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Title:

Salmon serum 22-kDa insulin-like growth factor binding protein (IGFBP) is IGFBP-1

Authors:

M Shimizu, J T Dickey, H Fukada and W W Dickhoff

Affiliations:

Northwest Fisheries Science Center, NOAA Fisheries, 2725 Montlake Boulevard East, Seattle, Washington 98112, USA, and School of Aquatic and Fishery Sciences, University of Washington, Seattle, Washington 98195, USA

Requests for offprints should be addressed to M Shimizu at Northwest Fisheries Science Center, NOAA Fisheries, 2725 Montlake Boulevard East, Seattle, Washington 98112, USA; e-mail: munetaka.shimizu@noaa.gov

Short title:

Cloning of salmon IGFBP-1

Key words:

insulin-like growth factor binding protein, salmon, purification, cloning

Abstract

Western ligand blotting of salmon serum typically reveals three insulin-like growth factor binding proteins (IGFBPs) at 22, 28 and 41 kDa. Physiological regulation of the 22-kDa IGFBP is similar to that of mammalian IGFBP-1; it is increased under catabolic states such as fasting and stress. On the other hand, its molecular weight on Western ligand blotting is closest to mammalian IGFBP-4. The conflict between physiology and molecular weight makes it difficult to conclude the identity of the 22-kDa IGFBP. This study therefore aimed to identify the 22-kDa IGFBP based on protein and cDNA sequences. The 22-kDa IGFBP was purified from Chinook salmon serum by a combination of IGF-affinity chromatography and reverse-phase chromatography. N-terminal amino acid sequence of the purified protein was used to design degenerate primers. Degenerate polymerase chain reaction (PCR) with liver template amplified a partial IGFBP cDNA, and full length cDNA was obtained by 5'- and 3'-rapid amplification of cDNA ends (RACE). The 1915-bp cDNA clone encodes a 23.8 kDa IGFBP and its N-terminal amino acid sequence matched that of purified 22-kDa IGFBP. Sequence comparison with six human IGFBPs revealed that it is most similar to IGFBP-1 (40% identity and 55% similarity). These findings indicate that salmon 22-kDa IGFBP is IGFBP-1. Salmon IGFBP-1 mRNA is predominantly expressed in the liver and its expression levels appear to reflect circulating levels. The 3'-untranslated region of salmon IGFBP-1 mRNA contains four repeats of the nucleotide sequence ATTTA, which is involved in selective mRNA degradation. In contrast, amino acid sequence analysis revealed that salmon IGFBP-1 does not have an Arg-Gly-Asp (RGD) integrin recognition sequence nor a Pro, Glu, Ser and Thr (PEST) rich domain (a segment involved in rapid turnover of protein), both of which are characteristics of mammalian IGFBP-1. These findings suggest that association with the cell surface and turnover rate may differ between salmon and mammalian IGFBP-1.

1 **Introduction**

2 Circulating insulin-like growth factor-I (IGF-I) is bound to a family of six IGF-binding
3 proteins (IGFBPs). Depending on the type of IGFBP and circumstance, they can either inhibit or
4 enhance growth-promoting actions of IGF-I. IGFBP-1 is the first member of the IGFBP family
5 sequenced for complete amino acids (for review, Shimasaki & Ling, 1991). IGFBP-1 has 12 and 6
6 cysteines in the N- and C-termini, respectively, which are well conserved among six IGFBPs and
7 responsible for the high-affinity IGF binding (for review, Shimasaki & Ling, 1991). The molecular
8 weight of human IGFBP-1 predicted from cDNA is 25 kDa and it appears as a 30-kDa band on
9 Western ligand blotting using labeled-IGF-I (Brinkman *et al.*, 1988). IGFBP-1 is one of the best-
10 characterized IGFBPs (for reviews, Lee *et al.*, 1993, 1997). IGFBP-1 usually has an inhibitory
11 effect on IGF-I action and increases under catabolic states. Unlike other IGFBPs, circulating
12 IGFBP-1 changes rapidly and shows diurnal rhythms according to food intake. This is because
13 insulin is the predominant inhibitor of IGFBP-1 transcription (Orlowski *et al.*, 1991; Powell *et al.*,
14 1991). Circulating IGFBP-1 and insulin levels therefore show an inverse relationship. Another
15 important regulator of IGFBP-1 is cortisol. Cortisol stimulates transcription of IGFBP-1 mRNA,
16 but its effect is secondary to the inhibitory effect of insulin (Unterman *et al.*, 1991). Since most
17 IGFBP-1 is unsaturated with IGFs and can bind free IGF-I, it is believed to regulate free IGF-I
18 levels (Frystyk *et al.*, 1997). These facts suggest that IGFBP-1 plays a crucial role in regulating
19 metabolic actions of circulating IGF-I.

20 IGFBP-1 is also present in fish based on molecular studies. Complete and partial
21 sequences for IGFBP-1 have been obtained in zebrafish (*Danio rerio*; Maures and Duan, 2002)
22 and longjaw mudsucker (goby) (*Gillichthys mirabilis*; Gracey *et al.*, 2001), respectively.
23 Candidates for fish IGFBP-1 protein have also been detected in the circulation by Western ligand
24 blotting. Fish plasma/serum typically reveals three IGFBP bands at 20-25 kDa, 28-30 kDa and 40-
25 50 kDa. The 40-50 kDa form may be fish IGFBP-3 since it has a molecular weight similar to
26 mammalian IGFBP-3 and is increased by growth hormone injection (Siharath *et al.*, 1995; Shimizu

