGTCCGTCAGTGCAGTCTGTACAATTACCACCTTCCAGGAATTTCCAGTCCGTCCG	-2109
	NF-kappaB
CACGCATCTTTGAATGCATATGTGGGTATTTTACGTCTGTGTCTGCAACATCTCTATAGGTGTGGCTTCCCAGAGAAAGTGAACACAA	-2021
	GRE
TGGGAGAAAAGGAAGTTCAGTTCAGGACACAATGATCGGACGCAGAAACAGATCCCTAAAGATCCCAGAAGGATTCTACAATTCATTT	-1933
GGCTTTTATGACTTTCCATAGTTTACAGCTTACTCATTGTGTTTACTCAAGGACAAAAGGTGCACTACCTTGTAGCCTTGTGCTATTTA	-1845
CdxA	
GATGGACGCACACTTAAGTACATGCAGTCTTCTCGGTGGGAACATCCTGGGGTTACAGCCTGCTGTAATAAATACTGCTCTACGTT	-1757
	ADRI
CTCTCCAGAGGATTGTCTGACTGTTTATAATATTATGATTTTTTTTCTCCTTTTAGAGAATTAGAGCCTGCATAAAAGTGTGTATT	-1669
	XFD-2
AATCCTGTATGTATCAAAACATTTTGTAGTAGATAAACTCGCTCATATATACTAGTGTAATAAATAAGAGAGAGCAAGTCAGGGACACA	-1581
	NIT2
TTATGGAGGGCGGGGGGGAGAGAGGGGATGTCAATTACTGGGAGACCTCACTATGTTTACAGCTCCAGTAAAGTAAAGAGGAACC	-1493
	ADRI
	Dfd
AGTGAACACTTTAGACACTATGTTAGGAGGTGAGGGCGTTCTGATCATCAAATCGTGTGTTTACAATATTTGTTTCATATGCAAAGCGTGT	-1317
	ERE
	HNF-3b
AAAAGTCACTAAGGTAGCAGCACAGGAATGACCATGTCATGTCCTGAGGAGAGGAGAATTGAATTTGTTGTATTATTAGATAGATAGA	-1317
	cap
TAGATAGATACTTTATTGATCCCGAGGAAATCAAGTATTATATTATATTATATTATATTATATTATAACATTCCTGATCTTATA	-1229
	CDP CR
ACCTTTAAGCTTCACATACACAGTTTTGAAATGTGGCTTTTATTGAGTGATGTAAGTAAATACATCTGCCATCACTGATCATCA	-1141
	MAIal
	HSF
GAATGAAAGTGCAAATCTAATTGATGATTAATAAATACTTCTTTAAAAACATTTAAACATTTTGTGGCTCTGATGCAAAGTAACTACT	-1053
GACTAAAGGTATCAGTTGAACATAGTAAGTAAATATTACCCCTCCAAATGTACTGAAAGTATAAAGTAACAGAAATGGAATACTT	-965
	XFD-1
TAGTGAACGTAGTTATTGTAGTTACATTCATTACTTATACATACTTGCGTCTTTCTACTTCTACTCCATCACATCTCAGCCATTAGC	-877
CAGTTAATCAATAACTGTTTAAAGTATCTTTAATCTAAATACATTTTCATAGCTCCAGCTTCACAAATGGAAATTGAGGCAAAATTGCT	-789
c-Myb	Nkx-2
TTTTCTTTTCAATATCTTTTAGAAACATTCTTTTAAATAAGGGCTAGGGTATGGGTATTTTACAAACCAAAAAATTAATTGATTAAT	-700
	CdxA
	Dfd
GCAGAAAATAATCAGGGGAATCAGTGGTAAGTTACTATTTTGTGACTATCATTTTTATAGTCATATTTCAAATGTAATGATATCTTTA	-612
	STRE
TTGTGTTATGAATATATATGTATGTATATATTATAAAAGGTATGTATAAAGGTAAACTCTTAGTTGGATACTAACACTCCCTC	-524
	CF2-II
	TATA
CACCACTGCACGGTTGCTTTAGGAGGAAATGCACTGACCCTCATTCTTTCTGGAAAGTTTCTAACCTCTAACCACTGCATGTGAC	-436
	STATx
ATCCAACCCCTGAAACACGCGCACACACACACACACACACAGAGGAATATATGATTAACCAATACAAAGCTCTACTGAAC	-348
CACAGCTACCAGCCCTTGTATCTCTTTTCTTAGAGGTCAAACCTCATCCAGCGTGACTGCTCATTGTATGGGCGTGGTTAAACTGG	-260
	NIT2
	ERE
	ADRI
GGGTGGGAGGGTCTATGCTCGTCCGCCACGCCCCCTCATAATGATAGCGCGGGCGGACTGAGAGGCGGTGGATGTTTCGACTTCTTG	-172
	Oct-1
TGGCAGACGCTCATGAGTGTACCATCGTCCCACGAGCCCGGTAAACCGGTTACACAAGTCGGTGAGTTCAGTTCGTTCTTCTGGGAGC	-84
	#
CGCCAGTGGACCCAGCACGGCCAGAACCCGAGGGAGAGACTGAAAGCTGCTGTCTGCTGAGCTGAAGCTGAGGCTTTGTGGTGCCACGATG	

