Title

Development of a radioimmunoassay for salmon 41-kDa insulin-like growth factor binding protein

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Short title

RIA for salmon IGFBP

Key words

insulin-like growth factor (IGF); IGF-binding protein; salmon; radioimmunoassay; smoltification

Abstract

Salmon plasma contains at least three insulin-like growth factor binding proteins (IGFBPs) with molecular masses of 41, 28 and 22 kDa. The 41-kDa IGFBP is similar to mammalian IGFBP-3 in size, type of glycosylation and physiological responses. In this study, we developed a radioimmunoassay (RIA) for the 41-kDa IGFBP. The 41-kDa IGFBP purified from serum was used for antibody production and assay standard. Binding of three different preparations of tracer were examined; ^{125}I-41-kDa IGFBP, ^{125}I-41-kDa IGFBP cross-linked with IGF-I (^{125}I-41-kDa IGFBP/IGF-I) and 41-kDa IGFBP/^{125}I-IGF-I. Only binding of 41-kDa IGFBP/^{125}I-IGF-I was not affected by added IGFs, and therefore it was chosen for the tracer in the RIA. Plasma 41-kDa IGFBP levels measured by RIA were increased by growth hormone treatment (178.9 ± 4.9 ng/ml) and decreased after fasting (95.0 ± 7.0 ng/ml). The molarities of plasma 41-kDa IGFBP and total IGF-I were comparable, and they were positively correlated, suggesting that salmon 41-kDa IGFBP is a main carrier of circulating IGF-I in salmon, as is mammalian IGFBP-3 in mammals. During the parr-smolt transformation (smoltification) of coho salmon, plasma 41-kDa IGFBP levels showed a transient peak (182.5 ± 10.3 ng/ml) in March and stayed relatively constant thereafter, whereas IGF-I showed peak levels in March and April. Differences in the molar ratio between 41-kDa IGFBP and IGF-I possibly influence availability of IGF-I in the circulation during smoltification.

Introduction

Insulin-like growth factor binding proteins (IGFBPs) are important modulators of IGF actions controlling IGF availability to IGF receptors in the target tissues. In mammals, six IGFBPs have been identified, and their regulation and functions have been extensively studied (for reviews, Shimasaki & Ling, 1991; Rechlar, 1993; Jones & Clemmons, 1995; Rajaram *et al*., 1997). Recent findings also indicate IGF-independent actions of IGFBPs on cell growth in many mammalian cell types (for review, Mohan & Baylink, 2002). IGF and IGFBP are evolutionary ancient proteins, since both molecules are found in lamprey (*Geotria australis*; Upton *et al*., 1993). In fish, IGFBPs corresponding to mammalian IGFBP-1 and -2 have been cloned in zebrafish (*Danio rerio*; Duan *et al.*, 1999; Maures & Duan, 2002) and gilthead sea bream (*Sparus aurata*, Funkenstein *et al.*, 2002), and an almost complete sequence has been reported for tilapia (*Oreochromis mossambicus*) IGFBP-3 (Cheng *et al*., 2002). Multiple IGFBPs are detected in fish plasma/serum by Western ligand blotting using labelled IGF-I and shown to be controlled by hormones, nutritional status and stress (Kelley *et al*., 1992; Niu & LeBail, 1993; Siharath *et al*., 1995, 1996; Shimizu *et al*., 1999; Park *et al*., 2000; Kelley *et al*., 2001). Duan and co-workers showed that fish IGFBPs expressed in animal cells or purified from culture medium inhibit IGF-stimulated DNA synthesis *in vitro* (Duan *et al*., 1999; Bauchat *et al*., 2001). These findings indicate that fish IGFBPs are conserved functionally as well as structurally.

To date, most studies on physiological regulation of fish IGFBPs have been conducted by use of Western ligand blotting. Although Western ligand blotting is a powerful tool to detect different types of IGFBPs and compare their relative concentrations, this technique is semi-quantitative and not suitable for processing a large number of samples. Development of an RIA for fish IGFBPs will greatly facilitate study of the physiological regulation of fish IGFBPs by providing a precise, high capacity tool. In salmon, at least three IGFBPs with molecular masses of 41, 28 and 22 kDa exist in plasma. The 41-kDa IGFBP is most likely the salmon IGFBP-3 since it has a molecular weight similar to mammalian IGFBP-3 and its plasma levels are influenced by growth hormone treatment and fasting as is mammalian IGFBP-3 (Shimizu *et al*., 1999). We recently purified the salmon 41-kDa IGFBP from serum (Shimizu *et al*., 2003) and generated specific antibody against it. Using this antibody and purified protein as assay components, we developed a radioimmunoassay for salmon 41-kDa IGFBP.

Materials and Methods

Fish

Spawning male chinook salmon (*Oncorhynchus tshawytscha*) were sampled for blood in the adult return pond on the University of Washington campus, Seattle, WA, USA in October and November. Fish were anesthetized in 0.05% tricane methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA). Blood was withdrawn by syringe from the caudal veins, allowed to clot overnight at 4°C and then centrifuged at 1350*g* for 30 min. Serum was collected and stored at -80°C until use.

Two-year-old coho salmon were reared in fresh water at the Northwest Fisheries Science Center in Seattle, WA, USA. They were maintained in recirculated fresh water in circular fiberglass tanks under natural photoperiod; flow rate was 25 L/min; temperature ranged from 10.5°C to 13.0°C. Fish were fed standard rations (0.6-1.0% body weight/day) of a commercial diet (Biodiet Grower; Bioproducts Inc., Warrenton, OR, USA). Some of two-year-old coho salmon were injected twice with salmon GH at a dose of 0.1 μg/g body weight or saline, and sampled 24 hr after second injection (48 hr after first injection) as described in Shimizu *et al*. (1999). Other groups were fasted or fed *ad libitum* for one month (Shimizu *et al*. , 1999). Blood was withdrawn by cutting the caudal peduncle and letting blood flow into a heparinized glass tube. Plasma was collected after centrifugation at 700*g* for 15 min and stored at -80°C until use.

One-year-old coho salmon were reared under same conditions as described above. From 2 March to 6 July 2000, twelve fish were sampled for blood every two weeks. Fork length and body weight of the fish at the beginning of the sampling were 12.3 ± 0.2 cm (mean ± SEM) and 21.7 ± 0.9 g, respectively, and those at the end of the sampling were 15.8 ± 0.3 cm and 47.2 ± 2.8 g, respectively. Condition factor was calculated as: (body weight) x 1000/(fork length)^3.

Purification of IGFBP

The 41-kDa IGFBP was purified from serum of spawning male chinook salmon as described in Shimizu *et al*. (2003). Briefly, salmon serum was acidified with 2 M acetic acid, 0.75 mM NaCl and mixed with SP-Sephadex C-25. The gel was settled out after incubating 1 hr and the supernatant was collected. The supernatant was neutralized with 7 M NaOH and a heavy precipitate was removed by centrifugation. Clarified supernatant was loaded onto an IGF-I affinity column and IGFBPs were eluted from the column with 0.5 M acetic acid. IGFBPs were further purified by reversed-phase high pressure liquid chromatography (HPLC) using a Vydac C-4 column (Separation Group, Hesperia, CA, USA).

Preparation of antiserum

Polyclonal antiserum against purified 41-kDa IGFBP (anti-41-kDa IGFBP) was raised in a rabbit. A total of 145 μg purified protein in 1 ml was emulsified in an equal volume of Freund's complete adjuvant (Iatoron; Tokyo, Japan). The rabbit was first immunized with 25 μg antigen by lymph node injection and boosted twice with 60 μg antigen at the backs three and five weeks after the first injection. Blood was withdrawn from the ear vein and antiserum was collected after centrifugation. The antiserum was stored at -30°C until use.