1 *et al.*, 2003). The two smaller fish IGFBPs at 20-25 kDa and 28-30 kDa are candidates for
2 IGFBP-1 based on the fact that both forms are increased under catabolic states such as fasting,
3 stress and diabetes (Kelley *et al.*, 2001, 2002). Hormonal control of these lower molecular weight
4 forms in fish are similar to those of human IGFBP-1. In tilapia (*Oreochromis mossambicus*),
5 exogenously administrated cortisol increases serum levels of 24- and 28-kDa IGFBPs (Kajimura *et*
6 *al.*, 2003). In the goby, the 24- and 30-kDa forms are suppressed by insulin treatment (Kelley *et*
7 *al.*, 2001). Kelley *et al.* (2001) pointed out that IGFBP sensitivities to various catabolic states differ
8 depending on species and/or conditions. In striped bass (*Morone saxatilis*), the circulating level of
9 25-kDa IGFBP was increased after 60 days of fasting and was reduced below the detection limit
10 after 14 days of fasting, whereas the 30-kDa form was barely detected throughout the experiment
11 (Siharath *et al.*, 1996). In goby serum, the 24-kDa IGFBP has a greater sensitivity to fasting and
12 diabetes than the 30-kDa form (Kelley *et al.*, 2001). In contrast, similar IGFBPs are present in jack
13 mackerel (*Trachurus symmetricus*) serum, but the larger (28-kDa) form is more sensitive to stress
14 (Kelley *et al.*, 2001). Comparing all fish studies to date, it appears that the smallest (20-25-kDa)
15 form generally has a greater sensitivity to nutritional status and hormone treatments, suggesting that
16 it is the physiological equivalent to mammalian IGFBP-1. However, the fish 28-30 kDa IGFBP is
17 closer to human IGFBP-1 in size. Bauchat *et al.* (2002) purified rainbow trout (*Oncorhynchus*
18 *mykiss*) 30-kDa IGFBP for the first time and sequenced the N-terminus. The partial N-terminal
19 amino acid sequence was most similar to human IGFBP-1. These facts suggest that the fish 28-30-
20 kDa form is structurally similar to human IGFBP-1. On the other hand, 20-25 kDa IGFBP is
21 closest to human IGFBP-4 in size. Since the fish 20-25 kDa IGFBP has not been purified or
22 sequenced in any fish species, it is not known at present which of the two low molecular weight fish
23 forms is a homolog of human IGFBP-1. Moreover, N-terminal amino acid sequences are not
24 necessarily reliable when comparing IGFBPs as this region is highly conserved and contains the
25 binding domain. To establish homologies between fish and mammalian IGFBPs, complete amino
26 acid sequence data are desirable.

As in other fish species, Chinook salmon (*Oncorhynchus tshawytscha*) serum contains at least three IGFBPs at 22, 28 and 41-kDa (Shimizu *et al.*, 2000). In this study, we purified salmon 22-kDa IGFBP from serum, sequenced the N-terminus in order to design degenerate primers and cloned its cDNA by polymerase chain reaction (PCR). Comparison of the complete amino acid sequence with human IGFBPs revealed that the salmon 22-kDa form is homologous to IGFBP-1.

Materials and methods

Fish

One-year-old Chinook salmon (*Oncorhynchus tshawytscha*) were reared in fresh water at 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 to 13°C. Fish were fed standard rations (0.6-1.0% body weight/day). From October, 1998, fish were either fed or fasted for six weeks. 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.

Under-yearling Chinook salmon were reared under the same conditions as described above. In October, fish were sorted into two groups by size. The large and small group were considered to be smolt and parr, respectively, which are two phenotypes differing in seawater-adaptability. The smolt has high osmoregulatory ability whereas the parr does not. The small group (parr) was directly transferred to either full-seawater (30 ppt) or a fresh water tank. Blood was collected 12 hr after transfer.

All experiments were carried out in accordance with the guidelines of the University of Washington Institutional Animal Care and Use Committee.

Purification of 22-kDa IGFBP

Serum was collected from spawning male Chinook salmon in the adult return pond on the

University of Washington campus, Seattle, WA, in October and November, 2000. Fish were anesthetized in MS-222. 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 stored at -80°C until use. Serum batches of 600 ml were mixed with an equal volume of 0.02 M phosphate-buffered saline (PBS), pH 7.4 and ammonium sulfate was added to make a 45% saturated solution. The sample was stirred for 30 min at room temperature and centrifuged at 1350 *g* for 20 min. The precipitate was collected, dissolved in 300 ml PBS, pH 6.5 and dialyzed against PBS, pH6.5 overnight. The sample was filtered through a filter paper, loaded onto an Affi-Gel 10 (Bio-Rad, Hercules, CA) column (1.0 x 7 cm) that had been covalently bound to 2 mg recombinant human IGF-I and run through the column at a flow rate of 36 ml/hr at 4°C. After washing with 800 ml PBS, pH 6.5, IGFBP was eluted from the column with 0.5 M acetic acid at 16 ml/hr. Eluted fractions were collected into pre-lubricated microcentrifuge tubes (PGC Scientifics, Frederick, MD). Fractions containing 22-kDa IGFBP (based on Western ligand blot) were pooled and applied directly to reversed-phase high pressure liquid chromatography (HPLC) using a Vydac C-4 column (0.46 x 5 cm; Separation Group, Hesperia, CA) equilibrated with 0.1% trifluoroacetic acid (TFA). IGFBPs were eluted by a linear gradient of 18-42% acetonitrile in 0.1% TFA at a flow rate of 48 ml/h. Acetonitrile and TFA in the fractions were removed by N₂ evaporation. The protein concentration of purified IGFBP was determined using the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

Electrophoresis and Western ligand blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel and 12.5% or 10% separating gel was carried out according to Laemmli (1970). Samples 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. Gels were stained with 0.1% Coomassie brilliant blue R250 (CBB; Bio-Rad). Estimation of molecular weight was performed using the Precision marker (Bio-Rad).

Western ligand blotting using digoxigenin-labeled 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 (Roche, Indianapolis, IN) at a dilution of 1:2500 for 1 hr at room temperature. IGFBP was visualized on autoradiography film by use of the enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Life Science Inc., Arlington Heights, IL).

