General and Comparative Endocrinology 173 (2011) 78–85

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Expression of insulin-like growth factor I receptors at mRNA and protein levels during metamorphosis of Japanese flounder (*Paralichthys olivaceus*)

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ARTICLE INFO

Article history:
Received 25 September 2010
Revised 22 April 2011
Accepted 30 April 2011
Available online 10 May 2011

Keywords:
IGF-I
IGF-IR
Expression
Metamorphosis
Japanese flounder
Paralichthys olivaceus

ABSTRACT

Insulin-like growth factor I (IGF-I) is an important regulator of fish growth and development, and its biological actions are initiated by binding to IGF-I receptor (IGF-IR). Our previous study has revealed that IGF-I could play an important role during metamorphosis of Japanese flounder, *Paralichthys olivaceus*. The analysis of IGF-IR expression thus helps further elucidate the IGF-I regulation of metamorphic processes. In this study, the spatial–temporal expression of two distinct IGF-IR mRNAs was investigated by real-time RT-PCR. The spatial distribution of two IGF-IR mRNAs in adult tissues is largely overlapped, but they exhibit distinct temporal expression patterns during larval development. A remarkable decrease in IGF-IR-2 mRNA was detected during metamorphosis. In contrast, a significant increase in IGF-IR-1 mRNA was determined from pre-metamorphosis to metamorphic completion. These indicate that they may play different function roles during the flounder metamorphosis. The levels and localization of IGF-IR proteins during larval development were further studied by Western blotting and immunohistochemistry. Immunoreactive IGF-IRs were detected throughout larval development, and the IGF-IR proteins displayed a relatively abundant expression during metamorphosis. Moreover, the IGF-IR proteins appeared in key tissues, such as thickened skin beneath the migrating eye, developing intestine, gills and kidney during metamorphosis. These results further suggest that the IGF-I system may be involved in metamorphic development of Japanese flounder.

1. Introduction

Insulin-like growth factor I (IGF-I) is a peptide hormone well known for stimulating growth and differentiation in vertebrates [1,9,51]. It is ubiquitously expressed in an autocrine/paracrine/endocrine manner [51], and is involved in a variety of physiological processes in fish including growth, development, metabolism, reproduction, osmoregulation and immune response [38,52]. The biological actions of IGF-I are mediated primarily through the binding to IGF-I receptor (IGF-IR) on target tissues [11,50], leading to intracellular signaling response. For this reason, IGF-IR is a key regulator of IGF-I signaling. The inhibition of IGF-IR expression in zebrafish (*Danio rerio*), using antisense morpholino oligonucleotides (MO), resulted in reduced embryonic growth, arrested development, increased lethality and induced neuronal apoptosis [40].

In fish, two distinct IGF-IR cDNAs have been identified in Japanese flounder (*Paralichthys olivaceus*) [30], Atlantic halibut (*Hippoglossus hippoglossus*) [17], rainbow trout (*Oncorhyndius mykiss*) [15], coho salmon (*Oncorhyndius kisutch*) [6], zebrafish [26] and common carp (*Cyprinus carpio*, AY144591 and AY144592) in addition to a single IGF-IR cDNA in other teleost species [12,21,34,35]. Both IGF-IR mRNAs are expressed in all tissues analyzed [12,15,21,26,30,34], further underling the pluripotency of IGF-I. Several previous studies have assessed IGF-IR expression during fish embryogenesis, showing that expression of both IGF-IR mRNAs seems to be developmentally regulated in zebrafish [26] and in rainbow trout [16]. At present, IGF-IRs have been cloned in at least 20 teleost species, but little is known about the physiological significance of IGF-IR expression during fish larval development.

The Japanese flounder is an important flatfish in aquaculture. During larval development, it undergoes a typical metamorphosis, which is characterized by dramatic morphological and physiological transformation from a symmetrical pelagic larva to an asymmetrical benthic juvenile concomitant with eye migration. The metamorphosis is largely controlled by thyroid hormones, and is also partially affected by other hormones such as cortisol, prolactin, and sex steroids [7,53]. But information on IGF-I in flatfish metamorphosis is scarce. It has only been reported that the growth hormone (GH)-IGF-I system could be important for metamorphic success in Atlantic halibut [17–19]. Our recent research has found that IGF-I mRNA increase just before metamorphosis, and decrease until the metamorphic climax, suggesting that IGF-I could be an important regulator of metamorphosis in Japanese flounder [55]. Owing to a pivotal role of IGF-IR on IGF signaling

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doi:10.1016/j.ygcen.2011.04.028

pathways, the expression of IGF-IR during larval development of Japanese flounder need to be clarified.

In this study, the spatial–temporal expression of two distinct IGF-IR mRNAs identified as IGF-IR-1 and IGF-IR-2 in adult tissues and during larval development was analyzed by real-time RT-PCR. Western blotting was carried out to determine whether the IGF-IR mRNAs are translated into proteins and assess the corresponding protein levels during larval development. The localization of IGF-IR proteins in key tissues was performed by immunohistochemical studies, and thus helps further investigate a potential role of the IGF-I system in metamorphosis of Japanese flounder.