Western ligand blotting and Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel and 12.5% separating gel was carried out according to Laemmli (1970). Purified salmon IGFBPs were treated with an equal volume of the 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.

Western ligand blotting using digoxigenin-labelled human IGF-I (DIG-hIGF-I) was carried out as described in Shimizu *et al*. (2000). After electroblotting, the nitrocellulose membrane was incubated with 9 ng/ml of DIG-hIGF-I for 2 hr at room temperature and then incubated with antibody against DIG conjugated with horse-radish peroxidase (Boehringer Mannheim, Indianapolis, IN, USA) at a dilution of 1:2500 for 1 hr at room temperature. IGFBPs were visualized on X-ray film by use of the ECL Western blotting reagents (Amersham Life Science Inc., Arlington Heights, IL, USA).

For immunoblotting, an electroblotted nitrocellulose membrane was incubated with anti-41-kDa IGFBP serum at a dilution of 1:1500 for 2 hr at room temperature. The membrane was then incubated with goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Bio-Rad; Hercules, CA, USA) for 1 hr at room temperature. Immunoreactive bands were visualized on X-ray film by use of the ECL Western blotting reagents.

Preparation of tracers

Purified 41-kDa IGFBP was iodinated with 0.5 mCi $Na^{125}I$ (Amersham Life Sicence Inc.) by chloramine-T method as described in Moriyama *et al*. (1994). Five micrograms of the 41-kDa IGFBP in 36 μl was mixed with 64 μl 0.5 M phosphate buffer, pH 7.4. The mixture was reacted with 20 μl of 0.4 mg/ml chloramine-T (Sigma; St. Louis, MO, USA) for 90 sec and 20 μl of 0.6 mg/ml metabisulfite was added to stop the reaction. Iodinated 41-kDa IGFBP (IGFBP*) was separated from free $Na^{125}I$ using Biogel P-6 (1 x 18 cm; Bio-Rad). Specific activity of the tracer estimated by the self-displacement assay (Catt, 1976) was 29.8 μCi/μg.

An aliquot of IGFBP* (1.8 μg) was incubated with 3.3 μg salmon IGF-I (GroPep Pty Ltd, Adelaide, Australia) for 2 hr and they were cross-linked by disuccinimidyl suberate (Pierce; Rockford, IL, USA) according to manufacturer's instruction. The IGFBP* cross-linked with salmon IGF-I (IGFBP*/IGF-I) was separated from non-reacted IGF-I by gel filtration using Sephadex G-50 (1 x 18 cm, superfine; Pharmacia, Uppsala, Sweden). Specific activity of the tracer was 49.2 μCi/μg.

A tracer consisting of unlabelled 41-kDa IGFBP and ^{125}I-salmon IGF-I was also prepared. Salmon IGF-I was iodinated by the chloramine-T method as described above and an aliquot of

^{125}I-salmon IGF-I (1 μg) was incubated with 6.7 μg purified 41-kDa IGFBP for 2 hr, and they were cross-linked by disuccinimidyl suberate. The 41-kDa IGFBP cross-linked with ^{125}I-salmon IGF-I (IGFBP/IGF-I*) was separated from non-reacted ^{125}I-salmon IGF-I by gel filtration using Sephadex G-50. Specific activity of the tracer was 6.6 μCi/μg.

Radioimmunoassay for 41-kDa IGFBP

Radioimmunoassay was carried out in 12 x 75 mm polystyrene test tubes. Purified 41-kDa IGFBP was used for the standard. One hundred microliters of standard or plasma diluted in 20 mM phosphate, 150 mM NaCl containing 1.0% bovine serum albumin and 0.05% Triton X-100 were incubated with 100 μl anti-41-kDa IGFBP at a dilution of 1:3000-5000 overnight at 4°C. Approximately 7000 cpm of tracer in 100 μl was added to the tubes and incubated overnight at 4°C. Free and antibody-bound tracers were separated by the addition of 0.5% Pansorbin (Calbiochem-Novabiochem Corp., La Jolla, CA, USA). After incubating overnight at 4°C, tubes were centrifuged at 1350*g* for 30 min and the supernatant was aspirated. Radioactivity in the pellets was measured by a gamma counter (Packard, Meriden, CT, USA).

Radioimmunoassay for IGF-I

IGF-I was extracted from plasma by acid-ethanol followed by cryoprecipitation according to the method of Breier *et al*. (1991). This extraction method has been validated for salmon plasma (Shimizu *et al*., 2000). IGF-I was measured by RIA as described in Shimizu *et al*. (2000).

Statistical analysis

Results of the experiments were analyzed using one-way ANOVA followed by the Fisher protected least-significant difference test (Dowdy & Weardon, 1991) using the Statview 512+ program (Abacus Concepts, Inc., Berkeley, CA, USA). Differences between groups were considered to be significant at $P < 0.05$.

Results

Specificity of a polyclonal antiserum against salmon 41-kDa IGFBP was assessed by immunoblotting of purified and semi-purified salmon IGFBPs (Fig. 1). Anti-41-kDa IGFBP immunostained doublet bands at 43 and 41 kDa in the purified 41-kDa IGFBP fraction. Doublet bands at higher molecular weight, presumably aggregation of the 43-41 kDa doublet bands, were also recognized by the antiserum. Anti-41-kDa IGFBP cross-reacted with 28-kDa IGFBP weakly but not with 22-kDa IGFBP. This antiserum was used for RIA of salmon 41-kDa IGFBP.

Three differently prepared tracers were examined for development of the RIA; purified 41-kDa IGFBP directly labelled with I-125 (IGFBP*), labelled 41-kDa IGFBP cross-linked with unlabelled salmon IGF-I (IGFBP*/IGF-I), and unlabelled 41-kDa IGFBP cross-linked with labelled salmon IGF-I (IGFBP/IGF-I*). All three tracers bound specifically to the antiserum and were displaced with unlabelled 41-kDa IGFBP in a concentration-dependent manner albeit total binding differed among tracers (Fig. 2). The tracers were next compared for interaction with added salmon IGF-I with and without the addition of 10 ng/ml unlabelled 41-kDa IGFBP (Fig. 3). When the combination of unlabelled IGF-I and 41-kDa IGFBP was added to the tracers, the percent of tracer bound increased for IGFBP* and IGFBP*/IGF-I, but remained constant for IGFBP/IGF-I*. Thus, added IGF-I in excess of 2 ng/ml reduced the displacement of two of the tracers. Total binding of two of the tracers (IGFBP* and IGFBP/IGF-I*) was not affected by added IGF-I, but the total binding of IGFBP*/IGF-I was increased slightly when IGF-I was added at 10 ng/ml or greater (Fig. 3). Therefore, IGFBP/IGF-I* was used as the tracer in the RIA.

Using the IGFBP/IGF-I* as tracer, specific and non-specific binding to the antiserum (1:3000 dilution) under the assay conditions were 20.83 ± 0.78% (mean ± SEM; n = 8) and 0.81 ± 0.04% (n = 8), respectively. The half-maximal displacement (ED_{50}) occurred at 10.01 ± 0.23 ng/ml (n = 8). The ED_{80} and ED_{20} were 3.43 ± 0.13 ng/ml and 27.13 ± 0.96 ng/ml (n = 8), respectively. The minimal detection limits of the assay, defined as the mean of the zero standard minus two standard deviations, was 0.44 ± 0.07 ng/ml (n = 8). The precision profile (Ekins, 1983) of the standard curve indicate that the functional sensitivity, defined as the concentration at which the inter-assay coefficients of variation is ≤ 20% (Spencer *et al*., 1995), was 1.56 ng/ml (n = 10). The intra- and inter-assay coefficients of variation estimated at 8.85 ng/ml using a control serum were 3.6% (n = 10) and 11.3% (n = 5), respectively. Recovery of purified 41-kDa IGFBP added to chinook salmon serum was 91.9 ± 4.5% (n = 3).

