RESEARCH ARTICLE | Hormones, Reproduction and Development

Intact rather than total circulating insulin-like growth factor binding protein-1a is a negative indicator of growth in masu salmon

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1Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan; 2Norwegian Research Centre (NORCE) Environment, NORCE Norwegian Research Centre AS, Bergen, Norway; and 3Department of Biological Sciences, University of Bergen, Bergen, Norway

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Kaneko N, Nilsen TO, Tanaka H, Hara A, Shimizu M. Intact rather than total circulating insulin-like growth factor binding protein-1a is a negative indicator of growth in masu salmon. Am J Physiol Regul Integr Comp Physiol 318: R329–R337, 2020. First published December 18, 2019; doi:10.1152/ajpregu.00099.2019.—Insulin-like growth factor binding protein (IGFBP)-1a is one of three major circulating forms in salmon and induced under catabolic conditions. However, there is currently no immunoassay available for this form because of a lack of standard and specific antibodies. We developed a time-resolved fluoroimmunoassay (TR-FIA) for salmon IGFBP-1a using recombinant protein for labeling, an assay standard, and production of antiserum. The TR-FIA had a low cross-reactivity (3.6%) with IGFBP-1b, another major form in the circulation. Fasting for 4 wk had no effect on serum immunoreactive (total) IGFBP-1a levels in yearling masu salmon, whereas 6-wk fasting significantly increased it. There was a significant, but weak, negative relationship between serum total IGFBP-1a level and individual growth rate (r2 = 0.12, P = 0.01). We next developed a ligand immuno-functional assay (LIFA) using europium-labeled IGF-I to quantify intact IGFBP-1a. In contrast to total IGFBP-1a, serum intact IGFBP-1a levels increased after 4 wk of fasting, and refeeding for 2 wk restored it to levels similar to those of the fed control. Serum intact IGFBP-1a levels showed a significant negative correlation with individual growth rate (r2 = 0.52, P < 0.001), which was as good as that of IGFBP-1b. Our findings using newly developed TR-FIA and LIFA suggest that regulation of intact IGFBP-1a levels has an important effect on growth in salmon and that intact IGFBP-1a is a negative index of salmon growth.

fasting; growth; immunoassay; insulin-like growth factor binding protein-1a; salmon

INTRODUCTION

Insulin-like growth factor binding proteins (IGFBPs) are important modulators of the actions of insulin-like growth factors (IGFs) (1, 2, 11). IGFBPs in the circulation prolong the half-lives of IGFs and carry them to target tissues (3, 42, 57). In target tissues, IGFBPs can either potentiate or inhibit the action of IGFs by sequestering free IGFs from the circulation (36, 37, 56). IGFBP-1 is usually unoccupied with endogenous IGFs and can act as an inhibitor of IGFs by sequestering free IGFs from the circulation (36, 37, 56). IGFBP-1 is highly phosphorylated, resulting in an IGF-binding affinity sixfold higher than that of nonphosphorylated forms (24). Under certain situations in which IGFBP-1 is nonphosphorylated and/or partially degraded, it can potentiate the action of IGF-1 (24, 55). In addition, Brandt et al. (8) reported that phosphorylated IGFBP-1 promoted the IGF-1 stimulated proliferation of human dermal fibroblasts when cells were sparsely plated at ~30% confluence. Thus, IGFBP-1 is an important regulator of IGF action.

In teleosts, there are two subtypes of IGFBP-1, which are products of the teleost-specific third round of the whole genome duplication event (13, 27, 38). Kamei et al. (27) was the first to identify two co-orthologs of IGFBP-1 in zebrafish (Danio rerio). The authors highlighted that two IGFBP-1s overlapped their function but were different in terms of temporal/spatial expression patterns, responses to fasting, IGF-binding affinity, and thus inhibitory actions on the IGF-induced cell proliferation (27). Such subfunction partitioning should increase the complexity of IGF regulation and enable fine-tuning of growth in teleosts.