N-terminal amino acid sequence

Approximately 4 µg of purified 22-kDa IGFBP was separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was stained with CBB and IGFBP bands were cut from the membrane. Excised bands were sequenced for N-terminal amino acids at Midwest Analytical (St. Louis, MO).

Total RNA isolation and first strand cDNA synthesis

Liver was collected from one-year-old Chinook salmon that had been transferred from fresh water to seawater. The fish were classified as parr based on their size and morphological appearance. Dissected liver was immediately frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted from the liver using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocol. First strand cDNA was synthesized from 7 µg total RNA using SuperScript™ II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA) and Oligo (dT)₁₂₋₁₈ Primer (Invitrogen).

Cloning of 22-kDa IGFBP

Forward degenerate primers (5' CAR GAR CCH ATH MGI TGY GCH CCH TG 3' where I indicates inosine; R = A or G, H = A, C or T, M = A or C, Y = C or T) were designed from the N-terminal amino acid sequence of purified 22-kDa IGFBP. The sequences of reverse primers (5' TGY CCR TAY TTR TCC ACR CAC CAG CA 3') were from the C-terminal region originally used for the cloning of tilapia IGFBP-3 cDNA (Cheng *et al.*, 2002). All gene specific primers used in the present study were synthesized by Bio-Synthesis, Inc. (Lewisville, TX). PCR was performed using a Perkin Elmer Gene Amp Thermal Cycler (Perkin Elmer Cetus, Foster City, CA) and components from Novagen (Madison, WI). PCR cycles consisted of 1 cycle of 94°C for 3 min; 36 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 1 min 30 sec; 1 cycle of 72°C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis. cDNA bands were excised from the gel and purified using the QIAEX II Gel Extraction Kit (Qiagen Inc., Valencia, CA). The purified cDNAs were blunt-end and ligated into the pSTBlue-1 Blunt Vector using the Perfectly Blunt Cloning Kits (Novagen). The vectors were then transformed into the NovaBlue Singles Competent Cells (EMI Biosciences) and spread on Luria-Bertani (LB) agar plates containing 40 µg/ml X-gal, 100 µM isopropylthiogalactoside (IPTG) and 50 µg/ml ampicillin. After overnight incubation at 37°C, positive clones were selected and grown in LB medium containing ampicillin. Plasmid DNA was purified using the QIAprep Miniprep Kits (Qiagen). Purified plasmid (200 ng) was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with T7 and SP6 promoter primers (Novagen) and run on ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Full-length cDNA was obtained by 5'- and 3'-rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA). Gene specific primers were designed from the sequence of the partial cDNA (forward primer, 5' GGG TGC CAG ACC ACA GCT CCC TAG CC 3'; reverse primer, 5' GCA GCT CAA TGT GGC AGG GAC CCT G 3'). An additional forward primer (5'CGT ATG TGC TTA CAA GTA TGG ACC ATT 3') was designed from the sequence of the 3'-RACE product to obtain the entire 3'-

untranslated region (UTR). Cloning and sequencing protocols were as described above.

Amino acid sequences of IGFBPs were aligned by the ClustalW method.

Expression of 22-kDa IGFBP

Several tissues (brain, pituitary, gill, heart, head kidney, liver, muscle, testis and ovary) were collected from three one-year-old coho salmon (*Oncorhynchus kisutch*). Liver samples were also collected from one-year-old Chinook salmon transferred to seawater or fresh water as described above. Total RNA was extracted from the tissues and reverse-transcribed as described above.

First-strand cDNA was used as template for reverse transcriptase (RT)-PCR. The primer pair used for amplification of 22-kDa IGFBP was as follows: forward primer, 5' GAG ATG CTT GGA TTA TAT AAG AAG TTG 3'; reverse primer, 5' TTA GAG CTC CAG CTG GCA CT 3'. Salmon β -actin was used as an internal control. The primer sequences for β -actin were as follows: forward primer, 5' CCT GAC CCT GAA GTA CCC CA 3'; reverse primer, 5' CGT CAT GCA GCT CAT AGC TC 3'. Conditions for PCR were essentially the same as described above except that the template dilution and PCR cycles were optimized for each primer set to allow comparison of relative expression levels.

Results

The response of circulating IGFBPs to fasting in one-year-old Chinook salmon was assessed by Western ligand blotting (Fig. 1a). After six weeks of fasting, the 22-kDa IGFBP band was more intense in the majority of fasted than in fed fish. When Chinook salmon parr were transferred directly to seawater, all fish in seawater exhibited an increase in the 22-kDa form 12 hr after transfer (Fig. 1b). In some fish in seawater, induction of the 28-kDa IGFBP was evident.

The 22-kDa IGFBP was purified from serum of spawning male Chinook salmon. Salmon serum was fractionated by ammonium sulfate, run through IGF-affinity chromatography and subjected to reverse-phase chromatography on HPLC (Fig. 2). The 22-kDa form was eluted at

37% acetonitrile from C-4 column as assessed by ligand blotting (data not shown). The yield of purified 22-kDa IGFBP from 600 ml of serum was 27 µg. Purified 22-kDa IGFBP appeared as a single band at 22-kDa on SDS-PAGE under non-reducing conditions, while other minor bands were also observed at higher molecular weight regions on ligand blotting (Fig. 2).

Partial N-terminal amino acid sequence of purified 22-kDa IGFBP was compared with other IGFBPs (Fig. 3). It was similar to zebrafish IGFBP-1 and trout 30-kDa IGFBP. The partial sequence showed some homology with human IGFBPs.