Figure 9 Sequence analysis of the 5'-flanking region of the *sbPRLR1* gene. The putative binding sites for transcription factors are underlined. These transcription factor-binding sites were identified using both TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=SEA-FR-QueryS>).

AAATAAATGAAGAAATAATAATAACAATAACAAACATTACTTTTATTATTATTATTATAGAGATTTGAATCTTAAGGGATCTCTGGA	-2179
HSF Sox-5	
TTGTGTAACAAGTAGCTGGAATGTGATTAATACTGTTCTTATAGTCAGTATTGTGTTTTGTAGATTGAGTGTCTCTCAAAAAATGTTTTA	-2086
CdxA cap	
TTCTGATGAGAAAGGACACCTGAAATGTGCAGGCTGCCTAAGATGTTCCTCTAAACTGAAAAGCCACACCTTGAGAATTTAGAGGATAAAA	-1993
STATx	
TCACCCAAAAATGGAATTCATCATCTACTTTTGAAGTTCACAAAAATGTCTACAGCTTCACAGCAAAACAGTCTTGACGATTTTCTCTAAAC	-1900
SRY	
AACCTGTAGATGGGGACCTACATTTACAAATGAGGGGTCTGCTCATTGTCCCATGAGAACCATCTCAAACACACTCCATCTCCACCGAGTGA	-1807
Ttk 69k HSF	
GAGAGAGAGGTCACTGCAATGCTCAGCTGATTACTTTTGTACAGTGTAGCATTATATATATACTTTCTTGCATTTTGTCTTTTTTCAACA	-1714
ERE AP4 CF2-II	
GGTCAGGTTAGAATACAGACATGCCTGTCATGAAACCAGCCAGTGTATGTAGTGGGATGCGGGTGTGGATCGTAGAAGATTAACAAGGTTAA	-1621
ERE	
CAATGATGTGCACACATATTAACCCCAACGATAAAACAATCATGTTGACTGTGAAGATATGCCCTCCTCACTGCACCTCAGGCCAGACAGG	-1528
Sox-5 HSF	
CTAACTCCATCCCTGGTAGAAGGGTGGAGTGAAAACTACCAGCCCTGTTGTTTACACGCTAGTGCTCAGTCAGCTGTGTACAAATCTTTGCT	-1435
GCTGTGAAGTCAACCTGTGTGATCCTCAACCTGGCTTTAAAGAAAAATCACAGTGGTGGTTATCATTGACAGCTGATGTTCACTCATTTTT	-1342
cap	
AATTTGACCATTTTCATATCAAAAAGGTAAAAAGGTTTGTGTTTTGGTGCACACTTTTTGTTCTTATCAAGGTGAAAAATGCTGCAGCTG	-1249
GATA-X	
CATTTATTTCCCACTTATTTATTTGTACATTTTGCACAACACTCTGTAAAAAGTATAGCTTACAGTCGCTAGTGTGGCATCAGTCTTGC	-1156
cap	
TTAGTCTTGTGGCTCTGCATTGCTTTGTTACTGTAGAATATCAATAGATACTACAGATGAAGAACGTTGCACCAGAGATGATTGATTTTC	-1063
HSF	
CGTGAATTCATAAATCTGTTTCATCATTTATTTGTGTTGGAGTTTTTGTAGGTCAACTTTTTCTTGCCAAATGCTCATTGACATCAGTTGA	-970
ERE	
ATAAAGAAGCAAAATGTAAAAAATAAATAAATGTTGCAGAAAAAAGTTTGTGGAGATGAAAAGGGGAAAAATAGGGTGGCACTTT	-877