These two subtypes are present in the circulation of salmon. In salmon circulation, three IGFBPs are detected at 22, 28, and 41 kDa (50), and the 22-kDa and 28-kDa forms have been identified as IGFBP-1b and IGFBP-1a, respectively (46, 49). Two low-molecular-weight IGFBPs, presumably corresponding to IGFBP-1s, are also consistently detected in circulation in other fish and reported to increase by fasting, osmotic stress, hypoxia, handling, and cortisol injection (26, 31–33, 39, 49, 52). Kelley et al. (31–33) proposed that they are markers of catabolic status in fish. To use fish IGFBPs for catabolic markers, unraveling their regulation and relation to IGFs and growth is essential.

Fish IGFBPs in the circulation are usually detected and semiquantified by ligand blotting using labeled IGF-I. The ligand blotting is useful because it detects different types of IGFBPs simultaneously based on their ability to bind IGF-I and does not require specific antisera. In addition, the ligand blotting does not detect IGFBP fragments that have lost the IGF-binding ability. Such a feature is important when enzy-
mantic degradation of IGFBPs is a concern (8, 9, 17, 55). However, the ligand blotting has the limitations of being semiquantitative and not being able to process a large number of samples. Thus, development of immunoassay for circulating fish IGFBPs is crucial to facilitate analyses of physiological regulation of fish IGFBPs in the circulation.

We have previously developed a radioimmunoassay (RIA) and a time-resolved fluoroimmunoassay (TR-FIA) for salmon IGFBP-1b and showed that circulating IGFBP-1b increased in fasting fish and negatively correlated with individual growth rates in immature coho salmon (Oncorhynchus kisutch), masu salmon (O. masou), and chum salmon (O. keta) (18, 29, 30, 44, 45). These findings led us to propose its utility as a quantitative negative index of growth. On the one hand, circulating IGFBP-1a semiquantified by ligand blotting also increased under long-term fasting or osmotic stress but appeared to be less sensitive to catabolic states than IGFBP-1b (30, 49). On the other hand, circulating IGFBP-1a still showed a negative correlation with growth rate in masu salmon, suggesting that it is also useful as a negative index of growth (30). However, there is currently no immunoassay available for fish IGFBP-1a because of a lack of purified protein and specific antiserum.

We have recently produced recombinant masu salmon IGFBP-1a using a bacterial expression system (54). By using the recombinant protein for assay components and antiserum production, the present study developed a TR-FIA for salmon IGFBP-1a. However, our initial analyses revealed that serum IGFBP-1a levels measured by the newly developed TR-FIA showed little response to fasting, whereas it clearly increased under long-term fasting or osmotic stress but appeared to be less sensitive to catabolic states than IGFBP-1b (30, 49). These findings led us to propose its utility as a quantitative negative index of growth. On the one hand, circulating IGFBP-1a semiquantified by ligand blotting also increased under long-term fasting or osmotic stress but appeared to be less sensitive to catabolic states than IGFBP-1b (30, 49). On the other hand, circulating IGFBP-1a still showed a negative correlation with growth rate in masu salmon, suggesting that it is also useful as a negative index of growth (30). However, there is currently no immunoassay available for fish IGFBP-1a because of a lack of purified protein and specific antiserum.

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MATERIALS AND METHODS

Production and purification of recombinant salmon IGFBP-1a. Recombinant salmon (rs) IGFBP-1a was produced using a bacterial expression system as described in Tanaka et al. (54). Briefly, a pET-32(+) expression vector (Novagen, Madison, WI) carrying the mature masu salmon igfbp-1a cDNA was transformed into a strain of Escherichia coli [Rosetta-gami B(DE3) pLysS (Novagen)], and rsIGFBP-1a was expressed as a fusion protein with a histidine tag and thioredoxin. The fusion protein was solubilized and isolated by Ni-affinity chromatography. The fusion partners were cleaved by enterokinase, and rsIGFBP-1a was purified by reversed-phase high-pressure liquid chromatography on a Vydac C-4 column (Separation Group, Hesperia, CA). Purified rsIGFBP-1a was aliquoted into low-absorption tubes (PGC Sciences, Frederick, MD) and stored at −80°C until use.