In order to obtain complete amino acid sequence of 22-kDa IGFBP, degenerate primers were designed from the N-terminal amino acid sequence. Degenerate PCR amplified several bands and one of the bands encoded IGFBP (data not shown). The partial cDNA was extended for both ends by 5'- and 3'-RACE and a full length of cDNA was obtained (Fig. 4). The sequence reported in this paper has been deposited in the GenBank data base (Accession No. AY662657). The cDNA was 1915 bp long consisting of 182 bp of 5'-UTR, 735 bp of open reading frame and 998 bp of 3'-UTR. A polyadenylation signal AATAAA was found upstream of the poly (A) tail (Fig. 4). The 3'-UTR contained four repeats of the ATTTA sequence, which is characteristic of mRNA with a short half-life. The N-terminal amino acid sequence deduced from the cDNA matched exactly with that of purified 22-kDa IGFBP, indicating it encodes the 22-kDa IGFBP. The predicted molecular weight of the mature protein was 23.8 kDa. The deduced 22-kDa IGFBP contained 12 cysteines and 6 cysteines in the N- and C-termini, respectively (Fig. 5), that are conserved in mammalian IGFBP-1 through -5. The amino acid sequence of 22-kDa form was compared with human IGFBPs and found to be most similar to IGFBP-1, being 40 % identical (Table 1). It also showed relatively high homology with human IGFBP-4 (37%) compared to other forms. These sequence comparisons indicate that salmon serum 22-kDa IGFBP is a homolog of mammalian IGFBP-1. Salmon 22-kDa IGFBP has high sequence homology with zebrafish IGFBP-1 (61%). Salmon 22-kDa IGFBP does not have an Arg-Gly-Asp (RGD) integrin recognition sequence found in the C-terminus of all mammalian IGFBP-1, nor a Pro-Glu-Ser-Thr (PEST) rich domain in

the mid region, which is characteristic of rapidly metabolized proteins (Fig. 5).

Gene expression of 22-kDa IGFBP in various tissues from one-year-old coho salmon was assessed by RT-PCR (Fig. 6). The 22-kDa form was mainly expressed in the liver and relatively low expression was observed in muscle. Expression of 22-kDa form was not evident in other tissues (Fig. 6) including ovary from one-year-old coho salmon (data not shown). Relative gene expression of 22-kDa IGFBP by RT-PCR was compared with the intensity of plasma 22-kDa band on Western ligand blotting of the same individuals (Fig. 7). Similar patterns of 22-kDa IGFBP mRNA and plasma levels were observed.

Discussion

Most studies of fish IGFBPs use Western ligand blotting to identify circulating forms or molecular studies of mRNA expression. No previously published study has linked the variously-sized circulating fish IGFBPs with identified expressed sequences in tissues. The aim of this study was to identify the smallest molecular weight circulating salmon IGFBP and establish its homology with a known member of the vertebrate IGFBP family.

Mammalian IGFBP-1 is known to respond to food intake; its level increases after fasting and decreases after re-feeding. When Chinook salmon were fasted for six weeks, most of the fasted fish had higher plasma 22-kDa IGFBP levels than did fed fish. An increase in circulating 20-25-kDa IGFBP after fasting is consistent with other fish species (Siharath *et al.*, 1996; Kelley *et al.*, 2001; Peterson *et al.*, 2004). This is a major reason why the fish 20-25-kDa form is considered to be IGFBP-1. The 22-kDa IGFBP was also increased by direct transfer of Chinook salmon parr to full-seawater. The increase in plasma 22-kDa form is presumably due to a stress-associated increase in cortisol, caused by salinity change rather than the result of seawater adaptation since some small fish died after transfer. In contrast, none of the large fish (smolt) died and only a small increase in the 22-kDa form in plasma was seen (M. Shimizu and WW Dickhoff, unpublished data), which might be due to an increase in cortisol associated with seawater adaptation. Response

1 of the 20-25-kDa IGFBP under “stressful conditions” has been reported in other fish species.
2 When tilapia was hypophysectomized, a 20-kDa form appeared in plasma (Park *et al.*, 2000).
3 Although cortisol levels were not measured in the present study nor in the study by Park *et al.*
4 (2000), high levels of cortisol could be expected in these conditions. In tilapia, cortisol injection
5 resulted in an induction of the 24-kDa IGFBP (Kajimura *et al.*, 2003). These results support our
6 assumption that salmon 22-kDa IGFBP is physiologically IGFBP-1.

7 In order to obtain partial N-terminal amino acid sequence, salmon 22-kDa IGFBP was
8 purified from serum for the first time. For the purification of IGFBP, serum is usually acidified,
9 run through IGF-affinity column and further separated by reverse-phase chromatography.
10 Acidification of serum is essential to separate endogenous IGF from IGFBP. However, our
11 preliminary experiments suggested that salmon 22-kDa form is acid-labile and unsaturated with
12 endogenous IGFs (data not shown). For these reasons, salmon serum that had been fractionated by
13 ammonium sulfate was loaded onto the IGF-affinity column without acidification. As we expected,
14 most 28- and 41-kDa IGFBP passed through the column and the 22-kDa form was the major
15 binding protein bound and subsequently eluted from the column. Salmon 22-kDa IGFBP was
16 further purified by reverse-phase HPLC. This step usually yields two main peaks and both contain
17 the 22-kDa form. These two peaks may represent different phosphorylated forms of 22-kDa
18 IGFBP as is the case for human IGFBP-1 (Busby *et al.*, 1988), although we have no data on
19 phosphorus content in the fractions from salmon serum. Since band patterns of the two fractions
20 were essentially same, the two fractions were pooled and used for further analysis.

21 CBB staining of purified 22-kDa IGFBP on SDS-PAGE revealed a single band at 22 kDa
22 whereas some other minor bands were also detected on Western ligand blotting. Some bands
23 migrated at the same position as 41-kDa IGFBP. They are presumably aggregates of the 22-kDa
24 form rather than the contamination of 41-kDa form since those bands were not immunoreactive to
25 antiserum against salmon 41-kDa form (M Shimizu, A Hara and WW Dickhoff, unpublished data).
26 Although there may be slight contamination with other IGFBPs, the 22-kDa IGFBP fraction was

1 pure enough to obtain partial N-terminal amino acid sequence.

