

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

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## Abstract

Western ligand blotting of salmon serum typically reveals three insulin-like growth factor (IGF) binding proteins (IGFBPs) at 22, 28 and 41 kDa. Physiologic regulation of the 22 kDa IGFBP is similar to that of mammalian IGFBP-1; it is increased in catabolic states such as fasting and stress. On the other hand, its molecular mass on Western ligand blotting is closest to mammalian IGFBP-4. The conflict between physiology and molecular mass makes it difficult to determine the identity of the 22 kDa IGFBP. This study therefore aimed to identify the 22 kDa IGFBP from 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. The N-terminal amino-acid sequence of the purified protein was used to design degenerate primers. Degenerate 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 characteristic of mammalian IGFBP-1. These findings suggest that association with the cell surface and turnover rate may differ between salmon and mammalian IGFBP-1.

*Journal of Endocrinology* (2005) **184**, 267–276

## Introduction

Circulating insulin-like growth factor-I (IGF-I) is bound to a family of six IGF-binding proteins (IGFBPs). Depending on the type of IGFBP and circumstance, they can either inhibit or enhance the growth-promoting actions of IGF-I. IGFBP-1 is the first member of the IGFBP family sequenced for complete amino acids (for review, see Shimasaki & Ling 1991). IGFBP-1 has 12 and 6 cysteines in the N- and C-termini respectively, which are well conserved among six IGFBPs and are responsible for high-affinity IGF binding (for review, see Shimasaki & Ling 1991). The molecular mass of human IGFBP-1 predicted from cDNA is 25 kDa, and it appears as a 30 kDa band on Western ligand blotting with labeled-IGF-I (Brinkman *et al.* 1988). IGFBP-1 is one of the best-characterized IGFBPs (for reviews, see Lee *et al.* 1993, 1997). IGFBP-1 usually inhibits IGF-I action and

increases in catabolic states. Unlike other IGFBPs, circulating IGFBP-1 changes rapidly and shows diurnal rhythms according to food intake. This is because insulin is the predominant inhibitor of IGFBP-1 transcription (Orlowski *et al.* 1991, Powell *et al.* 1991). Circulating IGFBP-1 and insulin levels therefore show an inverse relationship. Another important regulator of IGFBP-1 is cortisol. Cortisol stimulates transcription of IGFBP-1 mRNA, but its effect is secondary to the inhibitory effect of insulin (Unterman *et al.* 1991). Since most IGFBP-1 is unsaturated with IGFs and can bind free IGF-I, it is believed to regulate free IGF-I levels (Frystyk *et al.* 1997). These facts suggest that IGFBP-1 plays a crucial role in regulating the metabolic actions of circulating IGF-I.

IGFBP-1 is also present in fish according to molecular studies. Complete and partial sequences for IGFBP-1 have been obtained in zebrafish (*Danio rerio*) (Maures & Duan 2002) and longjaw mudsucker (goby) (*Gillichthys mirabilis*)

(Gracey *et al.* 2001) respectively. Candidates for fish IGFBP-1 protein have also been detected in the circulation by Western ligand blotting. Fish plasma/serum typically reveals three IGFBP bands at 20–25, 28–30 and 40–50 kDa. The 40–50 kDa form may be fish IGFBP-3, since it has a molecular mass similar to mammalian IGFBP-3 and is increased by growth hormone injection (Siharath *et al.* 1995, Shimizu *et al.* 2003). The two smaller fish IGFbps at 20–25 and 28–30 kDa are candidates for IGFBP-1 because both forms are increased in catabolic states such as fasting, stress and diabetes (Kelley *et al.* 2001, 2002). Hormonal control of these lower-molecular-mass forms in fish are similar to those of human IGFBP-1. In tilapia (*Oreochromis mossambicus*), exogenously administered cortisol increases serum levels of 24 and 28 kDa IGFbps (Kajimura *et al.* 2003). In the goby, the 24 and 30 kDa forms are suppressed by insulin treatment (Kelley *et al.* 2001). Kelley *et al.* (2001) pointed out that IGFBP sensitivities to various catabolic states differ depending on species and/or conditions. In striped bass (*Morone saxatilis*), the circulating level of 25 kDa IGFBP was increased after 60 days of fasting and was reduced below the detection limit after 14 days of refeeding, whereas the 30 kDa form was barely detected throughout the experiment (Siharath *et al.* 1996). In goby serum, the 24 kDa IGFBP has a greater sensitivity to fasting and diabetes than the 30 kDa form (Kelley *et al.* 2001). In contrast, similar IGFbps are present in jack mackerel (*Trachurus symmetricus*) serum, but the larger (28 kDa) form is more sensitive to stress (Kelley *et al.* 2001). Comparing all fish studies to date, it appears that the smallest (20–25 kDa) form generally has a greater sensitivity to nutritional status and hormone treatments, suggesting that it is the physiologic equivalent of mammalian IGFBP-1. However, the fish 28–30 kDa IGFBP is closer to human IGFBP-1 in size. Bauchat *et al.* (2001) purified rainbow trout (*Oncorhynchus mykiss*) 30 kDa IGFBP for the first time and sequenced the N-terminus. The partial N-terminal amino-acid sequence was most similar to human IGFBP-1. These facts suggest that the fish 28–30 kDa form is structurally similar to human IGFBP-1. On the other hand, 20–25 kDa IGFBP is closest to human IGFBP-4 in size. Since the fish 20–25 kDa IGFBP has not been purified or sequenced in any fish species, it is not known at present which of the two low-molecular-mass fish forms is a homolog of human IGFBP-1. Moreover, N-terminal amino-acid sequences are not necessarily reliable when comparing IGFbps, as this region is highly conserved and contains the binding domain. To establish homologies between fish and mammalian IGFbps, complete amino-acid sequence data are desirable.