Production of antiserum against rsIGFBP-1a. Polyclonal antiserum against purified rsIGFBP-1a (anti-IGFBP-1a) was raised in a rabbit. Immunization of the rabbit was conducted at a designated facility in accordance with the guidelines of the Animal Care Committee of Hokkaido University. The rearing experiment was approved from the independent, domestic committee for animal care. Project number was 30-3. Purified rsIGFBP-1a in phosphate-buffered saline (pH 7.0) was emulsified in an equal volume of TiterMax Gold Research Adjuvant (TiterMax USA, Norcross, GA). A rabbit was first immunized with 50 μg antigen by lymph node injection followed by subcutaneous boost injections with 100 and 150 μg antigen 2 and 3 wk after the first injection, respectively. One week after the last boost, blood was withdrawn from the ear vein and antiserum was collected after centrifugation. The antiserum was stored at −80°C until use.

TR-FIA for “total” IGFBP-1a. In the present study, “total” IGFBP-1a is all immunoreactive components recognized by the antiserum, which include “intact” (to be defined below) IGFBP-1a and its fragments. A competitive method was employed in the assay for quantifying total IGFBP-1a. Purified rsIGFBP-1a was labeled with biotin (EZ-link Sulfo-NHS-Biotin, Thermo Scientific, Rockford, IL). Thirty-four micrograms of purified protein were reacted with 31 μL of 1 mM NHS-Biotin at a molar ratio of 1:25. The mixture was incubated for 2 h on ice under dark. The reaction was stopped by adding 0.1 M Tris-HCl, pH 7.5, and incubation for 30 min. Biotinylated rsIGFBP-1a was dialyzed against 0.05 M Tris·HCl and 0.15 M NaCl, pH 7.5, using Slide-A-Lyzer 3.5K dialysis cassettes (Thermo Scientific). After dialysis, aliquots of the biotinylated rsIGFBP-1a were stored at −80°C until use.

A 96-well strip assay plate (DELFIA strip plate) or yellow plate (DELFIA yellow plate) coated with goat anti-rabbit IgG (PerkinElmer, Turku, Finland) was first washed with 200 μL DELFIA Wash Buffer (PerkinElmer). Each well received 80 μL DELFIA Assay Buffer (PerkinElmer), 20 μL anti-IGFBP-1a (1:8,000), and 40 μL standard (purified rsIGFBP-1a) or serum diluted with assay buffer. The plate was sealed and incubated at 4°C overnight with shaking at 600 revolutions/min on a shaker. The plate was flash centrifuged, and each well received 20 μL biotinylated rsIGFBP-1a (1:8,000) and was incubated at 4°C overnight with shaking at 600 revolutions/min on a shaker. After the plate was washed 3 times with 160 μL wash buffer, each well received 160 μL europium (Eu)-labeled streptavidin (1:2,000, PerkinElmer) and was incubated at room temperature for 1 h with shaking at 600 revolutions/min. The plate was washed with 160 μL 5 times and 200 μL 3 times with wash buffer. Each well received 200 μL DELFIA Enhancement Solution (PerkinElmer), which acclimated to room temperature before use, and the plate was shaken without sealing for 10 min at room temperature. Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (PerkinElmer) or SPARK multimode microplate reader (TECAN Group, Männedorf, Switzerland).

LIFA for “intact” IGFBP-1a. In the present study, “intact” indicates immunoreactive IGFBP-1a with IGF-binding ability in the reaction mixture. A LIFA for intact IGFBP-1a was developed by modifying the method for human IGFBP-3 (34); unextracted IGFBP-1a was first captured by anti-IGFBP-1a, reacted with Eu-labeled salmon IGF-I (GroPep Bioreagents Pty, Adelaide, SA, Australia), and quantified for its binding capacity. A 96-well strip assay plate (DELFIA strip plate) or yellow plate (DELFIA yellow plate) coated with goat anti-rabbit IgG (PerkinElmer) was first washed with 200 μL DELFIA Wash Buffer (PerkinElmer). Each well received 80 μL DELFIA Assay Buffer (PerkinElmer), 20 μL IGFBP-1a antiserum
was calculated as follows: HSI (%)

The specific growth rate (SGR) was calculated as follows: SGR

RESULTS

Specific binding of the biotinylated IGFBP-1a was displaced by increasing amounts of unlabeled IGFBP-1a in the TR-FIA. Sera from masu salmon and rainbow trout were serial diluted in parallel with that of the standard (Fig. 1). The half-maximal displacement (ED50) occurred at 61.5 ± 2.3 ng/mL (n = 8 standards). The ED50 and ED20 were 18.1 ± 2.5 ng/mL (n = 8 standards) and 226.9 ± 23.8 ng/mL (n = 8 standards), respectively. The minimum detection limit of the assay, defined as the mean count of the zero standard minus two standard deviations.