GCTCAGGAGGGAGAGCGGGTTGTACAGAATTCGGAAGGTGCTGTTTACTCCAGGATCCTCTGGCTGCAGGTCGAAGCATCCATGAGCAA	-784
GAAACTGAGCCCTGAGTTGCTCCTGATGAGCAGGTGGGAGCTGGCATGGCAACAGCCAACTCCATCAGTGTGTGAATGGCTGAATGTCCTA	-691
AGTCAGTACATAGGAAAAGAGTAAAGCAATGGAAGGAAAGAAAAATAGAAATAGAAAAATAACACAAAAATGAATAAATTCAGAAATGTATG	-598
ATTTATTTAAAAAATAAAGATAGATGAATTAATAAATGAAATTTATTAATTGGACTGTAAATTAATTGACAATTAATAAAGGTAAATAACA	-505
GR	
CGCAACAATAAATTAATGAATACATTTTATTTATTTTAAATCATTGGTGCATTTAACAGCAGCCAGTTTATCTATTAAGTAGTGTATT	-412
Sox-5 NIT2	
ACTTATTCATTACTACCAATATTAGTATTATTTGATTGAGCAGGTTTTGGTTCTCCGTACTAGCTCGCTTGGTAAAGTATAATCTGCCGGTG	-319
GGGCAGAAATTCGGTTTATAATAACCCAGAATAAAGCAGGAGTGACACATCTCGGTTACGCCACGCTCCCTCGGGTAGAGGAAATGTGACGG	-226
AATCAAGCCCCCTCCCCCTTCGTGGCTCAGCTCACCTCTCTCTCATTACATACTCCACCTCAGGTGACAGATGCGAGGGTTACCAGGTGTGA	-133
GAAGGTCGTCGACGACAATGTTGCGGCCGAGGAGGATTCTGTTCTGCTCTGTCGACGAGCAACATGGAGTTGAGTGACTCAGTGGTGTAGG	-40
#	
TCGACACTCAGCTGGACACACTGCGGGACAGTTGGTGCCATG	

Figure 10 Sequence analysis of the 5'-flanking region of the *sbPRLR2* gene. The putative binding sites for transcription factors are underlined. These transcription factor-binding sites were identified using TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and TESS (<http://www.cbil.upenn.edu/cgi-bin/teess/teess?RQ=SEA-FR-QueryS>).

receptors. In a previous study performed on mammalian PRLR (Rozakis-Adcock & Kelly 1992), this region was suggested to provide a target site for interaction with some accessory proteins necessary for the formation of a high-affinity receptor complex. Whether the same holds true for

the fish receptors remains to be established. The transmembrane domain of PRLR1 and PRLR2 comprises about 24 aa, which are similar to other PRLRs. Box 1 is the most conserved motif in the intracellular domain, which is consistent with its importance in the signal transduction