In order to further assess possible interference by IGFs in the RIA, the slopes of the IGFBP standards with or without adding IGFs were compared (Fig. 4). Addition of unlabelled sIGF-I up to 1:100 molar ratio did not affect the slope of the standard, and it was parallel to that of salmon serum. Similar results were obtained with human IGF-I and IGF-II (data not shown). Next, the effect of addition of IGFs to plasma on measured 41-kDa IGFBP was examined (Table 1). There was no statistical difference in measured plasma 41-kDa IGFBP with or without IGFs.

Coho salmon plasma from fish in different physiological states showed parallel displacement with the standard (Fig. 5). There was no difference in slope and binding of displacement curve between plasma and serum from coho salmon (data not shown). No displacement was observed with a partially purified salmon 28-kDa IGFBP containing 22-kDa IGFBP as a minor component.

Plasma levels of 41-kDa IGFBP in coho salmon from GH-injection and fasting experiments were measured by RIA. GH-treatment caused a significant increase of plasma 41-kDa IGFBP levels whereas fasting reduced plasma 41-kDa IGFBP levels (Fig. 6). There was a strong positive relationship between total IGF-I and 41-kDa IGFBP levels in individual samples (Fig. 7a). The correlation coefficient was higher with an exponential regression (r^2 = 0.85) than with a linear regression (r^2 = 0.78). A weaker, but significant, relationship was found between free IGF-I and 41-kDa IGFBP (Fig. 7b). The relationship was best represented by a polynomial regression (r^2 = 0.61).

Changes in plasma IGF-I and 41-kDa IGFBP levels during smoltification of coho salmon were measured by RIAs (Fig. 8). The condition factor, a morphological index of smoltification, declined from mid March to its lowest point in early May indicating the completion of smoltification (data not shown). Plasma IGF-I exhibited peaks in mid March and late April (Fig. 8). Plasma 41-kDa IGFBP, on the other hand, showed a transient peak in late March, corresponding to the first peak of IGF-I, and stayed relatively constant thereafter (Fig. 8).

Discussion

In this study, a radioimmunoassay for salmon 41-kDa IGFBP, a candidate of fish IGFBP-3, has been established and validated for the first time. In RIAs for mammalian IGFBP-3, the preparation of radio-tracer is one of the more critical aspects. Baxter & Martin (1986) reported that when purified human IGFBP-3 was labelled directly with iodine, an unacceptably high non-specific binding (> 15% of total radioactivity) was observed. In order to overcome this problem, IGFBP-3 is indirectly labelled by cross-linking with ^{125}I-IGF-I (Baxter & Martin, 1986), or labelled IGFBP-3 is purified by gel filtration (Blum *et al*., 1990). In the present study, three different tracers were prepared: ^{125}I-41-kDa IGFBP (IGFBP*), IGFBP* cross-linked with unlabelled salmon IGF-I (IGFBP*/IGF-I) and IGFBP/IGF-I*. Unlike mammalian IGFBP-3, none of tracers showed high non-specific binding in the RIA. There were, however, differences in the total binding among tracers. This may be partly because of the different specific activities of the tracers. It is also possible that unlabelled IGFBP in IGFBP/IGF-I*, which was not cross-linked with IGF-I*, influenced the total binding of the tracer. Despite the difference in the total binding, all three tracers were specifically displaced by adding unlabelled IGFBPs. In most RIAs for IGFs, IGFBPs interfere with the accurate measurement of IGFs and therefore IGF must be separated from IGFBPs prior to RIA (Bang *et al*., 1994). On the other hand, IGFs generally do not affect the RIAs for IGFBPs, with one exception: the RIA for IGFBP-6 (Baxter & Saunders, 1992). We thus examined the effect of IGF on the standard curve in our salmon RIA. Total binding was not influenced by addition of salmon IGF-I except for IGFBP*/IGF-I. However, when the tracers were competed with unlabelled 41-kDa IGFBP in the presence or absence of exogenous IGF-I, displacement of IGFBP* and IGFBP*/IGF-I with unlabelled IGFBP was diminished by the presence of IGF-I. An explanation for the interference by IGF-I may be that immunoreactivity of IGF-bound 41-kDa IGFBP is lower than that of unoccupied 41-kDa IGFBP. Although the exact mechanism of the interference by IGF is not clear, IGFBP/IGF-I* is practically the only tracer which is not affected by IGF-I under the assay conditions, and therefore chosen for the tracer.

In order to further assess the influence of IGF in the RIA using IGFBP/IGF-I*, various amounts of IGFs (salmon IGF-I, and human IGF-I and IGF-II) were added to the standard and plasma. Neither the slope of the standard curve nor measured plasma IGFBP levels were affected by addition of IGFs. These results confirm that RIA using IGFBP/IGF-I* is not influenced by varying levels of IGF. This implies that plasma can be directly assayed by the RIA without extraction to separate IGF and its binding proteins. No cross-reactivity was detected with other salmon IGFBPs (i.e. 28- and 22-kDa IGFBPs) in RIA for 41-kDa IGFBP despite the fact that anti-41-kDa IGFBP weakly recognized the 28-kDa IGFBP in Western blotting. These data demonstrate validity of the RIA for measuring 41-kDa IGFBP. Parallel displacement of coho salmon plasma with standard suggests that this RIA is applicable to other salmonid species.

Plasma IGFBP-3 levels are primarily influenced by GH and nutritional status: GH stimulates hepatic synthesis of IGFBP-3, probably indirectly through IGF-I (Villafuerte *et al*., 1994), and fasting or malnutrition causes a decrease in circulating IGFBP-3 (Clemmons & Underwood, 1991). In fish, candidates for IGFBP-3 have been detected based on molecular size, and responses to GH injection and fasting on Western ligand blotting (Kelley *et al*., 1992; Shiharath *et al*., 1995; Shimizu *et al*., 1999; Park *et al*., 2000). Recently, the cDNA sequence of most of a tilapia IGFBP-3 has been determined (Cheng *et al*., 2002). As determined by RNAse protection assay, the tilapia IGFBP-3 mRNA increased in response to GH treatment. Consistent with the observation by Western ligand blotting and RNAse protection assay, GH treatment increased measured 41-kDa IGFBP levels about 1.8-fold, and fasting decreased its levels by one third compared to the fed control. These findings suggest that the 41-kDa IGFBP is functionally IGFBP-3.