**Fasting/refeeding experiment.** A captive brood stock of yearling masu salmon from the Shiribetsu River in southern Hokkaido held at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan (41°54’N, 140°41’E), was used in the present study. In June 2012, fish were lightly anesthetized with an acid ethanol, as described in Shimizu et al. (50). IGF-I was quantified by TR-FIA using the method described in Small and Peterson (53), with recombinant salmon/trout IGF-I (GroPep Bioreagents Pty.,) as a standard. Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (PerkinElmer).

Serum IGFBP-1b levels were quantified by TR-FIA, as described in Fukuda et al. (18). Briefly, a competitive method was employed by following a procedure for DELFIA immunoassays (PerkinElmer). Serum samples were first incubated with antiserum against purified salmon IGFBP-1b (44) overnight at 4°C in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer). Biotinylated salmon IGFBP-1b was added to each well and incubated overnight at 4°C. After being washed with DELFIA Wash Buffer (PerkinElmer), each well received Eu-labeled streptavidin (PerkinElmer) followed by DELFIA Enhancement Solution (PerkinElmer). Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (PerkinElmer).

Fasting/refeeding experiment. A captive brood stock of yearling masu salmon from the Shiribetsu River in southern Hokkaido held at Nanae Freshwater Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan (41°54’N, 140°41’E), was used in the present study. In June 2012, fish were lightly anesthetized in water containing 2-phenoxethanol and individually marked with passive integrated transponder tags (Biomark, Boise, ID). They were randomly placed into one of three 350-l outdoor tanks (95 × 70 cm) and allowed to recover and acclimate for 1 wk with feeding. One week after tagging, their initial standard length (SL) and body weight (BW) were measured. During the experiment, one group was fed daily with a commercial diet (Marubeni Nissin Feed, Tokyo, Japan) to satiety for 6 wk (Fed). The second group (Fasted) was fasted throughout the experimental period (6 wk). The third group (Refed) was fasted for the first 4 wk and refed for the following 2 wk. They were reared using flow-through river water that ranged from 11.0°C to 16.0°C during the experiment (Suppl. Figure 1; https://doi.org/10.6084/m9.figshare.7957250.v1). The experiment was carried out in accordance with the guidelines of Hokkaido University Field Science Center Animal Care and Use Committee.

The SL and BW of all fish were measured 4 and 6 wk after the beginning of the experiment. The condition factor (K) was calculated as follows: BW (g) × 100/SL (cm)^3. The hepato-somatic index (HSI) was calculated as follows: HSI (%) = liver weight (g) × 100/BW (g). The specific growth rate (SGR) was calculated as follows: SGR (%/day) = ln(s2 - s1) × (d2 - d1)^{-1} × 100, where s1 is length or weight on day1, s2 is length or weight on day2, and d2 - d1 is the number of days between measurements. Blood samples were collected from 8 fish per treatment at 0 and 4 wk and 18–21 fish per treatment at 6 wk. Blood was withdrawn by a syringe from the caudal vein, allowed to clot overnight at 4°C, and centrifuged at 10,000 revolu-
tions, was 9.2 ng/mL ($n = 6$ standards). The intra- and interassay coefficients of variation estimated using the control sample were 5.3% ($n = 4$ samples) and 8.1% ($n = 4$ plates), respectively. The recovery of purified rsIGFBP-1a (50 ng/mL) added to rainbow trout serum was 96.3% ($n = 9$ fish).

Cross-reactivity of the polyclonal antibodies with other IGFBP subtypes purified from Chinook salmon serum was examined in the TR-FIA (Fig. 2). Both IGFBP-2b (41-kDa form) and IGFBP-1b (22-kDa form) showed some displacement at higher concentrations, and their cross-reactivity was calculated as 1.5% and 3.6%, respectively. Adding salmon IGF-I at a 1:10 molar ratio to masu salmon and rainbow trout sera did not considerably alter the displacement curves (Fig. 3).