Figure 11 (A) The basal activities of *sbPRLR1* and *sbPRLR2* promoters in transfected GAKS cells. The cells were transfected with 0.5 μ g pGL3-basic vector containing either the *sbPRLR1* or *sbPRLR2* gene promoter construct and 0.05 μ g pRL-CMV. The empty pGL3-Basic vector was used as a control. The transfected cells were incubated with the medium without any hormone for 24 h. Relative promoter activities are expressed as a percentage of the control. (B–D) The effect of E₂/cortisol/testosterone on the *sbPRLR1* and *sbPRLR2* gene promoter activities in transfected GAKS cells respectively. The cells were transfected with 0.5 μ g pGL3-basic vector containing either the *sbPRLR1* or *sbPRLR2* gene promoter construct and 0.05 μ g pRL-CMV. The transfected cells were incubated with different concentrations of E₂/cortisol/testosterone for 24 h. Relative promoter activities (i.e. those under hormonal stimulation) are expressed as a percentage of the control in the absence of the hormones (i.e. the basal promoter activity without hormonal stimulation). Results are mean values $\pm$ S.E.M. ($n=9$; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as compared with the respective control by one-way ANOVA).

pathway (Dinerstein *et al.* 1995, Pezet *et al.* 1997). A typical Box 2 is absent in PRLR2 (Fig. 4). To date, the function of Box 2 in PRLR is not well defined. Thus, the significance of the absence of this motif awaits further elucidation. In addition, PRLR2 has four other deletions in the intracellular domain as compared with PRLR1, contributing to the loss of some intracellular tyrosine residues (Fig. 4). For sbPRLR1, there are 11 intracellular tyrosine residues but for sbPRLR2, there are only 7 such tyrosine residues. Similarly, tilapia PRLR1 has 11 intracellular tyrosine residues, whereas tilapia

PRLR2 has only 7 tyrosine residues in the intracellular domain (Fig. 3). Due to the importance of these intracellular tyrosine residues in the signal transduction of cytokine receptors, such deletions might also result in changes in the biological activity of the receptor.

Expression of the cloned receptors in cultured eukaryotic cells followed by functional assays using specific promoter-driven reporter gene assays in the present study indicated that sbPRLR1 and sbPRLR2 are indeed functional entities capable of transducing post-receptor signaling events. It has

been reported previously that association of Janus kinase (JAK)2 to the Box 1 region is essential in mediating the signal transduction of mammalian PRLRs, followed by the activation of *c-fos* and β -casein expression (Lesueur *et al.* 1991, Berlanga *et al.* 1995). In our study, both sbPRLR1 and sbPRLR2 could activate the *c-fos* and β -casein promoters upon receptor stimulation. The two receptors exhibited similar activity towards activating the *c-fos* promoter but sbPRLR1 appeared to be more potent than sbPRLR2 in activating the β -casein promoter. In addition, only sbPRLR1 but not sbPRLR2 could activate the Spi 2.1 promoter. Such differences probably reflect the innate abilities of the receptors in mediating the signal transduction processes as they exhibited a very similar expression level in the transfected cells (Fig. 6D). The ligand specificity results indicated that the receptors could respond to PRL, but not to GH and SL. These results are consistent with the fact that the two receptors have a similar extracellular ligand-binding domain but with much differences in the intracellular domain. In fact, the Spi 2.1 pathway that we have demonstrated for sbPRLR1 is a novel one for PRL signaling. Spi 2.1 promoter activation has been demonstrated for mammalian GH receptor (GHR; Dinerstein *et al.* 1995, Gong *et al.* 1998) as well as for fish GHR (Tse *et al.* 2003, Jiao *et al.* 2006). To our knowledge, there is no previous report on the Spi pathway for PRLR.

PRL/SL/GH belong to the same cytokine family. They share many common features and so do their receptors. Recently, two SL receptor (SLR) sequences have been cloned from trout and medaka fish, and they are similar to the GHR in teleosts (Fukada *et al.* 2005, Fukamachi *et al.* 2005). This raises the question of whether the PRLRs cloned in the present study may also cross-react with GH or SL. The results in Fig. 6 clearly demonstrated that the cloned receptors could respond to the ligands in a specific manner indicating that they are truly PRLRs. This is in line with the phylogenetic analysis results showing that the cloned sequences are PRLRs but not GHRs or SLRs.