As in other fish species, chinook salmon (*O. 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 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 (*O. 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 was 10.5–13 °C. Fish were fed standard rations (0.6–1.0% body weight/day). From October 1998, fish were either fed or fasted for 6 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 groups were considered to be smolt and parr respectively, which are two phenotypes differing in seawater adaptability. Smolt have high osmoregulatory ability, but parr do not. The small group (parr) were directly transferred to either full seawater (30 ppt) or a fresh water tank. Blood was collected 12 h 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, 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 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 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, pH 6.5, overnight. The sample was put through filter paper, loaded onto an Affi-Gel 10 (Bio-Rad) column (1.0 × 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/h 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/h. Eluted fractions were collected into prelubricated micro-centrifuge tubes (PGC Scientifics, Frederick, MD, USA).

Fractions containing 22 kDa IGFBP (based on Western ligand blot) were pooled and applied directly to reversed-phase high pressure liquid chromatography (HPLC) on a Vydac C-4 column (0.46 × 5 cm; Separation Group, Hesperia, CA, USA) 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 N<sub>2</sub> evaporation. The protein concentration of purified IGFBP was determined with the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA).

#### *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 and 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 (CBB) R250 (Bio-Rad). Molecular mass was estimated with Precision Marker (Bio-Rad).

Western ligand blotting with digoxigenin-labeled human (h) IGF-I (DIG-hIGF-I) was carried out as described by Shimizu *et al.* (2000). After electroblotting, the nitrocellulose membrane was incubated with 9 ng/ml DIG-hIGF-I for 2 h at room temperature and then incubated with antibody against DIG conjugated with horseradish peroxidase (Roche, Indianapolis, IN, USA) at a dilution of 1:2500 for 1 h at room temperature. IGFBP was visualized on autoradiography film by use of the enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Life Science, Arlington Heights, IL, USA).

#### *N-terminal amino-acid sequence*

Approximately 4 µg purified 22 kDa IGFBP were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA). 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, USA).

#### *Total RNA isolation and first-strand cDNA synthesis*

Liver was collected from 1-year-old chinook salmon that had been transferred from fresh water to seawater. The fish were classified as parr by their size and morphologic appearance. Dissected liver was immediately frozen in liquid nitrogen and stored at –80 °C until use. Total RNA

was extracted from the liver with Tri Reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's protocol. First-strand cDNA was synthesized from 7 µg total RNA, using SuperScript TM II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA, USA) 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 (Lewisville, TX, USA). PCR was performed with a Perkin Elmer Gene Amp Thermal Cycler (Perkin Elmer Cetus, Foster City, CA, USA) and components from Novagen (Madison, WI, USA). PCR cycles consisted of one cycle of 94 °C for 3 min; 36 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min 30 s; and one 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 with the QIAEX II Gel Extraction Kit (Qiagen). The purified cDNAs were blunt-end and were ligated into the pSTBlue-1 Blunt Vector with the Perfectly Blunt Cloning Kit (Novagen). The vectors were then transformed into the NovaBlue Singles Competent Cells (Novagen, Madison, WI, USA) and spread on Luria-Bertani (LB) agar plates containing 40 µg/ml X-gal, 100 µM isopropylthio-galactoside (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 with the QIAprep Miniprep Kit (Qiagen). Purified plasmid (200 ng) was sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with T7 and SP6 promoter primers (Novagen) and run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Full-length cDNA was obtained by 5'- and 3'-rapid amplification of cDNA ends (RACE) with the SMART RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA, USA). 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 1-year-old coho salmon (*O. kisutch*). Liver samples were also collected from 1-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  $\beta$ -actin was used as an internal control. The primer sequences for  $\beta$ -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.