Specific binding of Eu-labeled IGF-I was increased by increasing amounts of the IGFBP-1a standard in LIFA. Serial dilutions of sera from masu salmon and rainbow trout were parallel with that of the standard, which was not affected by feeding status in masu salmon (Fig. 4). The minimum detection limit of the assay, defined as the mean count of the zero standard plus two standard deviations, was 1.2 BU ($n = 4$ standards). The intra- and interassay coefficients of variation estimated using the control sample were 6.4% ($n = 4$ samples) and 10.1% ($n = 4$ plates), respectively. The recovery of purified rsIGFBP-1a (10 ng/mL) added to Atlantic salmon plasma was 107.5% ($n = 8$ fish).

Responses to fasting and refeeding of serum IGF-I, IGFBP-1b, total IGFBP-1a, and intact IGFBP-1a in yearling masu salmon were examined (Fig. 5). Serum IGF-I levels in fasted fish were significantly lower than those in fed fish at week 4 ($P < 0.0001$; Fig. 5A). Refeeding for 2 wk significantly increased serum IGF-I ($P < 0.0001$), but its levels were intermediate between fed and fasted fish (Fig. 5A). Fasting for 4 wk had no significant effect on serum IGFBP-1b levels ($P = 0.0620$; Fig. 5B). It became significantly higher in fasted fish than fed and refed fish at week 6 ($P < 0.0001$; Fig. 5B). Total IGFBP-1a levels tended to be higher in fasted fish but were not different among groups ($P = 0.2615$; Fig. 5C). On the one hand, intact IGFBP-1a levels significantly increased in fish fasted for 4 wk ($P = 0.0132$) and maintained higher values until 6 wk (Fig. 5D). On the other hand, refeeding for 2 wk restored it to a level similar to that in the fed control ($P = 0.9288$; Fig. 5D).

Data from week 6 were used for correlation analyses. There was a positive correlation between serum IGF-I and SGR in weight, whereas serum IGFBP-1b showed a negative correlation with SGR in weight (Fig. 6, A and B). Both total and intact IGFBP-1a levels negatively correlated with SGR in weight, although the correlation coefficient of total IGFBP-1a was much weaker than that of intact IGFBP-1a (Fig. 6, C and D). There were no significant relationships between body size (SL and BW) and IGFBP-1b or total IGFBP-1a, whereas IGF-I and intact IGFBP-1a were positively and negatively correlated, respectively (Table 1). IGF-I showed a positive correlation with K, whereas IGFBP-1b and total and intact IGFBP-1a showed negative correlations (Table 1). Total and intact IGFBP-1a were positively correlated, but only intact IGFBP-1a showed a negative relationship with IGF-I (Table 2).

**DISCUSSION**

We developed two immunoassays to quantify “total” and “intact” IGFBP-1a in salmon as defined earlier. The findings of

![Fig. 2. Cross-reactivity of the antiserum against salmon insulin-like growth factor binding proteins (IGFBPs). Displacement of the tracer was assessed by adding increasing amounts of purified salmon IGFBP-1a, 1b, and 2b to the assay. Binding (B/B₀) is expressed as a percentage of specific binding.](image-url)

![Fig. 3. Effects of exogenous salmon insulin-like growth factor-I (IGF-I) on the displacement curves of masu salmon (A) and rainbow trout (B) serum dilutions in time-resolved fluoroimmunoassay (TR-FIA). Salmon IGF-I was added to serum at a molar ratio of 1:10. Binding (B/B₀) is expressed as a percentage of specific binding.](image-url)
the present study are relevant to other teleosts because IGFBP-1a is most likely present as a major circulating form in fish (47). In addition, the availability of immunoassays for both IGFBP-1a and 1b (18) enables us to further investigate how these duplicated IGFBP-1s are regulated and related to growth in salmon.