It should be mentioned that we have adopted a heterologous system in our study, viz. using salmon hormones to analyze the functional activity of seabream PRLRs in a human cell line. Since HEK293 cell line is a well-characterized cell line used in the functional expression of similar receptors, we have employed this established system to express our cloned receptors in order to avoid other uncertainties. The use of heterologous hormones in our system is not the most ideal. The availability of well-characterized and highly purified pituitary hormones from salmon and the lack of availability of the same hormones from seabream render this a feasible alternative. The message from the ligand specificity studies is clear although it could be argued that the amplitude of the response could be higher if one uses homologous hormones.

Real-time PCR was developed for the accurate quantitation of both receptors in seabream tissues. The sbPRLR1 and sbPRLR2 were found to be expressed in tissues implicated in the control of water and ion balance including

the gill, intestine, and kidney (Fig. 7), in line with the fact that these are the target organs of PRL. Interestingly, the expression of sPRLR1 is higher than that of sbPRLR2 in most tissues with the notable exception of the gill in which the expression of sPRLR2 is several fold higher than that of sbPRLR1, suggestive of a particular biological role of sbPRLR2 in the gill of the fish. The seabream gonad contains similar levels of both receptors, in line with the important role of PRL in reproduction (Bole-Feysot *et al.* 1998). The wide distribution of the receptors in different tissues of seabream is consistent with the multifaceted roles of PRL in fish. It is interesting to note that the black seabream muscle was found to express measurable amounts of PRLR, but not in the liver. This is in general agreement with previous finding in gilthead seabream (Santos *et al.* 2001), the significance of which is unknown at the moment. This diverge tissue distribution patterns of the two receptors in seabream indicate that these receptor paralogs in fish probably possess some different biological functions.

Reports on the actions of steroid hormones on the gene expression of PRLR in fish are scanty. In this study, the effects of steroid hormones on sbPRLR1 and sbPRLR2 expression were studied both in fish administered with steroid hormones *in vivo* and at the gene promoter level *in vitro*. The functionalities of the putative promoter regions of the *sbPRLR1* and *sbPRLR2* genes were tested by transient transfection in cultured cells by a reporter gene assay. The 5'-flanking region of the *sbPRLR1* gene exhibited about 30-fold induction of the promoter activity, while the 5'-flanking region of the *sbPRLR2* gene showed about 10-fold increase in the promoter activity as compared with the promoterless construct.

In our study, the expression level of sbPRLR1 in the kidney was up-regulated, while the sbPRLR2 was down-regulated by E_2 treatment of the fish (Fig. 8). Results of the promoter studies correlated with the gene expression studies as treatment of the transfected GAKS cells with increasing dose of E_2 activated the *sbPRLR1* gene promoter activity but inhibited the *sbPRLR2* gene promoter activity (Fig. 11B). Estrogen exerts its transcriptional regulation by binding to the ER and targeting to the estrogen-responsive element (ERE), which consists of a palindromic repeat of the 5'-TGACCT-3' half site (Zilliacus *et al.* 1995). By searching the sequences in our *sbPRLR* gene promoters, we have also located several such sequences (Figs 9 and 10). On the other hand, a new transcription mechanism that does not involve the ERE was recently found (Dong *et al.* 2006). Since E_2 could differentially alter the gene expression levels of sbPRLR1 and sbPRLR2 as well as the promoter activities, it is interesting to know whether the transcriptional regulation for these two receptors involves different mechanisms. Some other studies indicated that the PRLR expression level could be differently regulated by E_2 at different developmental stages in different species. In the gonad of adult gilthead seabream, the transcript level of PRLR, which is a PRLR2 according to the phylogenetic analysis (Fig. 5), increased significantly

(50-fold) after E₂ treatment, but a 24-fold decrease was found in the juvenile gonad (Cavaco *et al.* 2003). However, in mammals, E₂ stimulates the expression of PRLR in various tissues (Cheng *et al.* 1984, Tsim *et al.* 1985, Barash *et al.* 1992, Cassy *et al.* 2000). The mechanism for these differences awaits further studies to unravel.

