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Development of a time-resolved fluoroimmunoassay for salmon insulin-like growth factor binding protein-1b

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ABSTRACT

In salmon plasma/serum, three major insulin-like growth factor binding proteins (IGFBPs) are consistently detected at 22-, 28- and 41-kDa. The 22-kDa form has been identified as IGFBP-1b and shown to increase under catabolic conditions. We developed a competitive time-resolved fluoroimmunoassay (TR-FIA) for salmon IGFBP-1b. Purified salmon IGFBP-1b was used for biotin-labeling, assay standard and antiserum production. The TR-FIA did not cross-react with the 41-kDa form (IGFBP-2b) but showed 3% cross-reactivity with the 28-kDa form (IGFBP-1a). It measured IGFBP-1b levels as low as 0.4 ng/ml, and ED_{80} and ED_{20} were 0.9 and 24.6 ng/ml, respectively. There appears to be little interference by IGF-I. Using the TR-FIA, serum IGFBP-1b levels were measured in individually-tagged underyearling masu salmon fed or fasted for 5 weeks, or fasted for 3 weeks followed by refeeding for 2 weeks. Fasting for 3 weeks significantly increased circulating IGFBP-1b levels, while it returned to the basal levels after prolonged fasting for additional 2 weeks. Serum IGFBP-1b level negatively correlated with body weight, condition factor, specific growth rate and serum IGF-I level. During parr–smolt transformation of masu salmon, average circulating IGFBP-1b levels were the highest in May. There was a positive correlation between serum IGFBP-1b and IGF-I, which is in contrast to that in the fasting/feeding experiment. IGFBP-1b also showed a positive relationship with gill Na^+, K^+-ATPase activity. These results suggest that the relationship between circulating IGFBP-1b and IGF-I during smoltification differs from that during fasting and IGFBP-1b may play a role in the development of hypoosmoregulatory ability.

1. Introduction

Insulin-like growth factor binding proteins (IGFBPs) are a family of cysteine-rich proteins that are not structurally related to receptors for IGFs (Hwa et al., 1999). Despite the lack of sequence homology with the receptor, IGFBPs have high affinity for IGFs and are capable of regulating availability of IGF to target tissues (Hwa et al., 1999; Forbes et al., 2012). IGFBPs prolong the half-life of IGFs in the circulation, target them to certain tissues and either potentiate or inhibit action of IGFs depending on the type, physiological conditions and/or cellular environment (Rajaram et al., 1997; Firth and Baxter, 2002). They can also influence cell growth independent of IGFs through interacting on the cell surface receptors or translocating into the nucleus (Wheatcroft and Kearney, 2009; Forbes et al., 2012).

There are six members of the IGFBP family (IGFBP-1 to -6). They arose from local and whole genome duplications throughout the vertebrate evolution. A possible scenario is that an ancestral IGFBP gene was first duplicated locally followed by two rounds of whole-genome duplication (WGD) creating eight genes and two of them were lost (Daza et al., 2011). Supporting this, pairs of IGFBP-1 and -3, and IGFBP-2 and -5 are located on the same chromosomes, respectively, and IGFBP-4 and -6 are found on separate chromosomes (Daza et al., 2011). In teleosts, because of the 3rd round of WGD, there are two paralogs of each member of IGFBPs except IGFBP-4 (Daza et al., 2011). In addition, salmonids underwent an extra round of WGD and thus have four paralogs (Macqueen et al., 2013). Fate of duplicated genes depends on how selective pressure acts on them. One of duplicated gene is often lost (nonfunctionalization) but in rare case, gains new function(s) (neofunctionalization) (Postlethwait et al., 2004). Besides these fates, certain portion of duplicated genes undergoes partitioning of their original functions (subfunctionalization) (Postlethwait et al., 2004). Subfunction partitioning can be spatial and temporal. Duan and colleagues conducted a series of functional studies using developing zebrafish (*Danio rerio*) embryos and proved subfunctional partitioning of duplicated IGFBPs (Kamei et al., 2008; Zhou et al., 2008; Wang et al., 2009; Dai et al., 2010). Moreover, expression patterns of duplicated IGFBPs in response to changes in feeding status have been investigated in Atlantic salmon (*Salmo salar*; Bower et al., 2008), Arctic charr (*Salvelinus alpinus*; Macqueen et al., 2011), adult zebrafish (Amaral and Johnston, 2011, 2012) and Mozambique tilapia (*Oreochromis*

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mossambicus; Breves et al., 2014). These studies suggest that responses of duplicated IGFBPs are generally similar but differ under certain tissues and physiological conditions.