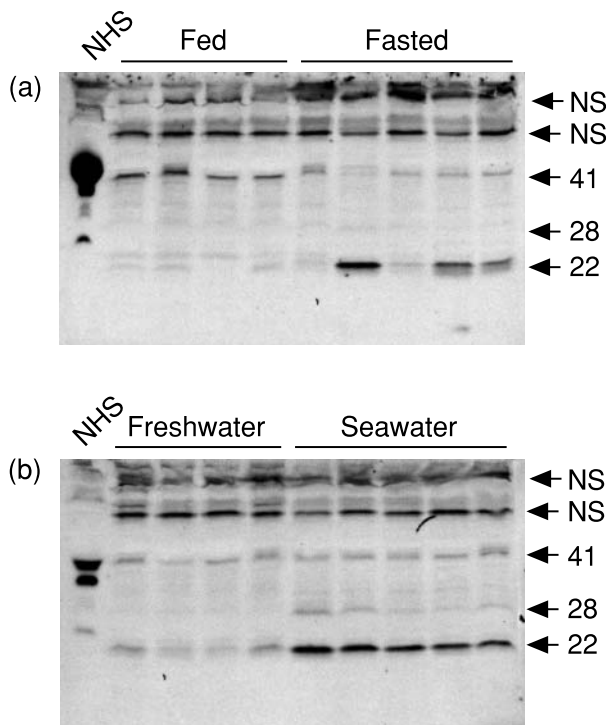
## Results

The response of circulating IGFbps to fasting in 1-year-old chinook salmon was assessed by Western ligand blotting (Fig. 1a). After 6 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 h 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 a C-4 column as assessed by ligand blotting (data not shown). The yield of purified 22 kDa IGFBP from 600 ml serum was 27  $\mu$ g. Purified 22 kDa IGFBP appeared as a single band at 22 kDa on SDS-PAGE under nonreducing conditions, while other minor bands were also observed at higher-molecular-mass regions on ligand blotting (Fig. 2).

The 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.

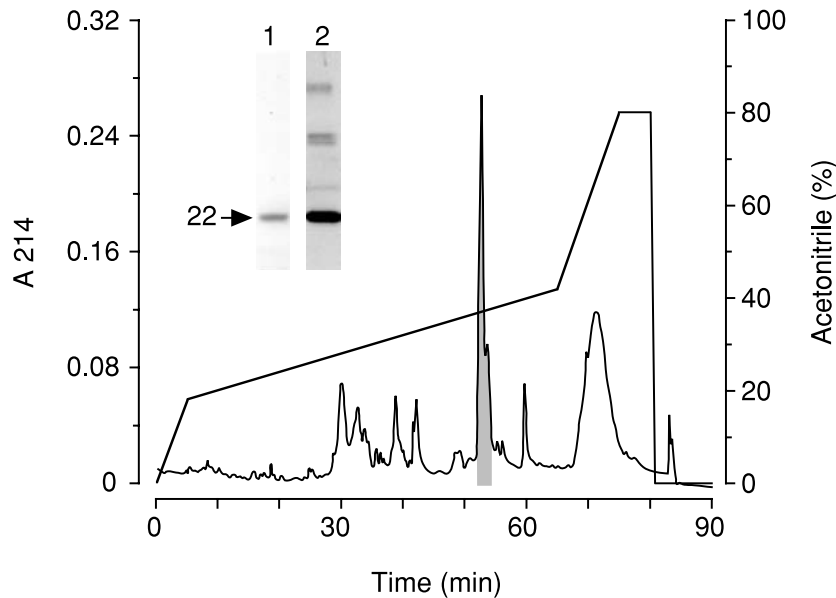
In order to obtain the complete amino-acid sequence of 22 kDa IGFBP, degenerate primers were designed from



**Figure 1** Western ligand blotting with digoxigenin-labeled human (h) IGF-I of plasma from chinook salmon. Fish were fed or fasted for 6 weeks (a), or transferred to fresh water or seawater for 12 h (b). Normal human serum (NHS; 1  $\mu$ l) and salmon plasma (2  $\mu$ l) were separated by 12.5% SDS-PAGE under nonreducing conditions and blotted onto nitrocellulose membranes. The membranes were incubated with digoxigenin (DIG)-labeled hIGF-I for 2 h and then with antibody against DIG at a dilution of 1:2500 for 1 h. Bands were visualized on radiographic film with the enhanced chemiluminescence reagents. Arrows indicate migration positions of salmon IGFbps with molecular mass (kDa) or nonspecific band (NS).