2 Degenerate primer was designed from the N-terminal amino acid sequence. A 1915-bp
3 cDNA encoding the 22-kDa IGFBP was obtained by degenerate PCR followed by RACE. The
4 complete amino acid sequence of the 22-kDa form was most similar to that of IGFBP-1 among six
5 human IGFBPs. The salmon 22-kDa form is unlikely to be IGFBP-4, because there are partial
6 sequences of IGFBPs that show higher identity to human IGFBP-4 in the fugu and zebrafish
7 genome and trout EST databases. These results demonstrate that the cDNA for 22-kDa IGFBP
8 encodes salmon IGFBP-1. This is the first report of the identification of the type of a circulating
9 IGFBP in teleosts.

10 Despite limited numbers of fish IGFBP sequence available at present, it appears that fish
11 IGFBPs are structurally similar to mammalian forms. This is true for salmon IGFBP-1; it has the
12 18 cysteines and its sequence homology with human IGFBP-1 is high in N- and C-termini (46%)
13 and low in the mid region (28%). However, some dissimilarity is seen. For instance, salmon
14 IGFBP-1 does not have the Arg-Gly-Asp (RGD) sequence in C-terminus, which is present in all
15 mammalian counterparts. The RGD sequence is important for localization of proteins on the cell
16 surface through interacting with $\alpha 5 \beta 1$ integrin (Ruoslahti and Piersbacher, 1987). The lack of
17 RGD sequence has been reported for zebrafish IGFBP-1 (Maures and Duan, 2002). As discussed
18 in Maures and Duan (2002), acquisition of RGD sequence to IGFBP-1 might have occurred after
19 divergence of teleosts from other vertebrates.

20 Unlike other IGFBPs, circulating human IGFBP-1 rapidly changes in response to
21 nutritional and physiological status. The rapid change can be explained, at least in part, by its
22 structure. Human IGFBP-1 mRNA contains AT-rich regions in 3'-UTR characterized by repeats
23 of the nucleotide sequence ATTTA (Luthman *et al.*, 1989). This sequence is involved in selective
24 mRNA degradation (Shaw and Kamen, 1986). Salmon IGFBP-1 mRNA has four repeats of the
25 sequence, suggesting that its mRNA is unstable as is mammalian IGFBP-1. Another factor
26 controlling the rapid change of IGFBP-1 is its turnover rate in tissues. Human IGFBP-1 has a Pro

(P), Glu (E), Ser (S) and Thr (T) rich segment in the mid region (Julkunen *et al.*, 1988). This region is called the PEST domain, and includes clusters of these four amino acids flanked on each side by a positively charged amino acid. A PEST domain is characteristic of rapidly metabolized proteins such as *c-fos*, *c-myc*, heat-shock proteins and ornithine decarboxylase (Rogers *et al.*, 1986). However, no such sequence is found in salmon IGFBP-1. Moreover, when the amino acid sequence of salmon IGFBP-1 was aligned with that of human IGFBP-1, a relatively large gap was introduced in the mid region corresponding to the PEST region. Based on these structural differences, salmon IGFBP-1 mRNA is presumably as unstable as mammalian counterpart but its protein turnover rate in tissues may differ.

Phosphorylation of human IGFBP-1 has been shown to affect binding affinity to IGF-I (Busby *et al.*, 1988). Rat IGFBP-1, on the other hand, is also phosphorylated, but phosphorylation does not affect IGF-binding (Peterkofsky *et al.*, 1998). Although the biological significance of the phosphorylation may differ among species, it is a common feature of mammalian IGFBP-1. In mammals, main sites of phosphorylation are serines in the mid region and their consensus sequence is Ser-X-Glu. Salmon IGFBP-1 does not contain this consensus sequence, suggesting that it may not be phosphorylated, while two discrete peaks of 22-kDa IGFBP on HPLC may represent differently phosphorylated forms. Analysis of phosphorylation of salmon IGFBP-1 awaits future study.

The tissue distribution of the 22-kDa IGFBP was assessed by RT-PCR. It was expressed primarily in the liver in one-year-old coho salmon. Muscle was also found to express 22-kDa IGFBP weakly, but its expression was not consistent when other individuals were examined (data not shown). This agrees with the finding in mammals that liver is one of the main sites of IGFBP-1 expression (Lee *et al.*, 1993). Other tissues expressing IGFBP-1 in mammals are uterine decidua and kidney. In zebrafish, kidney/stomach expresses IGFBP-1 weakly (Maures and Duan, 2002), but this is not seen in coho salmon. Inconsistency in the expression in tissues other than liver may depend on species, stage and/or physiological condition. Expression of fish IGFBP-1 is influenced

1 by fasting and hypoxia (Gracey *et al.*, 2001; Maures and Duan, 2002). In the present study, we
2 found that expression of 22-kDa IGFBP in the liver was influenced by possible stress during direct
3 seawater transfer. A notable finding is that the expression level of the 22-kDa form appears to
4 correlate well with plasma level in each individual. This implies that circulating 22-kDa IGFBP
5 levels may be regulated primarily at the level of transcription.

6 The present study shows that the 22-kDa IGFBP is salmon IGFBP-1. This raises another
7 question: What is the identity of the 28-kDa IGFBP? It is worth noting that 10 of 15 amino acid
8 residues in the N-terminus are identical between salmon 22-kDa IGFBP and trout 30-kDa IGFBP
9 despite the difference in their molecular weights. It is possible that the salmon/trout 28-30-kDa
10 IGFBP is an alternate form of IGFBP-1. However, taking account for the close structural
11 relationship between mammalian IGFBP-1 and -4, another possibility is that the salmon/trout 28-
12 30-kDa form is homologous to IGFBP-4. Identity of the 28-kDa IGFBP remains to be
13 demonstrated by cDNA cloning in future studies.

