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Tissue-specific and embryonic expression of the retinoid X receptors in *Sebastiscus marmoratus*

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ABSTRACT

Retinoid X receptors (RXRs) are highly conserved members of the nuclear receptor family and mediate various physiological processes in vertebrates. Most studies on RXRs have concentrated on their structure and function in mammals and their characterization and developmental expression in *Danio rerio*. However, there is little information concerning the distribution of RXRs in teleost tissues. In the present study, we cloned partial sequences of three RXR subtypes (RXRa, -b, -g) from *Sebastiscus marmoratus* by RACE PCR and analyzed the phylogeny of the teleost and the tetrapod RXR genes, and identified some inconsistencies with previous studies. The tissue-specific and embryonic expression profiles of each RXR gene were explored using real time quantitative PCR. This analysis demonstrated that these RXRs were expressed in all test tissues indicating their participation in many physiological processes. However, we found a great difference in the distribution of RXRg between teleosts and mammals. Furthermore, we followed expression of the three subtypes through various embryo developmental stages and found that the RXRa orthologues of teleosts might be involved in the development of the anterior hindbrain, tailbud and neural crest and in the formation of the pharynx and fin, that RXRb played ubiquitous roles in fish early development, and that RXRg probably played a role in brain and nervous system development and function.

1. Introduction

Retinoid X receptors (RXRs) are members of the nuclear receptor superfamily. RXRs form homodimers or heterodimers with other nuclear receptors, including retinoic acid receptors, thyroid-hormone receptors, liver X receptors, pregnane X receptor and others (Umesono and Evans, 1989; Berrodin et al., 1992; Gearing et al., 1993; Schrader et al., 1993; Willy et al., 1995; Waxman, 1999; Desvergne, 2007), indicating that RXRs mediate the expression of a large variety of hormone-responsive genes. RXRs are composed of at least four functional domains (Aranda and Pascual, 2001). The A/B domain, located in the least conserved N-terminus, is responsible for ligand-independent transactivation activity, comprising the activation function 1 (AF-1) element. The highly conserved DNA-binding domain (DBD) contains two zinc fingers that bind specifically to RXR elements in the regulatory region of RXR-responsive genes. A variable hinge region, the D domain, allows conformational changes of the protein. The ligand binding domain (LBD) is another conserved region among the nuclear hormone receptor superfamily members. The LBD includes the ligand-dependent activation function 2 (AF-2), which is located at the C-terminus of the receptors (Bastien and Rochette-Egly, 2004).

Three major RXR subtypes (RXRa, RXRb and RXRg) exist in vertebrates (Ross et al., 2001) of which multiple isoforms can be found as a result of alternate splicing and differential promoter usage (Leid et al., 1992; Bourguet et al., 1995).

Knock-out mice have provided important information on the physiological functions of RXRs in that RXRa null mice die at embryonic days 12.5–16.5 and exhibit a hypoplastic ventricular myocardium as well as conotruncal and ocular abnormalities (Kastner et al., 1994; Sucov et al., 1994). Approximately 50% of RXRb null mice die before or at birth, and the remaining male null mutants are sterile (Kastner et al., 1996). RXRg null mice are viable and do not display any abnormities (Krezel et al., 1996). RXRs are activated in vitro by 9-cis-retinoic acid (9-cis-RA), which binds with high affinity (Heyman et al., 1992; Levin et al., 1992). Whether 9-cis-RA is a true endogenous ligand to RXR has met with controversy. 9-cis-RA is not detected in mice (Horton and Maden, 1995). Docosahexaenoic acid (DHA) is identified a ligand for RXR in mouse brain (de Urquiza et al., 2000). However, 9-cis-RA is detected in the CNS of an invertebrate (Dmetrichuk et al., 2008).