The hormonal control of adaptation to seawater involves the pituitary interrenal axis with cortisol as the final steroid product (McCormick 2001). In this study, it was demonstrated that treatment of fish *in vivo* by cortisol increased the expression of sbPRLR1 but had no effect on the expression of sbPRLR2 (Fig. 8). At the promoter level, the sbPRLR1 gene promoter activity was increased, in contrast to the decreased sbPRLR2 gene promoter activity when challenged with increasing dose of cortisol (Fig. 11C). Glucocorticoids can regulate gene transcription by multiple mechanisms including direct GR binding to a gene promoter and those that involve GR acting on a gene promoter via interaction with a DNA-bound transcription factor (Newton 2000). Positive and negative glucocorticoid-responsive elements (GRE) have been identified in several gene promoters such as the Na⁺/K⁺-ATPase β1 subunit (Derfoul *et al.* 1998), Dax-1 (Gummow *et al.* 2006), and glucose-6-phosphatase catalytic subunit (Vander Kooi *et al.* 2005). However, negative GRE in fish have never been reported in any gene promoter. In our case, only positive GRE were identified (Figs 9 and 10) because negative GRE do not often match the consensus sequence (Vander Kooi *et al.* 2005). Under the stimulation of cortisol, the sbPRLR1 and sbPRLR2 gene promoters exhibited very different luciferase activity patterns. The mechanisms adopted by these two promoters in their cortisol response remain to be elucidated.

Apart from estrogen (Guzman *et al.* 2004), testosterone was also demonstrated to regulate osmoregulation through Na⁺/K⁺-ATPase. In a recent publication by Sangiao-Alvarellos *et al.* (2006), the role of testosterone on osmoregulation was studied on the euryhaline teleost gilthead seabream. Fish implanted with testosterone (5 µg/g body weight), the kidney Na⁺/K⁺-ATPase activity was enhanced on the first day but diminished by day 3. In our study, testosterone was demonstrated to regulate the expression of PRLRs in fish. The expression of sbPRLR1 was not significantly changed but the expression of sbPRLR2 was down-regulated (Fig. 8). These results were consistent with our promoter studies in which the sbPRLR1 gene promoter activity remained unchanged but the sbPRLR2 gene promoter activity was inhibited (Fig. 11D). The AR-mediated gene expression suppression might be due to the presence of a co-repressor of the ligand-bound AR, such as cyclin D1 (Burd *et al.* 2005) or Daxx (Lin *et al.* 2004). The discovery of these regulators could provide us with a more comprehensive explanation of the transcriptional regulation of PRLRs. In our study, only sbPRLR2 could respond to the exogenous androgen treatment suggesting that distinct physiological functions exist for these two receptors.

Evidence suggests that fish underwent a fish-specific genome duplication which accounts for the presence of

more duplicated genes in teleosts than in tetrapods (Hoegg *et al.* 2004, Meyer & Van de Peer 2005). The hypothesis is becoming more widely accepted based on evidence from the recent genome annotation work of the fugu and zebrafish genome sequences. In the present study, two PRLR subtypes encoded by two different genes were found in four different teleost species, viz. black seabream, Nile tilapia, fugu, and zebrafish. The results provide additional evidence for a genome duplication in the Actinopterygia (ray-finned fishes). Duplicated genes in a genome can only be maintained if they acquire a new function or maintain only some functions of the ancestral gene (Force *et al.* 1999). In this study, we have demonstrated that the two receptors exhibited distinct differences in several aspects. These functional differences probably explain and justify the preservation and coexistence of both PRLR subtypes during teleostean evolution after the genome duplication.

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