14 In conclusion, we purified salmon 22-kDa IGFBP from serum and cloned its cDNA using
15 N-terminal amino acid sequence information. Comparison of complete amino acid sequence with
16 human IGFBPs revealed that the 22-kDa IGFBP is salmon IGFBP-1.

18 **Acknowledgments**

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Figure legends

Fig. 1. Western ligand blotting using digoxigenin-labeled human IGF-I of plasma from Chinook salmon. Fish were fed or fasted for six weeks (a), or transferred to fresh water or seawater for 12 hr (b). Normal human serum (NHS; 1 μ l) and salmon plasma (2 μ l) were separated by 12.5% SDS-PAGE under non-reducing conditions and blotted onto nitrocellulose membranes. The membranes were incubated with digoxigenin (DIG)-labeled hIGF-I for 2 hr and then with antibody against DIG at a dilution of 1:2500 for 1 hr. Bands were visualized on X-ray film using the enhanced chemiluminescence reagents. Arrowheads indicate migration positions of salmon IGFBPs with molecular weight (kDa) or non-specific band (NS).

Fig. 2. Elution profile of salmon IGFBP on reverse-phase HPLC. Salmon IGFBP recovered from IGF-I affinity chromatography was applied to a Vydac C-4 column and eluted by a linear gradient of 18-42% acetonitrile in 0.1% TFA. Shaded area indicates fractions containing 22-kDa IGFBP. Inset: SDS-PAGE (1) and Western ligand blotting (2) of purified 22-kDa IGFBP. (1) Two micrograms of purified 22-kDa IGFBP were separated by 10% SDS-PAGE under non-reducing conditions and stained with CBB. (2) Ten nanograms of purified protein were separated by SDS-PAGE, blotted onto nitrocellulose membrane and probed with digoxigenin-labeled human IGF-I. Arrowhead indicates the migration position of purified 22-kDa IGFBP.

Fig. 3. Comparison of partial N-terminal amino acid sequence of salmon 22-kDa IGFBP with those of human and fish IGFBPs. Amino acid sequence data of human IGFBP-1 and -4, zebrafish IGFBP-1 and trout 30-kDa IGFBP were obtained from Póvoa *et al.* (1984), Shimasaki *et al.* (1990), Maures and Duan (2002) and Bauchat *et al.* (2001), respectively. Amino acid residues identical to those of salmon 22-kDa IGFBP are shaded.

Fig. 4. Nucleotide and deduced amino acid sequences of salmon 22-kDa IGFBP. The nucleotides are numbered at the left. Amino acid number starting from the N-terminus of the purified protein (+1) is shown under amino acid residues. The ATTTA sequence involved in mRNA stability and the AATAAA polyadenylation sequence are underlined. The GenBank database accession number is AY662657.

Fig. 5. Comparison of amino acid sequence of salmon 22-kDa IGFBP with those of mammalian and fish IGFBP-1. Amino acid sequence of human (Accession No. P08833), bovine (P24591), rat (A36082), mouse (NP_032367) and zebrafish (AAM83038) IGFBP-1 were obtained from GenBank. They were aligned by the ClustalW method. Consensus amino acid residues are boxed and the cystein residues conserved in the IGFBP family are asterisked. The position of the Arg-Gly-Asp (RGD) integrin recognition sequence conserved in mammalian IGFBP-1, is underlined.

Fig. 6. Tissue distribution of salmon 22-kDa IGFBP expression. Tissues were collected from one-year-old male coho salmon. A representative result from one of three individuals was shown in the figure. First strand cDNA reverse-transcribed from total RNA was used as template of RT-PCR. Salmon β -actin was used as internal control.

Fig. 7. Comparison of band intensities of 22-kDa IGFBP between Western ligand blotting of plasma and RT-PCR of liver samples. Smolt, precociously maturing male (preco) and parr were transferred directly to full-seawater (SW) or fresh water (FW). Blood and liver samples were analyzed by Western ligand blotting using digoxigenin-labeled IGF-I and RT-PCR using gene specific primers for 22-kDa IGFBP or β -actin.