Mammalian IGFBP-3 carries most of the circulating IGF-I. In general, there is a positive relationship between IGFBP-3 and IGF-I levels (Baxter & Martin, 1986; Frystyk *et al*., 1998). In the present study, plasma 41-kDa IGFBP levels positively correlated with total IGF-I levels and molarity of the 41-kDa IGFBP was comparable to that of total IGF-I. These comparisons indicate that the 41-kDa IGFBP is a main carrier of circulating IGF-I as is IGFBP-3 in mammals. Free IGF-I levels also positively correlated with 41-kDa IGFBP levels, but the regression coefficient was not as high as for total IGF-I. This might be due to other IGFBP regulation of free IGF-I levels (Frystyk *et al*., 1997)

In spring, juvenile salmon migrate to the ocean to grow. Prior to down-stream migration, juvenile salmon undergo the parr-smolt transformation (smoltification), which is a pre-adaptation to ocean life involving behavioral, morphological and physiological changes (for review, Hoar, 1988). The GH-IGF-I endocrine axis plays a central role in regulation of smoltification since this axis promotes growth and seawater adaptability of juveniles, both of which are important for successful smoltification (Dickhoff *et al*., 1997). In the present study, plasma IGF-I showed peaks in March and April. Discrete peaks of IGF-I in plasma during smoltification have been reported in chinook salmon (Beckman *et al*., 1998), coho salmon (Larsen *et al*., 2001) and Atlantic salmon (*Salmo salar*; Ágústsson *et al*., 2001). Those IGF-I profiles seem to be influenced by environmental factors such as photoperiod, water temperature and feeding ration (Beckman *et al*., 1998; McCormick *et al.*, 2000; Larsen *et al*., 2001). Plasma 41-kDa IGFBP, on the other hand, showed a single peak in March and stayed relatively constant thereafter whereas IGF-I showed a second peak in April. This suggests that the molar ratio of IGF-I to 41-kDa IGFBP fluctuated during smoltification. The difference in the molar ratio could influence availability of IGF-I to target tissues. In this regard, the 41-kDa IGFBP may regulate salmon smoltification via controlling IGF-I availability. However, we did not measure free IGF-I levels, which is believed to be a biologically active form available to the receptor, nor other IGFBP levels in those fish. In order to fully understand regulation of salmon smoltification by the GH-IGF-I axis including IGFBPs, more detailed studies are needed.

In conclusion, we established a radioimmunoassay for salmon 41-kDa IGFBP. Quantification of 41-kDa IGFBP in plasma from fish in different physiological states suggests that the 41-kDa IGFBP is salmon IGFBP-3 and it acts as a main carrier of circulating IGF-I.

Differences in the molar ratio between IGF-I and 41-kDa IGFBP possibly influence availability of IGF-I from the circulation to the target tissues during smoltification.

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References

Ágústsson, T, Sundell, K, Sakamoto, T, Johansson, V, Ando, M & Björnsson, BTh 2001 Growth hormone endocrinology of Atlantic salmon (*Salmo salar*): pituitary gene expression, hormone storage, secretion and plasma levels during parr-smolt transformation. *Journal of Endocrinology* **170** 227-234.

Bang, P, Baxter, RC, Blum, WF, Breier, BH, Clemmons, DR, Hall, K, Hintz, RL, Holly, JM, Rosenfeld, RG & Zapf, J 1994 Valid measurements of total IGF concentrations in biological fluids. Recommendations from the 3rd International Symposium on Insulin-like Growth Factors. *Journal of Endocrinology* **143** C1-2.

Bauchat, JR, Busby, WH Jr, Garmong, A, Swanson, P, Moore, J, Lin, M & Duan, C 2001 Biochemical and functional analysis of a conserved IGF-binding protein isolated from rainbow trout (*Oncorhynchus mykiss*) hepatoma cells. *Journal of Endocrinology* **170** 619-628.

Baxter, RC & Martin, JL 1986 Radioimmunoassay of growth hormone-dependent insulinlike growth factor binding protein in human plasma. *Journal of Clinical Investigation* **78** 1504-1512.

Baxter, RC & Saunders, H 1992 Radioimmunoassay of insulin-like growth factor-binding protein-6 in human serum and other body fluids. *Journal of Endocrinology* **134** 133-139.

Beckman, BR, Larsen, DA, Moriyama, S, Lee-Pawlak, B & Dickhoff, WW 1998 Insulin-like growth factor-I and environmental modulation of growth during smoltification of spring chinook salmon (*Oncorhynchus tshawystscha*). *General and Comparative Endocrinology* **109** 325-335.

Blum, WF, Ranke, MB, Kietzmann, K, Gauggel, E, Zeisel, HJ & Bierich, JR 1990 A specific radioimmunoassay for the growth hormone (GH)-dependent somatomedin-binding protein: its use for diagnosis of GH deficiency. *Journal of Clinical Endocrinology and Metabolism* **70** 1292-1298.

Breier, BH, Gallaher, BW & Gluckman, PD 1991 Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls. *Journal of Endocrinology* **128** 347-357.

Catt, KJ 1976 Receptors for gonadotropic hormones. In *Methods in Receptor Research* 1, pp 175-250. Ed M Blecher. New York, NY: Marcel Dekker.

Cheng, R, Chang, K-M & Wu, J-L 2002 Different temporal expressions of tilapia (*Oreochromis mossambicus*) insulin-like growth factor-I and IGF binding protein-3 after growth hormone induction. *Marine Biotechnology* **4** 218-225.

Clemmons, DR & Underwood, LE 1991 Nutritional regulation of IGF-I and IGF binding proteins. *Annual Review on Nutrition* **11** 393-412.

Dickhoff, WW, Beckman, BR, Larsen, DA, Duan, C & Moriyama, S 1997 The role of growth in endocrine regulation of salmon smoltification. *Fish Physiology and Biochemistry* **17** 231-236.

Dowdy, S & Weardon, S 1991 In *Statistics for Research*, pp 303-305. New York, NY: Wiley.

Duan, C, Ding, J, Li, Q, Tsai, W & Pozios, K 1999 Insulin-like growth factor binding protein 2 is a growth inhibitory protein conserved in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America* **96** 15274-15279.

Ekins, RP 1983 The precision profile: its use in assay design, assessment and quality control. In Immunoassays for Clinical Chemistry, pp 76-105. Eds WM Hunter & JET Corrie. Churchill Livingstone, Edinburgh.

Frystyk, J, Grofte, T, Skjaerbaek, C & Orskov, H 1997 The effect of oral glucose on serum free insulin-like growth factor-I and -II in health adults. *Journal of Clinical Endocrinology and Metabolism* **82** 3124-3127.

Frystyk, J, Gronbaek, H, Skjaerbaek, C, Flyvbjerg, A, Orskov, H & Baxter, RC 1998 Developmental changes in serum levels of free and total insulin-like growth factor I (IGF-I), IGF-binding protein-1 and -3, and the acid-labile subunit in rats. *Endocrinology* **139** 4286-4292.

Funkenstein, B, Tsai, W, Maures, T & Duan, C 2002 Ontogeny, tissue distribution, and hormonal regulation of insulin-like growth factor binding protein-2 (IGFBP-2) in a marine fish, *Sparus aurata*. *General and Comparative Endocrinology* **128** 112-122.

Hoar, WS 1988 The physiology of smolting salmonids. In *Fish Physiology*, vol 11B, pp 275-343. Eds WS Hoar & D Randall. Orland, FL: Academic Press.

Jones, JI & Clemmons, DR 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Review* **16** 3-34.

Kelley, KM, Siharath, K & Bern, HA 1992 Identification of insulin-like growth factor-binding proteins in the circulation of four teleost fish species. *Jouranal of Experimental Zoology* **263** 220-224.

Kelley, KM, Haigwood, JT, Perez, M & Galima, MM 2001 Serum insulin-like growth factor binding proteins (IGFBPs) as markers for anabolic/catabolic condition in fishes. *Comparative Biochemistry and Physiology* **129B** 229-236.

Laemmli, UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680-685.

Larsen, DA, Beckman, BR & Dickhoff, WW 2001 The effect of low temperature and fasting during the winter on metabolic stores and endocrine physiology (insulin, insulin-like growth factor-I, and thyroxine) of coho salmon, *Oncorhynchus kisutch*. *General and Comparative Endocrinology* **123** 308-323.

Maures, TJ & Duan, C 2002 Structure, developmental expression, and physiological regulation of zebrafish IGF binding protein-1. *Endocrinology* **143** 2722-2731.

McCormick, SD, Moriyama, S & Björnsson, BTh 2000 Low temperature limits photoperiod control of smolting in Atlantic salmon through endocrine mechanisms. *American Journal of Physiology* **278** R1352-R1361.