The present study is the first to report the development of immunoassays for salmon IGFBP-1a in teleosts. Availability of an immunoassay for quantifying fish IGFBPs is limited to salmon IGFBP-1b and 2b (18, 44, 48). One of the challenges in establishing an immunoassay for fish IGFBPs is to prepare enough purified IGFBP as antigen for immunization because circulating levels of IGFBPs are low, being ~300 ng/mL or less (46, 50). Purifying salmon IGFBP-1a from serum was possible, but the final yield was <25 μg from 1 L of serum (49), making protein purification an impractical method for antigen preparation. We have recently produced recombinant masu salmon IGFBP-1a using a bacterial expression system (54). The present study used the recombinant protein for antiserum production, assay tracer, and a standard to establish a TR-FIA for salmon IGFBP-1a.

Fig. 4. Standard curves in the ligand immuno-functional assay (LIFA) for intact insulin-like growth factor binding protein (IGFBP)-1a and dilution curves of serum dilutions from fed, fasted, and refed masu salmon (A) and rainbow trout (B). The count is the time-resolved fluorescence of Eu-labeled insulin-like growth factor-I (IGF-I) bound to the IGFBP-1a standard or serum. BU, binding unit.

Fig. 5. Effects of fasting and refeeding on serum insulin-like growth factor-I (IGF-I) (A), insulin-like growth factor binding protein (IGFBP)-1b (B), total IGFBP-1a (C), and intact IGFBP-1a (D) in yearling masu salmon. Values are expressed as means ± SE. The number of fish sampled in each group and time point is shown under the corresponding bar. Symbols sharing the same letter are not significantly different from each other (Fisher’s least-significant difference, P < 0.05). BU, binding unit.
The TR-FIA was validated for its stability using recombinant protein and sera from masu salmon and rainbow trout, as well as specificity and cross-reactivity using other subtypes of IGFBP purified from Chinook salmon serum. Purified rsIGFBP-1a was biotinylated and used as a tracer. This labeling appeared to have little effect on binding to the antiserum and possible interaction with IGFs in the assay. The cross-reactivity of the TR-FIA with other IGFBPs, particularly IGFBP-1b, was a concern because IGFBP-1a and 1b are paralogs sharing 61% sequence homology (49). Indeed, antiserum against IGFBP-1b showed cross-reactivity with IGFBP-1a in the TR-FIA (3.1%) (18). In the present study, anti-IGFBP-1a also cross-reacted with IGFBP-1b but showed little cross-reactivity with IGFBP-2b. Because the cross-reactivity with IGFBP-1b was low (3.6%), as is the case for the TR-FIA for IGFBP-1b, it should not severely affect the quantification of IGFBP-1a. The TR-FIA using components from masu salmon IGFBP-1a can be used for quantifying IGFBP-1a in other salmonids, such as rainbow trout, be-

Table 1. Correlation coefficients between endocrine parameters and morphological parameters in week 6

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<th></th>
<th>SL</th>
<th>BW</th>
<th>K</th>
<th>HSI</th>
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<tr>
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NS, not significant; r, correlation coefficient. n = sample size. IGFBP-1a and 1b values were natural log transformed.

Table 2. Correlation coefficients among endocrine parameters in week 6

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<tr>
<td>Total BP-1a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>NS</td>
<td>0.38</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.251</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>56</td>
<td>56</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Intact BP-1a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>−0.57</td>
<td>0.60</td>
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<td></td>
</tr>
<tr>
<td>P value</td>
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<td>0.004</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>n</td>
<td>57</td>
<td>57</td>
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</tbody>
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NS, not significant; r, correlation coefficient. n = sample size. IGFBP-1a and 1b values were natural log transformed.

Fig. 6. Correlations between specific growth rate (SGR) in body weight and serum insulin-like growth factor-I (IGF-I) (A), insulin-like growth factor binding protein (IGFBP)-1b (B), total IGFBP-1a (C), and intact IGFBP-1a (D). Pearson’s correlation coefficients and sample size were shown in each figure (P < 0.05). Values of IGFBP-1a and 1b are natural log transformed.
cause a serial dilution of trout serum was conducted in parallel with the masu salmon standard.