Fig. 1

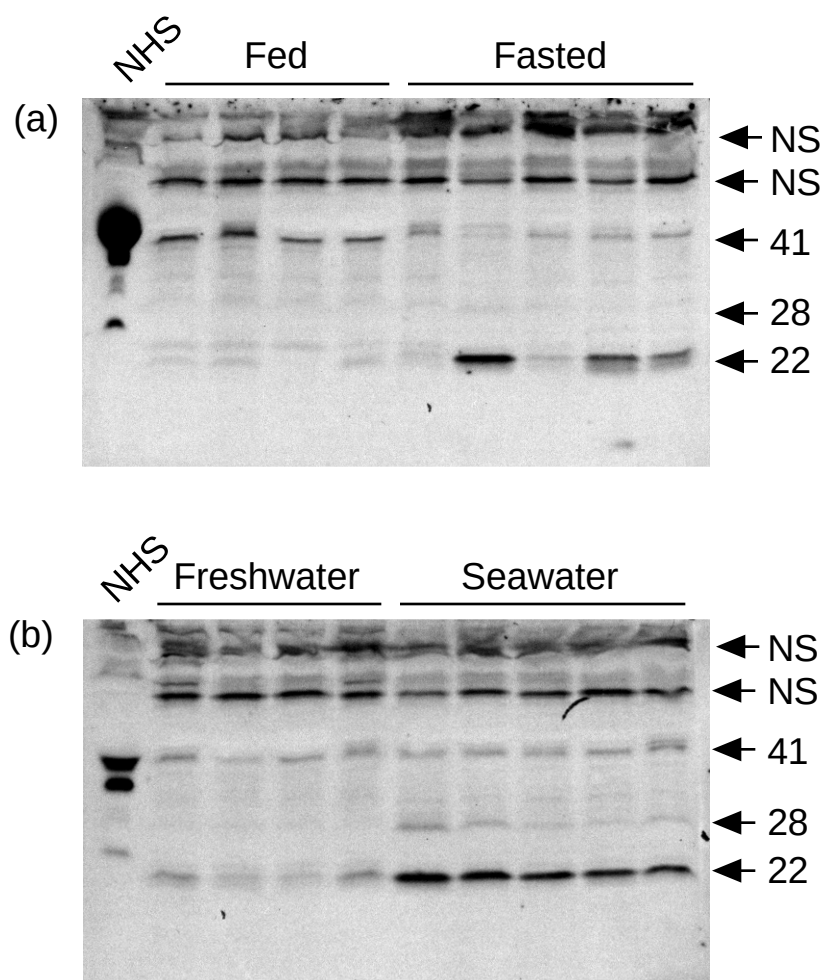


Fig. 2

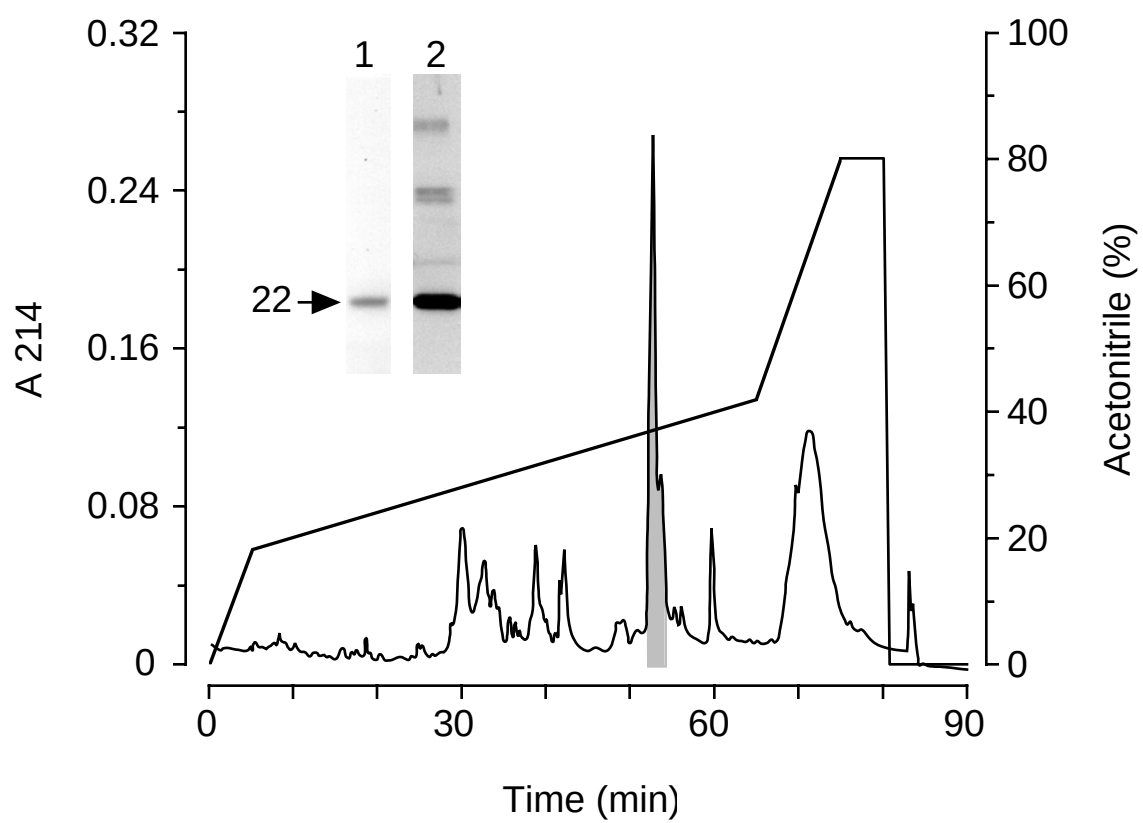


Fig. 3

human IGFBP-1								A	P	W	Q	C	A	P	C	S	
human IGFBP-4								D	E	A	I	H	C	P	P	C	S
zebrafish IGFBP-1	S	P	V	V	G	Q	E	P	I	R	C	A	P	C	S		
trout 30-kDa IGFBP	K	P	V	L	A	Q	E	P	I	R	C	A	P	C	S		
salmon 22-kDa IGFBP	S	P	V	V	G	P	E	P	I	R	C	A	P	C	T		