Mohan, S & Baylink, DJ 2002 IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. *Journal of Endocrinology* **175** 19-31.

Moriyama, S, Swanson, P, Nishii, M, Takahashi, A, Kawauchi, H, Dickhoff, WW & Plisetskaya, EM 1994 Development of a homologous radioimmunoassay for coho salmon insulin- like growth factor-I. *General and Comparative Endocrinology* **96** 149-161.

Niu, P-D & Le Bail, P-Y 1993 Presence of insulin-like growth factor binding protein (IGF-BP) in rainbow trout (*Oncorhynchus mykiss*) serum. *Journal of Experimental Zoology* **265** 627-636.

Park, R, Shepherd, BS, Nishioka, RS, Grau, EG & Bern, HA 2000 Effects of homologous pituitary hormone treatment on serum insulin-like growth-factor-binding proteins (IGFBPs) in hypophysectomized tilapia, *Oreochromis mossambicus*, with special reference to a novel 20-kDa IGFBP. *General and Comparative Endocrinology* **117** 404-412.

Rajaram, S, Baylink, DJ & Mohan, S 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocrine Review* **18** 801-831.

Rechler, MM 1993 Insulin-like growth factor binding proteins. *Vitamin and Hormone* **47** 1-114.

Shimasaki, S & Ling, N 1991 Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and -6). *Progress in Growth Factor Research* **3** 243-266.

Shimizu, M, Swanson, P & Dickhoff, WW 1999 Free and protein-bound insulin-like growth factor-I (IGF-I) and IGF-binding proteins in plasma of coho salmon, *Oncorhynchus kisutch*. *General and Comparative Endocrinology* **115** 398-405.

Shimizu, M, Swanson, P, Fukada, H, Hara, A & Dickhoff, WW 2000 Comparison of extraction methods and assay validation for salmon insulin-like growth factor-I using commercially available components. *General and Comparative Endocrinology* **119** 26-36.

Shimizu, M, Swanson, P, Hara, A & Dickhoff, WW 2003 Purification of a 41-kDa insulin-like growth factor binding protein from serum of chinook salmon, *Oncorhynchus tshawytscha*. *General and Comparative Endocrinology* (in press).

Siharath, K, Nishioka, RS, Madsen, SS & Bern, HA 1995 Regulation of IGF-binding proteins by growth hormone in the striped bass, *Morone saxatilis*. *Molecular Marine Biology and Biotechnology* **4** 171-178.

Siharath, K, Kelley, KM & Bern, HA 1996 A low-molecular-weight (25-kDa) IGF-binding protein is increased with growth inhibition in the fasting striped bass, *Morone saxatilis*. *General and Comparative Endocrinology* **102** 307-316.

Spencer, CA, Takeuchi, M, Kazarosyan, M, MacKenzie, F, Beckett, GJ & Wilkinson, E 1995 Interlaboratory/intermethod differences in functional sensitivity of immunometric assays of thyrotropin (TSH) and impact on reliability of measurement of subnormal concentrations of TSH. *Clinical Chemistry* **41** 367-474.

Upton, Z, Chan, SJ, Steiner, DF, Wallace, JC & Ballard, FJ 1993 Evolution of insulin-like growth factor binding proteins. *Growth Regulation* **3** 29-32.

Villafuerte, BC, Koop, BL, Pao, CI., Gu, L, Birdsong, GG & Phillips, LS 1994 Coculture of primary rat hepatocytes and nonparenchymal cells permits expression of insulin-like growth factor binding protein-3 in vitro. *Endocrinology* **134** 2044-2050.

Figure legends

Fig. 1 Ligand blotting using digoxigenin-lebeled hIGF-I (DIG-hIGF-I) (left) and immunoblotting using anti-41-kDa IGFBP (right) of purified salmon IGFBPs. Fifity nanograms of each IGFBP fraction was separated by 12.5% SDS-PAGE under non-reducing conditions and transferred to a nitrocellulose membrane. For ligand blotting, the nitrocellulose membrane was incubated with 9 ng/ml of DIG-hIGF-I for 2 hr and then with anti-DIG conjugated with HRP at a dilution of 1:2500 for 1 hr. For immunoblotting, the nitrocellulose membrane was incubated with anti-41-kDa IGFBP at a dilution of 1:1500 for 2 hr and then with anti-rabbit IgG conjugated with HRP. Bands were visualized on X-ray film using ECL reagents. Arrowheads indicate migration positions of salmon IGFBPs.

Fig. 2 Displacement curves of radio-labelled tracers with unlabelled 41-kDa IGFBP. Approximately 7000 cpm of ^{125}I-41-kDa IGFBP (IGFBP*), ^{125}I-41-kDa IGFBP/IGF-I (IGFBP*-IGF-I) and 41-kDa IGFBP/^{125}I-IGF-I (IGFBP-IGF-I*) were incubated with anti-41-kDa IGFBP (1:5000 dilution) and different concentrations of unlabelled 41-kDa IGFBP (0.4 to 200 ng/ml). Binding (B/T) is expressed as a percentage of total binding. All values are means of duplicate determinations.

Fig. 3 Effect of salmon IGF-I on tracer binding with and without addition of unlabelled 41-kDa IGFBP. Approximately 7000 cpm of ^{125}I-41-kDa IGFBP (IGFBP*; squares), ^{125}I-41-kDa IGFBP/IGF-I (IGFBP*-IGF-I; triangles) and 41-kDa IGFBP/^{125}I-IGF-I (IGFBP-IGF-I*; circles) were incubated with anti-41-kDa IGFBP (1:5000 dilution) and different concentrations of salmon IGF-I (0.4 to 400 ng/ml) in the presence (closed symbols) or absence (open symbols) of 10 ng/ml unlabelled 41-kDa IGFBP. Binding (B/Bo) is expressed as a percentage of specific binding. All values are means of duplicate determinations.

Fig. 4 Effect of salmon IGF-I on the standard curve. Approximately 7000 cpm of 41-kDa IGFBP/^{125}I-IGF-I was incubated with anti-41-kDa IGFBP (1:3000 dilution) and 41-kDa IGFBP standard (0.8 to 100 ng/ml). Salmon IGF-I was added to the standard at a molar ratio of 1:1, 1:10 or 1:100. Serial dilution (1:32 to 1: 512) of serum from spawning chinook salmon is shown for comparison. Binding (B/Bo) is expressed as a percentage of specific binding. All values are means of duplicate determinations.

Fig. 5 Displacement curves of purified salmon IGFBPs and salmon plasma. Approximately 7000 cpm of 41-kDa IGFBP/^{125}I-IGF-I was incubated with anti-41-kDa IGFBP (1:3000 dilution) and serial dilution (1:8 to 1:512) of plasma from coho salmon under different physiological states. Displacement curves of plasma are compared with that of the standard. Binding (B/Bo) is expressed as a percentage of specific binding. All values are means of duplicate determinations.

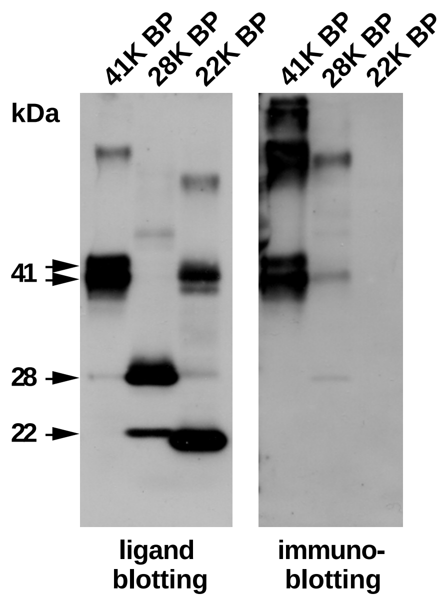
Fig. 6 Plasma 41-kDa IGFBP levels measured by RIA. Plasma from coho salmon injected twice with saline (Cont; n = 10) or 0.1 μg/g GH (n = 9), and coho salmon fed (n = 10) or fasted (n = 10) for a month were measured for 41-kDa IGFBP by RIA. Vertical bars represent S.E. Asterisks indicate significant difference between treatments of each experiment (P < 0.05).

