



Somatotropic axis genes are expressed before pituitary onset during zebrafish and sea bass development

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ARTICLE INFO

Article history:

Received 4 March 2013

Revised 28 August 2013

Accepted 31 August 2013

Available online 19 September 2013

Keywords:

Growth hormone GH

Insulin-like growth factor-1 IGF-1

Pituitary

Development

Zebrafish

Sea bass

ABSTRACT

The somatotrophic axis, or growth hormone-insulin-like growth factor-1 (GH-IGF-1) axis, of fish is involved in numerous physiological process including regulation of ionic and osmotic balance, lipid, carbohydrate and protein metabolism, growth, reproduction, immune function and behavior. It is thought that GH plays a role in fish development but conflicting results have been obtained concerning the ontogeny of the somatotrophic axis. Here we investigated the developmental expression of GH, GH-receptor (GHR) and IGF-1 genes and of a GH-like protein from fertilization until early stages of larval development in two Teleosts species, *Danio rerio* and *Dicentrarchus labrax*, by PCR, *in situ* hybridization and Western blotting. GH, GHR and IGF-1 mRNA were present in unfertilized eggs and at all stages of embryonic development, all three displaying a similar distribution in the two species. First located in the whole embryo (until 12 hpf in zebrafish and 76 hpf in sea bass), the mRNAs appeared then distributed in the head and tail, from where they disappeared progressively to concentrate in the forming pituitary gland. Proteins immunoreactive with a specific sea bass anti-GH antibody were also detected at all stages in this species. Differences in intensity and number of bands suggest that protein processing varies from early to later stages of development. The data show that all actors of the somatotrophic axis are present from fertilization in these two species, suggesting they plays a role in early development, perhaps in an autocrine/paracrine mode as all three elements displayed a similar distribution at each stage investigated.

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1. Introduction

The pituitary constitutes an anatomical and physiological link between the nervous and endocrine systems. In fish, it controls diverse physiological functions including somatic growth, metabolism, osmoregulation, reproduction, and behavior (Herzog et al., 2003). Among the hormones produced by the pituitary, growth hormone (GH) belongs to a hormonal superfamily which also includes somatolactin (SL) and prolactin (PRL). In fish GH has pleio-

tropic actions, including ionic and osmotic balance, reproduction and immune function, as well as different aspects of behavior (Peréz-Sanchez, 2000; Reinecke et al., 2005).

The general organization of the somatotrophic axis or growth hormone-insulin-like growth factor-1 (GH-IGF-1) axis is similar between mammals and fish, and includes GH, GH receptor (GHR), insulin-like growth factor (IGF), IGF receptor (IGF-R) and IGF binding protein (Gabillard et al., 2003; Moriyama et al., 2000; Reinecke, 2010). GH is expressed and synthesized in somatotrophic cells from the adenohypophysis in adults, but expression has also been detected in several extra hypophyseal tissues (Miura et al., 2011). Because of its involvement in growth, GH has also been studied during development. Studies in birds and mammals indicated GH gene expression may appear before pituitary differentiation (Murphy and Harvey, 2001; Pantaleon et al., 1997). In fish, there is evidence that it is transcribed and translated early during development either before or after pituitary differentiation (Ayson et al., 1994; Herzog et al., 2003; Ozaki et al., 2006; Yang et al., 1999). However, the role GH plays during fish embryonic and larval

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development remains unclear (Fuentes et al., 2008; Gabillard et al., 2003; Zhu et al., 2007). A clear-cut picture cannot yet be provided because the studies relate to a very limited number of species, focusing either on mRNA or on protein detection, seldom on both, and barely consider the somatotrophic system as a whole (i.e., the GH/GHR/IGF system). This probably accounts for the differences observed from a study to another. In addition, conflicting results have been reported concerning a same species (e.g., in trout Jones et al., 2001; Yang et al., 1999). More information is thus clearly needed to further elucidate the role GH/IGF play in fish larval growth and differentiation. In the present study we investigated the expression of the somatotrophic axis genes encoding GH, GHR and IGF-1 during early stages of development in the zebrafish *Danio rerio* and sea bass *Dicentrarchus labrax*. These species were chosen because they have high value for the scientific community (zebrafish) as well as for the farming industry (sea bass), and tools are available for both species. A better knowledge of the GH/GHR/IGF system may help improving the conditions for embryo and larval development in these species. Here we investigated the expression of GH, GHR and IGF-1 transcripts at different stages of early development, including unfertilized eggs. Localization of the sites of expression was achieved using whole-mount *in situ* hybridization. Finally, we developed an antibody directed against GH protein from sea bass in order to see whether the presence of transcripts was associated with GH protein production.

2. Material and methods

2.1. Animals

Adult zebrafish, *D. rerio* were reared at 28 °C on a 12 h light/12 h dark (12L/12D) cycle. Fertilized eggs were incubated under similar LD and temperature conditions. Mature adult sea bass, *D. labrax*, were reared at the “Station IFREMER” of Palavas (France). After fertilization, embryos and larvae were reared under a 19L/5D cycle and natural temperature. Embryos to be used in whole-mount *in situ* hybridization (ISH) studies were placed at 24 hours post-fertilization (hpf) in 0.2 mM phenylthiourea to prevent pigmentation.