2. Materials and methods

2.1. Fish

The Japanese flounder larvae and adult fish were collected at Beidaihe Center Experimental Station, Chinese Academy of Fishery Sciences, China. Adult tissue samples ($n = 3$ specimens) were rapidly dissected, immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

For larval studies, fertilized flounder eggs were incubated in a 300 L circular tank at 15–16 °C for 3 days. Newly hatched larvae were transferred to a 2000 L tank at 14–16 °C. Larvae were fed with rotifers (*Brachionus plicatilis*) from 3 to 20 dph (days post hatching). From 14 dph, larvae were fed with enriched brine shrimp (*Aremia salina*) nauplii. The larvae ($n = 3$ pools, 5–10 specimens/pool) were periodically collected until the completion of metamorphosis, as characterized in a previous study [28]. Sampled larvae were washed with DEPC water, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

In addition, flounder larvae at 3, 13, 17, 21, 24, 29, 33, 36 dph (100 mg per stage) were collected, immediately stored at −80 °C until Western blotting analysis. For immunohistochemical studies, flounder larvae ($n = 10$ per stage) at 17 dph (pre-metamorphosis), 27 dph (mid-metamorphosis) and 33 dph (post-metamorphosis), and adult tissues were fixed in Bouin's solution overnight at 4 °C, and then transferred to 70% ethanol for storage at 4 °C.

2.2. RNA extraction and cDNA synthesis

Total RNA from whole larvae and adult tissues was extracted using the TRIzol Reagent (Invitrogen). All RNA extraction procedures were carried out in accordance with the manufacturer's instruction. To avoid the contamination of genomic DNA, total RNA was treated with DNase I (Qiagen) for 30 min. RNA concentration was determined by spectrophotometry and RNA integrity was assessed by agarose-gel electrophoresis and A_{260}/A_{280} ratios > 1.8. Total RNA (500 ng) from each sample was reverse-transcribed using PrimeScript™ 1st Strand cDNA Synthesis kit (TaKaRa).

2.3. Real-time PCR

The gene-specific primers (Table 1) were designed using Primer 5.0 software for IGF-IR-1 (GI: 18150107) and IGF-IR-2 (GI: 181 50109) as well as for the internal control β-actin (GI: 311294698). IGF-IR-1 and IGF-IR-2 transcripts were effectively amplified by designing primer sets. The primers selected did not produce any non-specific anneal when testing with IGF-IR-1 primers on IGF-IR-2 plasmid templates, and vice versa.

Real-time PCR was carried out on an iCycler (Bio-Rad). Reaction volumes were 20 μl and consisted of cDNA from 10 ng of original RNA template, 400 nM of each of the gene-specific forward and reverse primers (Table 1), and 10 μl SYBR® Premix Ex Taq™ (TaKaRa). The amplification procedure was as follows: initial denaturation for 4 min at 95 °C, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s. Each assay was done in triplicate. For normalization of cDNA loading, all samples were run in parallel with the internal control gene β-actin. To estimate amplification efficiencies, a standard curve was generated for each gene based on 10-fold serial dilutions of larvae cDNA ranging from 100 ng to 0.01 ng of total RNA. All calibration curves exhibited correlation coefficients higher than 0.99, and the corresponding real-time PCR efficiencies (E) were higher than 0.95. Relative mRNA expression for each IGF-IR gene was determined using the $2^{-\Delta\Delta CT}$ method [24]. The 3 dph and liver was used as a calibrator in larval development and tissues analysis, respectively.

Table 1
Primer sequences for real-time PCR.

Gene (bp)	Primer sequence	Amplicon size
IGF-1R-1-f	5′-TGGGTGAGGTAAGAAATGAC-3′	123
IGF-1R-1-r	5′-CCAAATGCTTTAGCAGGTT-3′	
IGF-1R-2-f	5′-GAGGAACTGCCGCCGTAT-3′	118
IGF-1R-2-r	5′-GAGGGTTTGCCGTCTGGT-3′	
β-actin-f	5′-GGAAATCGTGCGTGACATTAAG -3′	155
β-actin-f	5′-CCTCTGGACAACGGAACCTCT -3′	

Comparisons between groups were made by one-way analysis of variance (ANOVA, SAS release 8.02), followed by a Duncan's Multiple Range Test for identification of the statistically distinct groups. All experimental data were shown as the mean ± SEM ($n = 3$). Differences were considered significant at $P < 0.05$.

2.4. Antisera

The IGF-IR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz). This antibody was a rabbit polyclonal antibody against human IGF-IR β subunit, which is highly conserved among vertebrates. A mouse polyclonal antibody against human β-actin (Sigma) was used as the internal control for assessing IGF-IR protein levels during larval development.

2.5. Western blotting

Western blotting was performed to assess IGF-IR expression at protein level during larval development. Briefly, proteins from whole larvae were prepared, and the concentration of proteins was determined using BCA protein assay kit (Pierce). Proteins (25 μg per sample) were separated on 10% SDS–PAGE gels. After electrophoresis, proteins in the gel were transferred onto PVDF membrane (Millipore) for 90 min at a constant current (350 mA) using a Tris–glycine/methanol buffer in Mini Trans-Blot system (Bio-Rad). Following transfer, the gel was washed in TRIS-buffered saline (TBS) containing 0.1% Tween 20 (TBST), and then blocked for 1 h at room temperature in TBST containing 5% defatted milk power. Immunodetection was carried out by incubating blocked membrane with the primary IGF-IR antibody overnight at 4 °C. Membrane was then washed in several changes of TBST, incubated with HRP-conjugated goat anti-rabbit IgG (KPL) for 1 h at room temperature, and washed in TBST. All samples were run in parallel with β-actin, by incubating with mouse anti-β-actin and HRP-conjugated goat anti mouse IgG (KPL), respectively. Detection of immunoreactive protein was performed on multiple function gel imaging system (UVP) using a SuperSignal^R West Pico Chemiluminescent Substrate (PIRECE).

2.6. Immunohistochemistry

Flounder larvae from pre-, mid-, post-metamorphosis and adult tissues were processed for paraffin embedding using a gradient of