Fig. 7 Relationship between plasma 41-kDa IGFBP and total IGF-I levels (a), and plasma 41-kDa IGFBP and free IGF-I levels (b). Data on plasma total and free IGF-I levels are from Shimizu *et al*. (1999).

Fig. 8 Changes in plasma 41-kDa IGFBP and IGF-I levels during smoltificatin of coho salmon. n

1 = 12 per each point. Vertical bars represent S.E.

Fig. 1


41K BP
28K BP
22K BP
41K BP
28K BP
22K BP
kDa
41
28
22
ligand blotting
immuno-blotting

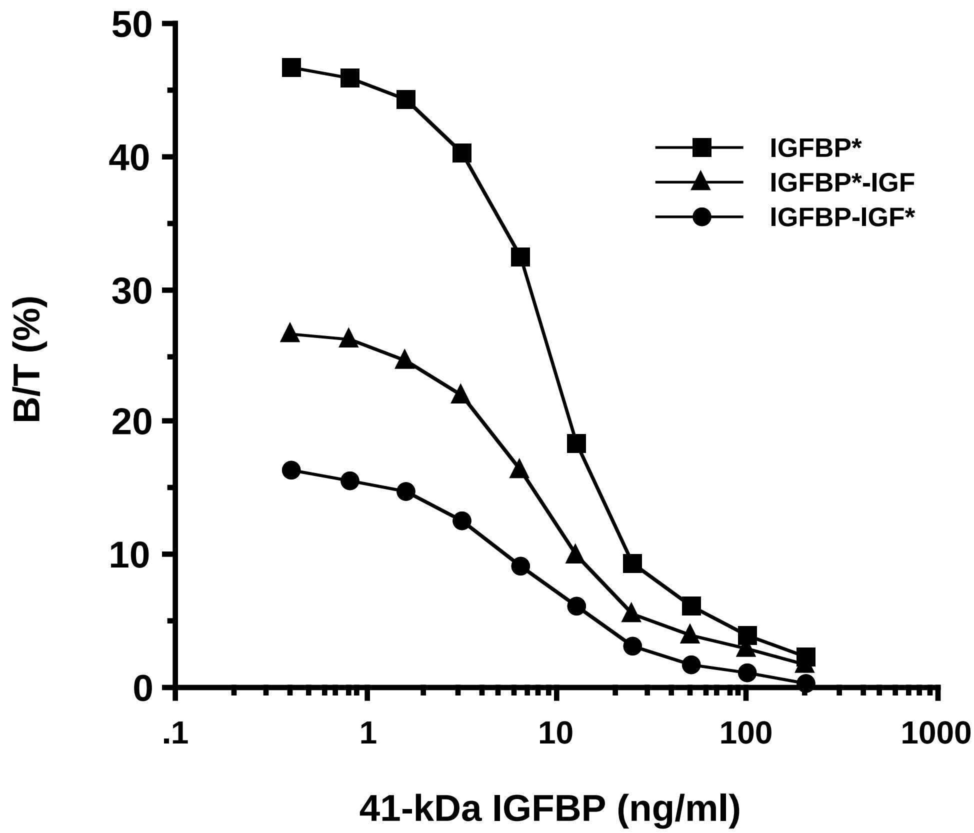
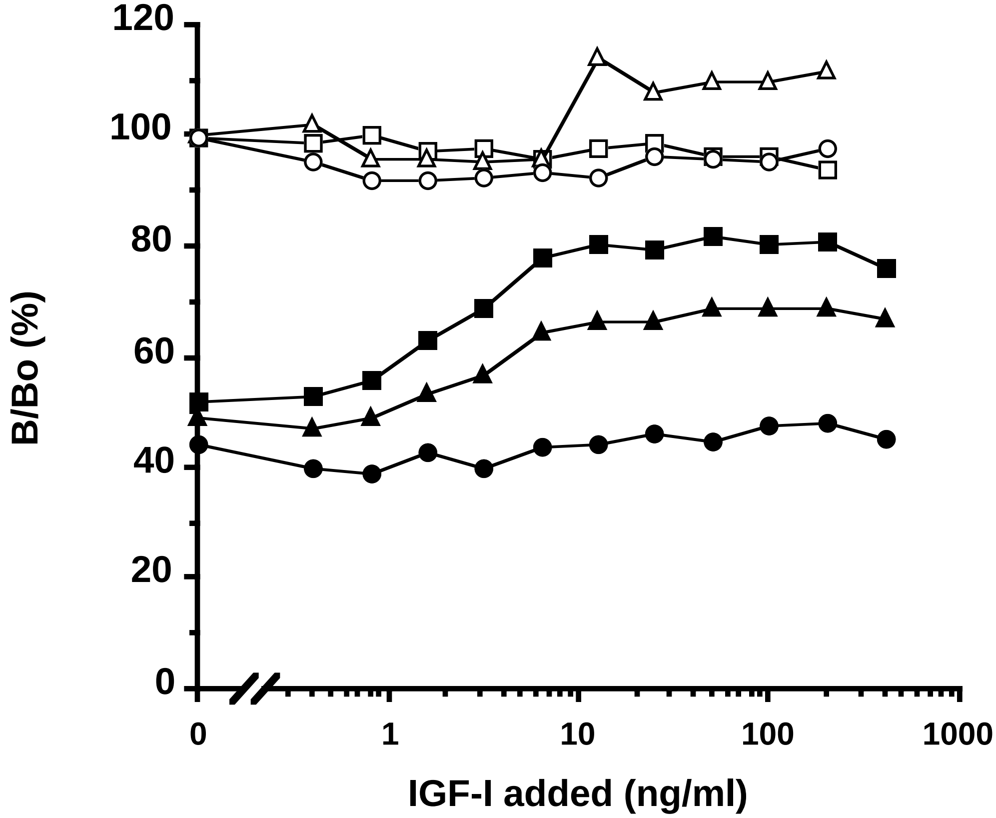