Macqueen and colleagues identified up to 22 genes for IGFBPs in salmonids (19, 38). A larger number of IGFBP repertoires came from an additional round of whole genome duplication among salmonids. As a result, four IGFBP-1 paralogs are present, and the recombinant IGFBP-1a used in the present study corresponds to IGFBP-1a1. Because IGFBP-1a1 and 1a2 share 79% sequence homology (38), the TR-FIA using antiserum against IGFBP-1a1 should cross-react with IGFBP-1a2. However, it is not known whether IGFBP-1a2 is present in the circulation and, if so, how much. In any case, we assume it is possible to measure both IGFBP-1a subtypes in our TR-FIA.

Because IGFs are tightly associated with IGFBPs in the circulation, IGFBPs interfere with an accurate measurement of IGFs in immunoassay (12, 15). Therefore, separation of IGF-I from IGFBPs by acid-ethanol extraction is important for measuring salmon IGF-I (47). In contrast, IGFs generally do not interfere with the performance of IGFBP assays, although in some RIA, the interference by IGF-I was reported (4, 48). In the present study, the effect of IGF-I on the serial dilution curves of masu salmon and rainbow trout sera was examined by adding excess IGF-I. As a result, the serum dilution curves were not affected by the presence of IGF-I, indicating that interference by IGF-I in the TR-FIA is minimal.

Despite the validity of the TR-FIA for IGFBP-1a, measurement of total IGFBP-1a levels did not provide useful information on the catabolic status or growth retardation of masu salmon. IGFBP-1 is believed to be an inhibitor of IGF-I actions in fish by increasing under catabolic conditions and sequestering IGF-I from the circulation (25). There have been attempts to utilize circulating protein levels or hepatic mRNA levels of IGFBP-1 as an index of negative growth and/or stress (30, 31, 32, 41). Kawaguchi et al. (30) semiquantified serum IGFBP-1a levels by ligand blotting using labeled IGF-I and found a negative correlation with SGR in weight in yearling masu salmon. Our assumption was that quantification of circulating IGFBP-1a by TR-FIA should establish a consistent negative relationship between serum IGFBP-1a and growth rate. However, total IGFBP-1a was less sensitive to fasting, and only a weak negative relationship with growth rate was observed, which conflicts with the findings by ligand blotting (30). Ligand blotting detects IGFBPs based on the ability to bind to labeled IGF, whereas TR-FIA measures immunoreactive components regardless of their IGF-binding ability. A possibility is that IGFBP-1a in serum of fed fish was partly degraded by enzymes, whereas that of fasted fish remained intact. We thus hypothesized that the selective measurement of intact IGFBP-1a that retained IGF-binding ability was more reflective of the degree of growth retardation and developed a LIFA.

LIFA is a combination of immunoassay and ligand-binding assay, as reported by Lassarre and Binoux (34), for measuring intact IGFBP. In a LIFA for human IGFBP-3, immunoreactive IGFBP-3 containing both fragments and intact protein was first captured by a monoclonal antibody, and its ability to bind to 125I-labeled IGF-I was quantified, which made an accurate and sensitive measurement of intact IGFBP-3 possible (34). In the present study, we modified the protocol (34) to avoid a risk of denaturation of intact IGFBP-1a during acid-ethanol extraction and used Eu-labeled IGF-I instead of radio-labeled IGF-I. The IGF-I binding by the standard rsIGFBP-1a increased in a dose-dependent manner, and serial dilutions of masu salmon and rainbow trout sera showed parallelism with the standard, indicating that the LIFA quantifies intact IGFBP-1a in salmonids. It is of note that the LIFA does not distinguish intact IGFBP-1a that is occupied or unoccupied with endogenous IGF, since the incubation time with excess Eu-IGF-I may be long enough to bring it an equilibrium state and displace with endogenous IGFs for intact IGFBP-1a. In human, a complex of IGFBP-1 and IGF-I was formed by incubation for 2.5 h at 37°C (15) and overnight at 4°C (10). Despite its limitation, intact IGFBP-1a measured by the LIFA showed biologically meaningful responses to fasting and refeeding in masu salmon.

As we expected, intact IGFBP-1a was more sensitive to fasting treatment by increasing after 4 wk of fasting when total IGFBP-1a was unchanged. Intact IGFBP-1a also responded to refeeding, and its level was restored to a level comparable with that in the fed control. Such changes were in good agreement with those reported by Kawaguchi et al. (30). The difference in the responses between total and intact IGFBP-1a suggests that the measurement of both fractions is of biological value. Although the significance of different patterns of total and intact IGFBP-1a is unknown at present, our finding invites future study on the regulation of circulating IGFBP-1a in salmon.