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 database (accession no. AY662657). The cDNA was 1915 bp long, comprising 182 bp of 5'-UTR, 735 bp of open reading frame and 998 bp of 3'-UTR. A polyadenylation signal AATAAAA 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 that of purified 22 kDa IGFBP, indicating that it encodes the 22 kDa IGFBP. The predicted molecular mass 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-1–5.





**Figure 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 nonreducing 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. Arrow indicates the migration position of purified 22 kDa IGFBP.

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 with 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 the 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 midregion, as characteristic of rapidly metabolized proteins (Fig. 5).

Gene expression of 22 kDa IGFBP in various tissues from 1-year-old coho salmon was assessed by RT-PCR (Fig. 6). The 22 kDa form was mainly expressed in the liver; relatively low expression was observed in muscle. Expression of the 22 kDa form was not evident in other tissues (Fig. 6), including ovary from 1-year-old coho salmon (data not shown). Relative gene expression of 22 kDa IGFBP by RT-PCR was compared with the intensity of the plasma 22 kDa band on Western ligand

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

**Figure 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.

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1  GAATTCAGTTCAGAAAGAAAGAAAGAAACCTATAGATGCGCTATCTACAGTATATTTGTGCTTCTCATTAAGTACTTGGAGC
81  TAAACAAAACCTACACTTGAAGATTAAAGACTGCACTTGAAGAAAATTCACATCTACACCTGACTGTAGATTTTTAGTGT
161  TTTCTACACCTGAAGATTGAG  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  GGC  CAG  TCT  TCC  OCT  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  TOC  OCA  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  OCA  AGA  GCT
      A  S  C  G  V  Y  T  A  H  C  A  Q  G  G  L  K  C  S  P  R  A
      +50      +60
465  GGG  GAT  OCT  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  AIT  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  AIT  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  OCC  GGC  TTA  GAG  TGC  CTG  GAG  CTC  TAA  ATCATGGACACTCTGACCCCAATTCACAGGCACA
      L  P  G  L  E  C  Q  L  E  L  *
      +210      +219
952  TGCATGCACACGTAATTGACACAGTCTTTTCTTACTCCACTTAAACACATATATAGGTGTCTCCTCAAACCTACACACATCCA
1032  CATCATATATATATACCTGACTTCTTTTACAAACACGTATAAATACACACTCAITCAAATAAGCACACATTTTATTTT
1112  GATCTAATTAAGTGTAAATTTAATTTACGTTGATGATCACCCTTATTTTATTCAAAATGCGTATGTGCTTACAAGTATGGAC
1192  CATTTGTAAATTTTATGATTTGTTATGTTTCTTATTTTCTGATTTGTAAACAGGGCAACAGTGTATGCAITTTGTGTTCTTGA
1272  CTAATAAATCACTCCATCTCTGAAGACCACAGGTCTCCAGCTCTGCCAAACCCAGTTTCAGGGTCTTCAGAAATCTGTTTGTGAT
1352  TCAATTCACACAGGTGGACCGTGCACAGTAAATGAAACACACAGCCATTCATTCATTCACATTCACACATGTTGACAGTITTC
1432  GGAAGGTATTTCTCATTTGACATTTCTCAATGGGTATTTGGGAAATGCTTAAAGTTCCACAAGTATGCATCTGGTGGCCAG
1512  GCACAACATATCACTGCTCATCCAAGCCCTGTTTGGTTTTGGGGAGTTTTTCTGTCTTCATCACTCACTTACTGTTCT
1592  GTGCAGGTCTTCTGGTTTTGGCAGATGTTTTTTGGGACAGTCTTTTTTCTCCCACTCTCTCCGACATPACACACCCCTCTCT
1672  ATGTGAGGTCATTTGTTTCATTTGTCTGAAAGGAAGTCTCTTTTGTGTTGATGTAATGTCTGTTTATATATGTCAAAC
1752  TAITTCTGACAGCAAGTCAGAAATATGTGATGAGATATGCTGCTATTACTTTTTTGTTTGGTTTGTATCAAAATCATGTATA
1832  GCCTATGTTAGTTACTCTGTGTTGTAATTTTGTGCTTTCAATAAACACAGTTAAACCCGCAAAAAAAAAAAAAAAAAAAAA
1912  AAAA

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**Figure 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. GenBank database accession no.: AY662657.