50
40
30
20
10
0
B/T (%)
.1
1
10
100
1000
41-kDa IGFBP (ng/ml)
IGFBP*
IGFBP*-IGF
IGFBP-IGF*

Fig. 3



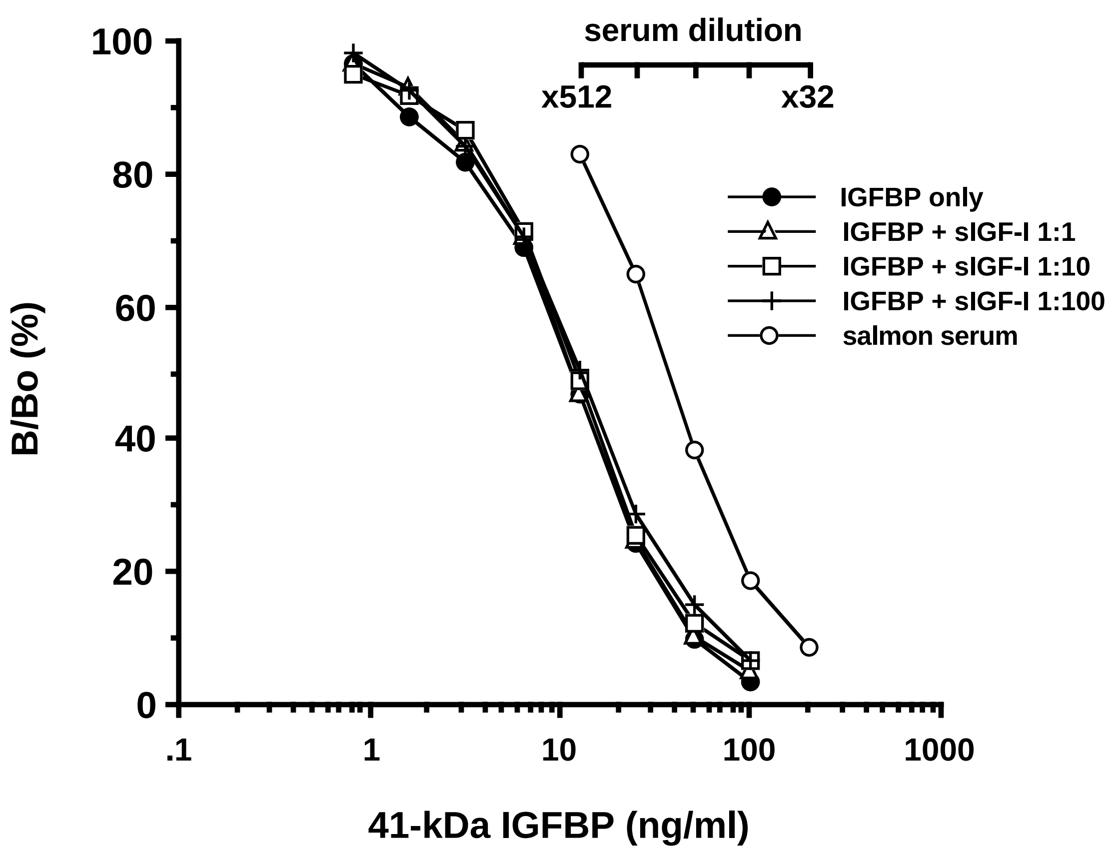
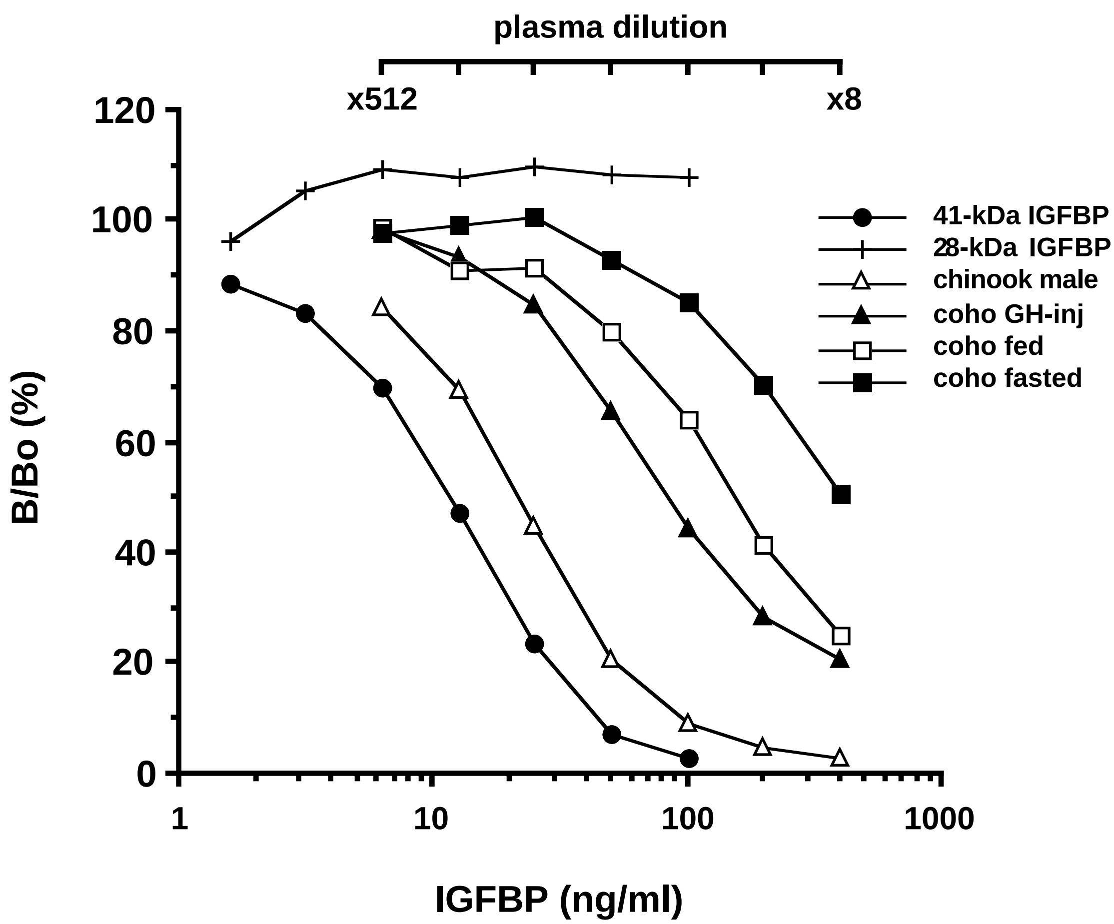

serum dilution
x512
x32
100
80
60
40
20
0
B/Bo (%)
.1
1
10
100
1000
41-kDa IGFBP (ng/ml)
IGFBP only
IGFBP + sIGF-I 1:1
IGFBP + sIGF-I 1:10
IGFBP + sIGF-I 1:100
salmon serum

Fig. 5


plasma dilution
x512
x8
120
100
80
60
40
20
0
B/Bo (%)
1
10
100
1000
IGFBP (ng/ml)
41-kDa IGFBP
28-kDa IGFBP
chinook male
coho GH-inj
coho fed
coho fasted