IGFBP-1 is the first member to be identified in humans and is one of major circulating IGFBPs (Lee et al., 1988; Lee et al., 1993,1997). It shows diurnal fluctuation often reflective of meal and insulin status (Lee et al., 1993,1997). Insulin is a potent inhibitor of IGFBP-1. Fasting induces IGFBP-1 in the circulation probably due to relaxation of the suppressive effect of insulin. An amino acid deficiency also leads to an induction of IGFBP-1 in the hepatocytes through affecting mRNA stability (Jousse et al., 1998; Averous et al., 2005). IGFBP-1 is unsaturated in circulation and usually inhibits IGF actions by sequestering free IGFs from the circulation (Lee et al., 1997; Wheatcroft and Kearney, 2009). This response is believed to be protective from energy expenditure by the actions of IGFs during shift from anabolism to catabolism. Cortisol, on the other hand, induces IGFBP-1 while its effect is not as strong as insulin (Unterman et al., 1991). IGFBP-1 is thus a fine tuner of energy partitioning and growth velocity under fluctuating metabolic status (Kajimura and Duan, 2007).

Candidates of IGFBP-1 in fish circulation have been detected by ligand blotting using labeled human IGF-I (Kelley et al., 1992, 2001). In fish blood, three major IGFBPs are consistently detected at 20–25 kDa, 28–32 kDa and 40–50 kDa and the two low-molecular-weight forms have been thought to be IGFBP-1, -2 or -4 (Kelley et al., 1992, 2001). Analyses of these fish IGFBPs using ligand blotting revealed that they are up-regulated under catabolic conditions such as fasting, stress and cortisol treatment as seen in mammals (Siharath et al., 1996; Park et al., 2000; Kelley et al., 2001, 2006; Kajimura et al., 2003; Peterson and Small, 2004, 2005; Hevrøy et al., 2011). We have identified 22- and 28-kDa IGFBPs in salmon plasma as co-orthologs of IGFBP-1 through protein purification and cDNA cloning (Shimizu et al., 2011a). Our finding suggests that other fish IGFBPs with similar molecular weights and physiological responses are also IGFBP-1s.

We previously developed a radioimmunoassay (RIA) for salmon IGFBP-1b (22-kDa form) and reported that it was increased by fasting or restricted ration of feed and negatively correlated with individual growth rate (Shimizu et al., 2006). Plasma IGFBP-1b also showed a consistent negative relationship with condition factor and quick response to a single meal (Shimizu et al., 2009). Availability of RIA has thus provided important information on physiological regulation and possible roles of IGFBP-1b in salmon. However, there is a trend to switch from RIA to non-radioisotopic (RI) immunoassay because of safety, stableness and ease of use. Indeed, RIA for fish IGF-I is being replaced by time-resolved fluoroimmunoassay (TR-FIA) (Andoh, 2005; Small and Peterson, 2005). In contrast, there is no non-RI immunoassay available for fish IGFBP. The present study reports development of a competitive TR-FIA for salmon IGFBP-1b.

2. Materials and methods

2.1. Fish and blood collection

Yearling masu salmon (*Oncorhynchus masou*, Shiribetsu River strain) were reared in freshwater at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan. Blood was withdrawn by a syringe from the caudal vein, allowed clotting overnight at 4 °C and centrifuged at 8050 *g* for 10 min. Serum was collected and stored at −30 °C until use.

Underyearling Atlantic salmon (NLA strain) and rainbow trout (*Oncorhynchus mykiss*, NLA strain) were reared in seawater at Matre Research Station, Institute of Marine Research, Matredal., Norway. Blood was collected from the caudal vein with the use of a heparinized syringe. Plasma was collected after centrifugation at 1250 *g* for 10 min. Plasma was stored at −80 °C until use.