The result that intact IGFBP-1b levels were higher than those of total IGFBP-1a in fasted fish is puzzling, but a different degree of phosphorylation of IGFBP-1a under feeding and fasting conditions may account for it. The LIFA developed in the present study relies both on the immunoreactivity to antiserum against recombinant masu salmon IGFBP-1a and on the affinity to Eu-labeled IGF-I. If the affinity of IGFBP-1a to IGF-I was increased by fasting, it would result in a higher binding of the label and thus higher measured value. The results of the LIFA were thus expressed as a binding unit (BU) relative to recombinant masu salmon IGFBP-1a in the present study. Indeed, phosphorylation of human IGFBP-1, but not rat IGFBP-1, is known to increase the affinity for IGF-I (24, 40). There are potential phosphorylation sites in the deduced amino acid sequences of Chinook and masu salmon IGFBP-1a (49, 54). Recombinant masu salmon IGFBP-1a used as a standard is unlikely to be phosphorylated because it was produced by bacterial cells (54). Thus, a hypothesis is that fasting altered the phosphorylation status of IGFBP-1a, increased its affinity to IGF-I, and resulted in apparent high levels in LIFA. However, whether salmon IGFBP-1a is actually phosphorylated needs to be confirmed.

Correlation analysis on the relationships between circulating total and intact IGFBP-1a with individual growth rate suggests that intact IGFBP-1a but not total IGFBP-1a can be used as a negative index of salmon growth. The utility of circulating IGF-I and IGFBP-1b as positive and negative growth indices, respectively, has been reported in salmonid species (5–7, 28–30, 44) and confirmed in the present study. Correlation analysis suggests that intact IGFBP-1a is comparable with IGFBP-1b as a growth index. However, it is not known whether dual measurements of IGFBP-1a and 1b would give a better estimate of growth retardation because a stepwise regression model that combines intact IGFBP-1a and/or IGFBP-1b with IGF-I provided an improved but similar coefficient of regression to explain growth variance (data not
shown). Comparing responses of intact IGFBP-1a and 1b under other developmental and physiological situations, such as smoltification, maturation, disease, and stress, may reveal differential regulation of these two subtypes and their relations with growth rate.

We provide data showing that intact IGFBP-1a levels increased in fasting fish and suggest that a fraction of circulating IGFBP-1a is degraded under normal feeding situations. Although there is no empirical evidence of fragmentation of IGFBP-1a in fish, specific enzymes play an important role in controlling the binding affinity of IGFBPs for IGFs in mammals (9, 17, 55). For instance, a significant portion of IGFBP-3 in the serum of pregnant women is enzymatically degraded and has a reduced IGF-binding affinity so that more IGFs are readily available to the receptor (21, 35). Examining the presence of specific enzymes for IGFBP-1a in the circulation is a subject for future study.

Perspectives and Significance

The present study developed two immunoassays: TR-FIA for total immunoreactive IGFBP-1a (fragments + intact) and LIFA for intact IGFBP-1a with IGF-binding ability in salmon. Intact IGFBP-1a was more sensitive to fasting and refeeding treatment than total IGFBP-1a and had a higher negative relationship with individual growth rate, suggesting that degradation of IGFBP-1a is an important mechanism to regulate IGF-I activity in circulation. Thus, intact rather than total IGFBP-1a is useful as a negative index of salmon growth. In addition, the availability of immunoassays for both IGFBP-1a and IGFBP-1b will help unravel how circulating IGF-I is regulated by two subtypes of IGFBP-1 in salmon.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.K. and M.S. conceived and designed research; N.K., H.T., A.H., and M.S. performed experiments; N.K., T.O.N., and M.S. analyzed data; N.K., T.O.N., A.H., and M.S. interpreted results of experiments; N.K. prepared figures; N.K. drafted manuscript; N.K., T.O.N., and M.S. edited and revised manuscript; N.K., T.O.N., H.T., A.H., and M.S. approved final version of manuscript.

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