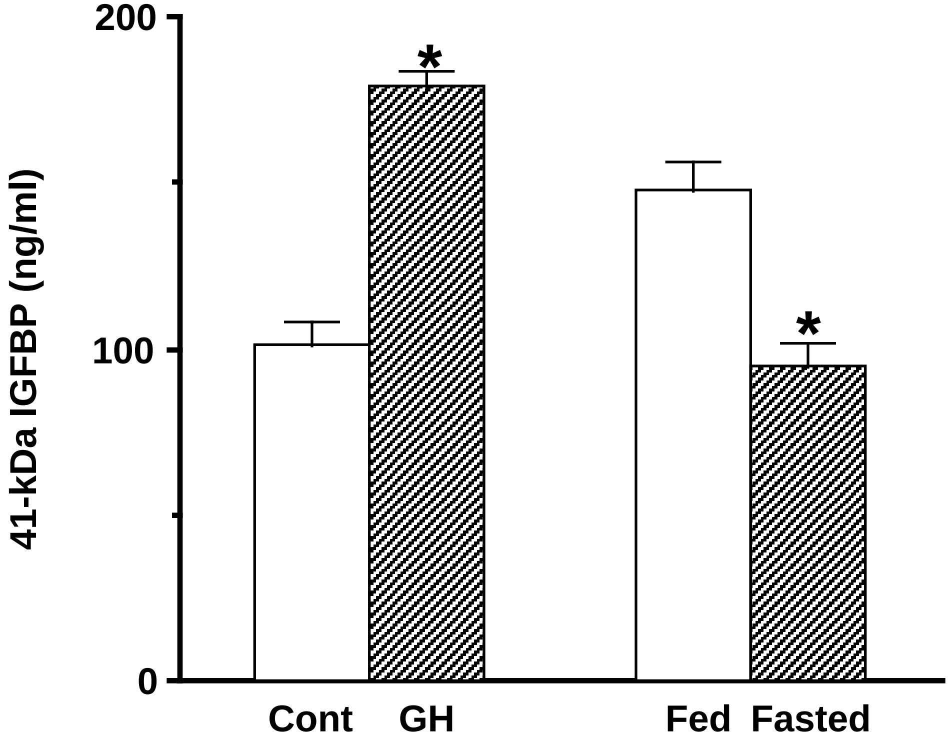
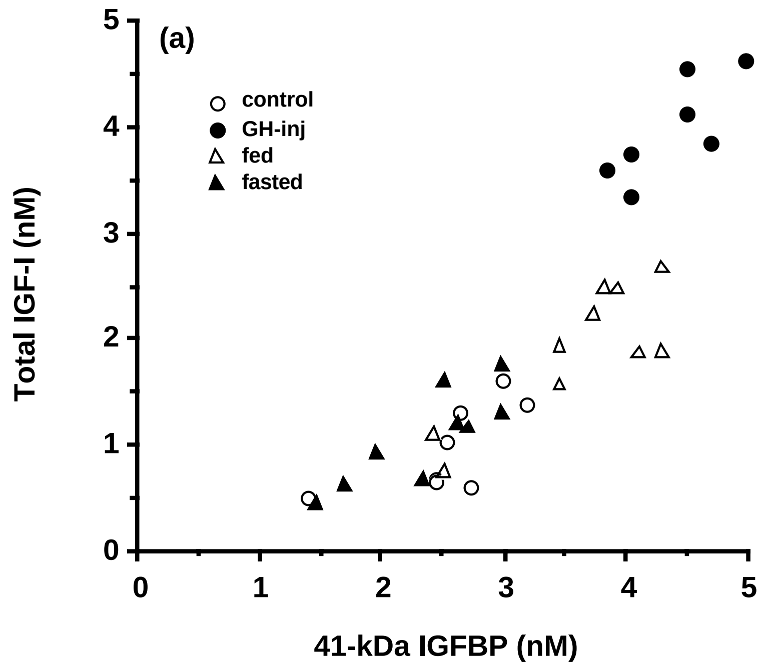

200
100
0
41-kDa IGFBP (ng/ml)
*
*
Cont
GH
Fed
Fasted

Fig. 7


(a)
control
GH-inj
fed
fasted
Total IGF-I (nM)
41-kDa IGFBP (nM)
0
1
2
3
4
5

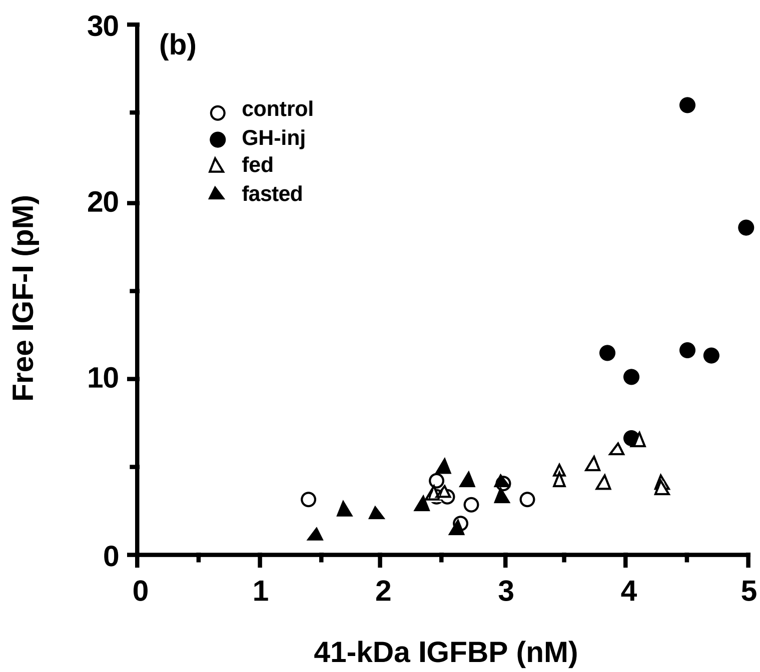
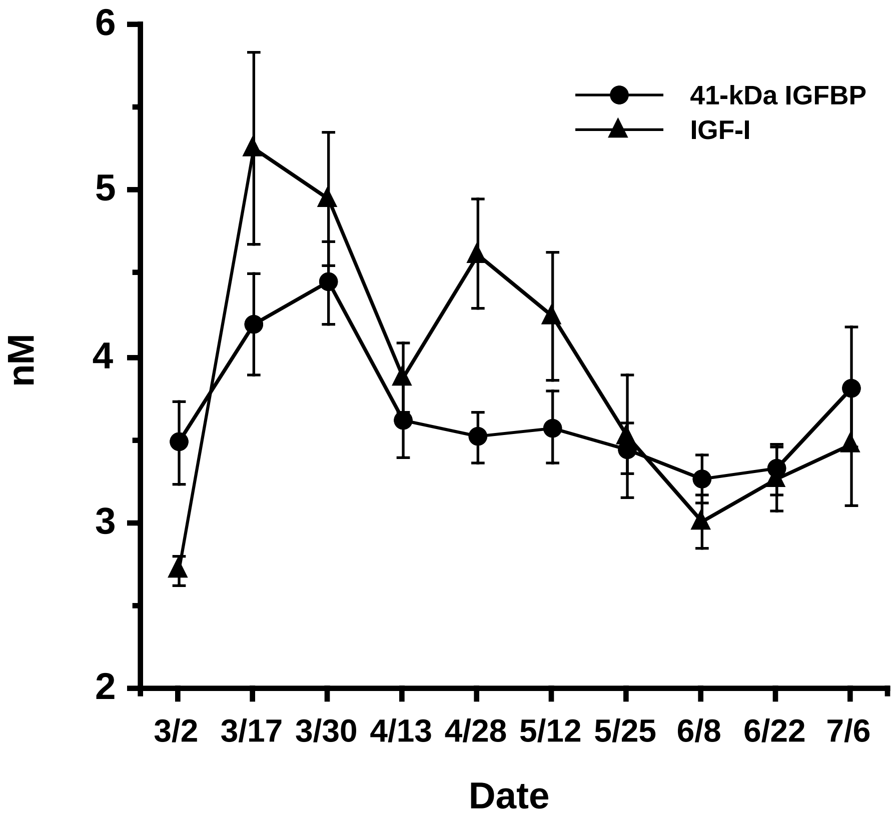

(b)
control
GH-inj
fed
fasted
Free IGF-I (pM)
41-kDa IGFBP (nM)
0
10
20
30
0
1
2
3
4
5

Fig. 8



41-kDa IGFBP
IGF-I
nM
6
5
4
3
2
3/2
3/17
3/30
4/13
4/28
5/12
5/25
6/8
6/22
7/6
Date

Table I Effect of addition of IGFs on measured 41-kDa IGFBP in plasma

Added to plasma	Concentration (ng/ml)	IGFBP (ng/ml)
plasma only		107.9 ± 12.3
salmon IGF-I	1	109.8 ± 13.3
	10	106.9 ± 11.8
	100	108.6 ± 12.6
human IGF-I	1	103.5 ± 11.1
	10	102.0 ± 9.7
	100	108.7 ± 11.7
human IGF-II	1	110.9 ± 13.5
	10	107.6 ± 11.7
	100	104.0 ± 11.0

Data are expressed as mean ± S.E., $n = 5$