In fish, several RXR subtypes have been identified and cloned, such as *Danio rerio* RXRaa, -ab, -ba, -bb, -ga and gb (Tallafuss et al., 2006; Waxman and Yelon, 2007), *Chelon labrosus* RXRa (Raingeard et al., 2009) and several RXR sequences which were submitted to NCBI

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directly, such as: *Oryzias latipes* RXRa (**ABQ09280**), RXRb (**BAD93255**), *Paralichthys olivaceus* RXRa (**BAB71758**), *Carassius auratus* RXRa (**AAO22211**), *Latris lineate* RXRa (**ABO15684**), *Scophthalmus maximus* RXRb (**AAK76400**). Furthermore, the developmental expression of each subtype has been detected in *D. rerio* via in situ hybridization and semi-quantitative RT-PCR (Tallafuss et al., 2006; Waxman and Yelon, 2007). However, in marine fish, the RXRs tissue distribution and embryo developmental expression have received only limited study (Raingeard et al., 2009).

In the present study, RXRa, RXRb and RXRg have been cloned in *Sebastiscus marmoratus*, false kelpfish, which is a marine viviparous teleost and is distributed throughout the coastal waters of China (Wu et al., 2007). Most studies on RXRs concentrate on their structure and function in mammals (Bourguet et al., 1995; Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004; Kawakami et al., 2005). However, there is some information on the distribution of RXRs in teleost tissues. Therefore, this study explored the adult fish tissue-specific expression of each gene. Further, we followed expression of the three subtypes through various embryo developmental stages in order to understand their different roles in teleost development.

2. Materials and methods

2.1. Animals and sample collection

Adult *S. marmoratus* were captured from the coastal area in Xiamen, Fujian Province, China. For the cloning of the RXR subtypes, 6 adult fish (>1 year old) were anesthetized by buffered tricaine methanesulfonate (MS-222). Liver, gonad and brain tissues were excised, immediately flash frozen in liquid nitrogen, and stored at −80 ºC until RNA was isolated. For the tissue expression study, fish were collected ($n = 6$–8) in the period of reproduction (approximately January). Brain, eye, gill, heart, intestine, kidney, liver, muscle, ovary, skin, spleen and testis were excised, and frozen as above. As for developmental expression analysis, embryos were incubated at 18 ± 1 ºC in a saline solution for marine fish, containing double distilled water with: 230 mM NaCl, 8.0 mM KCl, 2.25 mM $CaCl_2$, 1.7 mM $MgCl_2$ and 0.24 mM $NaHCO_3$. Approximately 50 embryos were collected per developmental stage (see Table 1) from period 1 (blastula) to 6 (hatching) (Lin and You, 2002), and frozen as above.

2.2. Isolation of total RNA

Total RNA was extracted from fish tissues using a Trizol Kit (TaKaRa, Dalian, China) according to the manufacturer's procedure. The quantity of RNA isolated was determined by measuring the optical density in a Smartspec™ plus spectophotometer (BIO-RAD, USA) at 260 nm and its purity was established by calculating the absorbance ratio 260/280 nm (1.8–2.0). The quality of the RNA was examined using 1.2% agarose gel electrophoresis. Additionally, the isolated RNA was treated with RNase-Free DNase (TaKaRa, Dalian, China) to avoid any contamination by genomic DNA.

Table 2
Oligomeric primers used in PCR.