Fig. 4

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1 GAATTCAGTTCAGAAAGAAAGAAGGAAACCTATAGATGCCTATCTACAGTATATTGTGCTTCTCATAAGTACTTGAGGAC
81 TAAGCAAAACTACACTTGAAGATTAAGACTGCACCTTGAAGAAAATTCACATCTACACCTGACTGTAGATTTTAGTGTC
161 TTTCTACACCTGAAGATTTCGAG ATG CTT GGA TTA TAT AAG AAG TTG ACC CTG CTG GCA GCG ATG
      M L G L Y K K L T L L A A M
      -25 -20
225 TCA TTG TCC CTC CTG ACC ACT CTG GCC CAG TCT TCC CCT GTG GTG GGC CCG GAG CCT ATT
      S L S L L T T L A Q S S P V V G P E P I
      -10 -1 +1
285 CGC TGT GCC CCC TGT ACC CAG GAG AAG CTG GAT GAA TGC CCG GCC ATC TCC CCA GAC TGT
      R C A P C T Q E K L D E C P A I S P D C
      +10 +20
345 AAG CAG GTT CTC AGG GAG CCA GGC TGT GGC TGC TGC ATG GCA TGT GCC CTG GAG AAG GGG
      K Q V L R E P G C G C C M A C A L E K G
      +30 +40
405 GCC TCC TGT GGA GTA TAC ACG GCC CAC TGT GCT CAG GGG TTA AAA TGC AGC CCA AGA GCT
      A S C G V Y T A H C A Q G L K C S P R A
      +50 +60
465 GGG GAT CCT AGA CCC CTC CAC TCC CTC ACC AGA GGA CAG GCC ATC TGC ACT GAG GAC CAG
      G D P R P L H S L T R G Q A I C T E D Q
      +70 +80
525 GGA CAA GAG AAA GTA GAA GGG GTG CCA GAC CAC AGC TCC CTA GCC TAC TTC CTG GGA CTC
      G Q E K V E G V P D H S S L A Y F L G L
      +90 +100
585 AAC ACC CCC TTT GAC ACC AAA AAT GAG GGT GCT CAG GAG AGC ATC AAG GCC AAG GTC AAC
      N T P F D T K N E G A Q E S I K A K V N
      +110 +120
645 ACC ATC CGC AAG AAA CTG GTG GAA CAG GGT CCC TGC CAC ATT GAG CTG CAT GCA GCT CTG
      T I R K K L V E Q G P C H I E L H A A L
      +130 +140
705 GAC AAG ATA ACC AGC TCT CAG CAG GAA CTA GGA GAG AAG TTC ACC AAC TTC TAC CTA CCC
      D K I T S S Q Q E L G E K F T N F Y L P
      +150 +160
765 AAC TGT GAC AAA CAT GGC TTC TAC AAG GCC AAG CAG TGT GAG TCG TCT CTG GTG GGA CCC
      N C D K H G F Y K A K Q C E S S L V G P
      +170 +180
825 CAT GCC AGG TGT TGG TGT GTG TCC TCC TGG AAT GGG AAG AAG ATT CTG GGG TCC AAT TAT
      H A R C W C V S S W N G K K I L G S N Y
      +190 +200
885 CTA CCC GGC TTA GAG TGC CAG CTG GAG CTC TAA ATCATGGACACTCTGACCCACATTCACAGGCACA
      L P G L E C Q L E L *
      +210 +219
952 TGCATGCACACGTATTGACACAGTCTTTTTTACTCCACTTAAACACATATATAGGTGTCCTCAAACCTACACACATCCA
1032 CATCATATATATATACTGACTTTCTTTTACAAACACGTATAAATACACACTCATTCAAATAGCACACATTTTTTATTTT
1112 GATCTAATTACTGTAAATTTATTTTACGTGATGTATCACACCTTATTTATTCAAATGCGTATGTGCTTACAAGTATGGAC
1192 CATTTGTAATTTTATGATTGTTTATGTTTCTATTTTCTGATTGTAAACAGGGCAACAGTGTATGCATTTGTGTTTCTGA
1272 CTAATAACTCCATCTCTGAAGACCACAGGTCTCCAGCTCTGCCAAACCCAGTTTCAGGGTCTTCAGAATCTGTTTGTGAT
1352 TCATTCAACCAGGTGGACCCGTGCACAGTAAATGAACAACACAGCCATTTCATTTCATTCAACATGTTGACAGTTTC
1432 GGAAGGTATTCTCATTGACATTTCCCTCAATGGGTATTGGGGAAATGCTTAAAGTTCCACAAGTATGCATCTGGTGGCCAG
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1672 ATGTGAGGTCAATTGTTTCATTTGTCTGAAAGGAAGTCTCTTTTGTGTTGATGTAATGTCTGTATTTATTTATATGTCAAC
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1912 AAAA

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Fig. 6

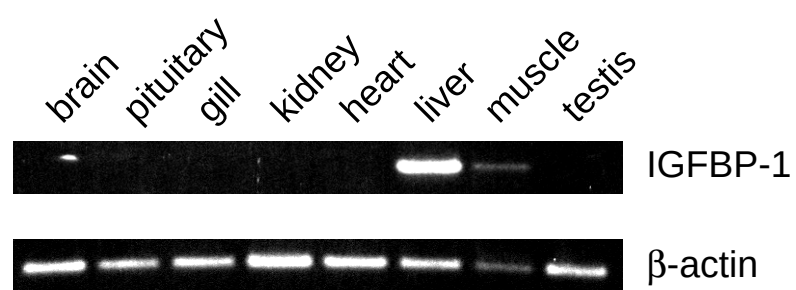


Fig. 7

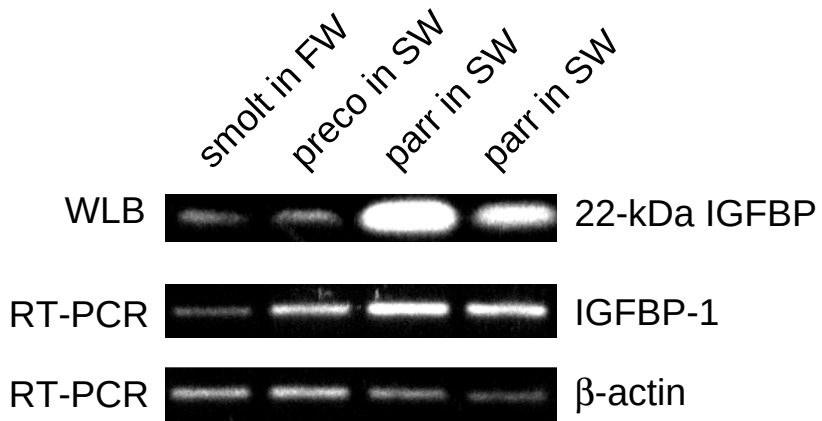


Table 1. Amino acid sequence identity and similarity of salmon 22-kDa IGFBP with human IGFBPs

BP type	Identity (%)	Similarity (%)
IGFBP-1	40	55
IGFBP-2	30	42
IGFBP-3	30	42
IGFBP-4	37	51
IGFBP-5	33	49
IGFBP-6	31	42