2.2. Eggs, embryos and larvae sampling

Unfertilized eggs as well as embryos and larvae were collected at different times of their development. They were either frozen in liquid nitrogen (Western blotting or PCR purposes) or immersed in RNA Later solution (Ambion Biosystems; Austin, TX) (PCR purposes only); they were then stored at –80 °C. Other samples were fixed overnight in freshly prepared 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) at +4 °C, for ISH or immunocytochemical (ICC) studies. ISH samples were stored in methanol at –20 °C. ICC samples were cryo-protected by successive baths of glycerine/sucrose in PBS at +4 °C as follows: 2 × 30 min in 4% sucrose, 30 min in 5% glycerine/10% sucrose, 1 h in 10% glycerine/15% sucrose, and overnight in 10% glycerine/30% sucrose; finally they were frozen in –50 °C isopentane.

2.3. Amplification from different developmental stages

The sequences of the genes investigated in this study were available from the data bases (Supplementary Table 1; partial sequence in the case of *dlIGF-1*). We therefore designed specific primer sets for further amplification from the respective cDNAs (Supplementary Table 1). Total RNA of embryos and larvae from each developmental stage was extracted using the Trizol® method (Invitrogen; Cergy Pontoise, France). One µg of total RNA was incubated with 1 unit of DNase I (Roche; Meylan, France) for 30 min at

37 °C. After DNase inactivation (10 min at 65 °C), RNA was reverse transcribed using Powerscript Reverse transcriptase (Invitrogen; Cergy Pontoise, France) in a total volume of 20 µl. The polymerase chain reaction (PCR) was performed on the obtained cDNA in a total volume of 25 µl, using the primers and conditions described in Supplementary Tables 1 and 2, respectively. In the controls, the template cDNA was replaced by water. The PCR products were loaded in a 2% agarose gel, in the presence of DNA size markers (DNA ladder 1 kb, Promega; Charbonnières, France). Fragments of the expected size were extracted, subcloned in a pGEM-T Easy vector (Promega; Charbonnières, France) and sequenced by Cogenics (Meylan, France) for verification.

2.4. Western blots

Total protein content from pooled sea bass larvae were sonicated at 4 °C in PBS solution containing protease inhibitors (Complete Solution; Roche, Meylan, France). A pool of 3 adult sea bass pituitaries was used as a positive control. Protein content in the homogenates was quantified using the Bradford assay and bovine serum albumin (BSA) as a standard (Bradford, 1976).

Proteins were resolved on a 12.5% polyacrylamide gel in a Tris/glycine electrophoresis system (Biorad; Marnes-La-Coquette, France). Gels were run at 100 V for 1 h. Markers (Precision-Plus Protein Standard's; Biorad) were used to determine the molecular weight of the proteins. The proteins were transferred on a polyvinylidene Immobilon-P membrane (Millipore; Molsheim, France) in a Tris–glycine/20% methanol buffer with a semi-dry Fisherbrand blotting system (ThermoFisher; Illkirch, France) following the manufacturer's protocol. After transfer, the membranes were placed for 2 h PBS (pH 7.4) containing 10% nonfat dry milk and 0.2% Tween (PBST). They were then incubated overnight (18 h at 4 °C) in the primary antibody dilution (1/5000) in PBS containing 1 mg BSA fraction V. After washing in PBST and then in PBS, the membranes were submitted to horseradish peroxidase conjugated goat antirabbit IgG (0.0083 µg/ml in PBST containing 0.1% normal goat serum). The membranes were then washed in PBST (3 × 10 min) and in PBS (3 × 5 min) and immunodetection was performed using the ECL⁺ detection kit (Life Technologies, Saint Aubin, France), and finally observed under a Vilbert Lourmat apparatus (Marne-la-Vallée, France).

The antibody directed against sea bass GH (dlGH-antibody) was obtained and validated by Eurogentec (Liège, Belgium) by rabbit immunization against two peptide sequences from sea bass GH (GANQDGAEMFPDSSL and WEFPSRSLSPGAARN).

2.5. In situ hybridization (ISH)

For all species, embryos and larvae were fixed overnight by immersion in 4% paraformaldehyde in PBS at 4 °C, washed in buffer, transferred to 100% methanol and stored at –20 °C. Whole-mount ISH was performed using digoxigenin-labeled riboprobes made using a commercially available kit (Roche, Meylan, France) according to the manufacturer's instructions. The antisense and sense probes (Supplementary Table 2) were generated using a cDNA fragment of the genes of interest. Sea bass IGF-1 antisense and sense probes were produced from a cDNA fragment (291 bp) obtained by a 5',3'-rapid amplification of cDNA ends (RACE)-PCR (SMART RACE cDNA Amplification kit, Clontech; Mountain View, CA) with the specific primer dlIGF-1-F GGTCGACACGCTG-CAGTTTGTCG under the following conditions: 95 °C (1 min), then 10 cycles of 94 °C (20 s), 67 °C (1 min), 68 °C (1 min), followed by 20 cycles of 94 °C (10 s), 65 °C (1 min), 68 °C (90 s), then 68 °C (7 min) and finally 4 °C. The amplified fragment was purified, subcloned in PGEM-T Easy Vector (Promega, Charbonnières, France) and sequenced.

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