Designation	Primer sequence	Use
De-RXRa-F	5'-CAAACACATCTGTGCCATYTG-3'	Initial amplification, FP
De-RXRa-R	5'-CYCCRATSAGCTTGAAGAAG-3'	Initial amplification, RP
De-RXRb-F	5'-AGCAGCCCHCAGATYAACTC-3'	Initial amplification, FP
De-RXRb-R	5'-AAGGTGTCRATDGGGGTGT-3'	Initial amplification, RP
De-RXRg-F	5'-CAGTACTGCCGCTAYCAGAAGT-3'	Initial amplification, FP
De-RXRg-R	5'-CATCTCCATRAGGAAGGTGTC-3'	Initial amplification, RP
RA-RXRa-F1	5'-CAACAAGCACTGTGTGATTGATAAGCGC-3'	RACE first-round, FP
RA-RXRa-F2	5'-GGTCACCGCAAATGGCACAGATGTGTT-3'	RACE nested, FP
RA-RXRb-R1	5'-GGTTGAAGAGGATAATGGCTGGAAGG-3'	RACE first-round, RP
RA-RXRb-R2	5'-CAAGCCCAACGGAGGTTTCACATCATC-3'	RACE nested, RP
RA-RXRg-F1	5'-CGGAGACCGTTCATCAGGAAAGCAC-3'	RACE first-round, RP
RA-RXRg-R1	5'-GCGAGGGATGCAAAGGCTTCTTCAAG-3'	RACE nested, RP
RA-RXRg-F2	5'-GAGCGATGCGAGAAAGAGGCGATAAG-3'	RACE first-round, FP
RA-RXRg-R2	5'-GTGACGGGGTCGTTGGTTGAGTTGC-3'	RACE nested, RP
RT-RXRa-F	5'-AGCGAGCCAAGGAGAGAAATG-3'	Real time RT PCR, FP
RT-RXRa-R	5'-ACCGAGGTTGGTCTCAATGTAG-3'	Real time RT PCR, RP
RT-RXRb-F	5'-ACGAACGGCAGAGGAACAAG-3'	Real time RT PCR, FP
RT-RXRb-R	5'-CTGCCTGACAGATGTTGGTGAC-3'	Real time RT PCR, RP
RT-RXRg-F	5'-CCTCAGGAAAGCACTACGGC-3'	Real time RT PCR, FP
RT-RXRg-R	5'-TTGTCGATCAGGCACTCCTTAC-3'	Real time RT PCR, RP
RT-18 S-F	5'-GGGTCCGAAGCGTTTACT-3'	Real time RT PCR, FP
RT-18 S-R	5'-CACCTCTAGCGGCACAATACh-3'	Real time RT PCR, RP

FP, forward primer; RP, reverse primer.

2.3. Molecular cloning of retinoid X receptors

Reverse transcription (RT) was carried out using equal total RNA of each group and a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's procedure. One microlitre of RT reaction mix was used for PCR in a final volume of 50 μL containing 1 U Taq DNA polymerase (TaKaRa, Dalian, China), 5 mmol/L Tris–HCl (pH 9.0), 10 mmol/L NaCl, 0.1 mmol/L DDT, 0.01 mmol/L EDTA, 5% (w/v) glycerol, 0.1% (w/v) Triton X-100, 0.2 mmol/L of each dNTP, 1.0–2.0 mmol/L $MgCl_2$, and 0.5 μmol/L degenerated primers for respective target genes. The PCR primers for RXRs were designed from conserved nucleotide sequences, using software Primer Premier 5.0, and the primers were synthesized by the Sangon Biological Engineering Technology & Services Company (Shanghai, China). The nucleotide sequence primers are shown in Table 2. PCR cycling conditions for RXRs were: 5 min of denaturation at 94 ºC, followed by 35 cycles of denaturation for 30 s at 94 ºC, annealing for 30 s at 58 ºC, and extension for 60 s at 72 ºC. An extra extension step consisting of 7 min at 72 ºC was added after completion of 35 cycles. Amplification products were separated by electrophoresis on a 1.2% agarose gel containing anthocyanin, purified using Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa, Dalian, China), and cloned into a pMD18-T vector (TaKaRa, Dalian, China). Sequencing was performed by the Invitrogen Biological Engineering Technology & Services Company (Shanghai, China). Identification of the sequences was carried out using basic local alignment search tool (nucleotide blast) analyses against National Centre for Biotechnology Information

Table 1
Stages of *Sebastiscus marmoratus* embryonic development used (Lin and You, 2002).

Period (hpf)	Stage	Description
Blastula (4.1–13.7)	1. dome	Yolk cell bulging toward animal pole as epiboly begin
	2. 30%-epiboly	Blastoderm an inverted cup of uniform thickness
Gastrula (13.7–26.4)	3. 50%-epiboly	Blastoderm remains of uniform thickness
	4. 90%-epiboly	Axis and neural plate; brain and notochord rudiments
Segmentation (26.4–79.2)	5. 7–9 somites	Polster prominent; optic vesicle, Kupffer's vesicle, neural keel
	6. 11–15 somites	Pronephros forms, otic placode, brain neuromeres
	7. 20–25 somites	lens, otic vesicle, hindbrain neuromeres
Pharyngula (79.2–94)	8. early pharyngula	early pigmentation, heartbeat
	9. late pharyngula	retina pigmented, early motility, tail pigmentation
Hatching (94–120)	10. hatching	Developing the pectoral fins, the jaws, and the gills