2.2. Assay components

IGFBP-1b was purified from serum of spawning Chinook salmon (*Oncorhynchus tshawytscha*; Shimizu et al., 2005). Briefly, salmon serum was first fractionated by ammonium sulfate precipitation and loaded onto an IGF-affinity column. IGFBP-1b was eluted from the column with 0.5 M acetic acid and further purified by reversed-phase high pressure liquid chromatography (HPLC) on a Vydac C-4 column (Separation Group, Hesperia, CA). Purified IGFBP-1 was aliquoted into prelubricated microcentrifuge tubes (PGC Scientifics, Frederick, MD) and stored at −80 °C until use. Chinook salmon IGFBP-1a and -2b were also purified as described in Shimizu et al. (2011a) and Shimizu et al. (2003b), respectively. Polyclonal antiserum against purified IGFBP-1b (anti-IGFBP-1b) was raised in a rabbit as described in Shimizu et al. (2006).

2.3. Ligand blotting and western blotting

Sodium dodecyl sufate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel and 12.5% separating gel was carried. Purified salmon IGFBPs (50 ng) were treated with an equal volume of a sample buffer containing 2% SDS, 10% glycerol at 85 °C for 5 min. Gels were run in a solution of 50 mM Tris, 400 mM glycine and 0.1% SDS at 50 V in the stacking gel and at 100 V in the separating gel until the bromophenol blue dye front reached the bottom of the gel. For western blotting, an electroblotted nitrocellulose membrane was incubated with anti-IGFBP-1b serum at a dilution of 1:200 for 2 h at room temperature. The membrane was then incubated with goat anti-rabbit IgG-HRP conjugate (Bio-Rad, Hercules, CA) at a dilution of 1:2000 for 1 h at room temperature. Immunoreactive bands were visualized on an X-ray film by use of ECL western blotting reagents.

The result of western ligand blotting using digoxigenin-labeled human IGF-I (DIG-hIGF-I) of purified salmon IGFBPs was reproduced from Shimizu et al. (2003a) with permission.

2.4. Biotinylation of IGFBP-1b

Purified IGFBP-1b was labeled with a biotin (EZ-link sulfo-NHS-LC-biotin, Thermo Scientific, Rockford, IL). Two micrograms of purified protein was mixed with 15 μl 0.5 M phosphate buffer, pH 7.4 and reacted with 2 μl 1 mM NHS-LC-biotin at a molar ratio of 1:25. A low-adsorption 0.5 ml tube (PGC Scientifics) containing the mixture was incubated at room temperature under dark with occasional flipping. Reaction was stopped by adding 32 μl 0.1 M Tris–HCl, pH 8.0 for 15 min and 64 μl 0.05 M Tris–HCl, 0.15 M NaCl, 0.2% BSA (Nacalai tesque, Kyoto, Japan) were added to the tube. The biotinylated IGFBP-1b was dialyzed against 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.5 using Slyde-A-Lyzer 3.5 K dialysis cassette (Thermo Scientific). After dialysis, aliquots of the biotinylated IGFBP-1b were stored at −80 °C until use.

2.5. TR-FIA for IGFBP-1b

A competitive method was employed in the assay. A 96-well assay plate coated with goat anti-rabbit IgG (DELFIA® Yellow Plate; Perkin Elmer, Turku, Finland) was first washed with 200 μl DELFIA® Wash Buffer (Perkin Elmer) and each well received 40 μl DELFIA® Assay Buffer (Perkin Elmer), 40 μl anti-IGFBP-1b (1:1250–5000) and 40 μl standard (purified IGFBP-1b) or serum diluted with Assay Buffer. The plate was sealed by Thermal Seal RT™ (Excel Scientific, Victorville, CA) and incubated at 4 °C overnight with shaking at 150 rpm on a shaker. The plate was flash centrifuged, and each well received 40 μl biotinylated IGFBP-1b (1.3–10 ng/ml) and incubated at 4 °C overnight at 150 rpm or at room temperature for 3 h at 600 rpm. After plate was washed three times, each well received 100 μl europium–avidin conjugated with HRP (Perkin Elmer) and incubated at room temperature for 1 h with shaking at 600 rpm. The plate was washed six times and room-