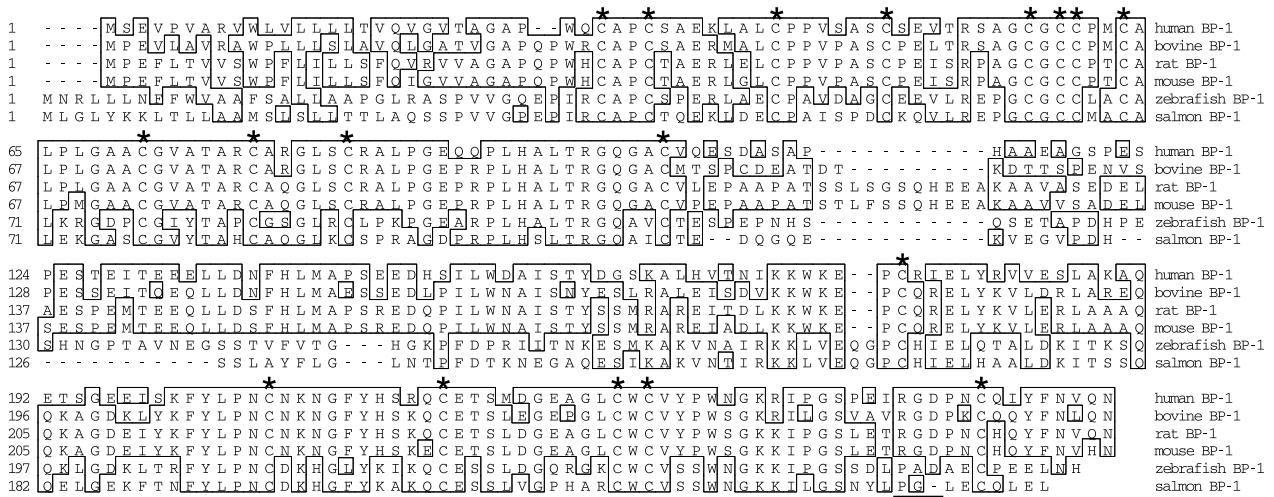
blotting of the same individuals (Fig. 7). Similar patterns of 22 kDa IGFBP mRNA and plasma levels were observed.

## Discussion

Most studies of fish IGFbps use Western ligand blotting to identify circulating forms or molecular methods for 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 circulating salmon IGFBP with the smallest molecular mass 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 refeeding. When chinook salmon were fasted for 6 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



**Figure 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.

species (Siharath *et al.* 1996, Kelley *et al.* 2001, Peterson & Small 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 the plasma 22 kDa form is presumably due to a stress-associated increase in cortisol, which is caused by salinity

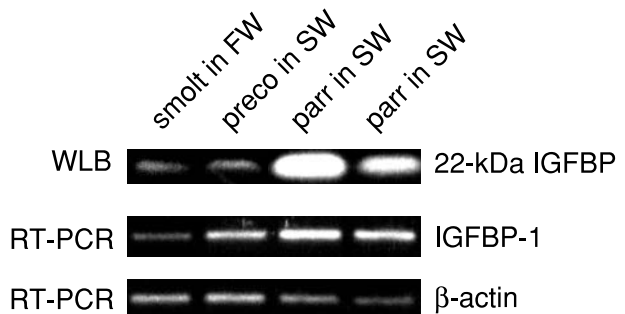
change rather than being the result of seawater adaptation, since some small fish died after transfer. In contrast, none of the large fish (smolt) died, and only a small increase in the 22 kDa form in plasma was seen (M Shimizu & WW Dickhoff, unpublished data), which might have been due to an increase in cortisol associated with seawater adaptation. The response of the 20–25 kDa IGFBP under ‘stressful conditions’ has been reported in other fish species. When tilapia were hypophysectomized, a 20 kDa form appeared in plasma (Park *et al.* 2000). Although cortisol levels were not measured in the present study nor in the study by Park *et al.* (2000), high levels of cortisol could be expected in these conditions. In tilapia, cortisol

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

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

**Figure 6** Tissue distribution of salmon 22 kDa IGFBP expression. Tissues were collected from 1-year-old male coho salmon. A representative result from one of three individuals is shown. First-strand cDNA reverse-transcribed from total RNA was used as template of RT-PCR. Salmon β-actin was used as internal control.



**Figure 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 with digoxigenin-labeled IGF-I and RT-PCR, using gene-specific primers for 22 kDa IGFBP or β-actin.

injection resulted in an induction of the 24 kDa IGFBP (Kajimura *et al.* 2003). These results support our assumption that salmon 22 kDa IGFBP is physiologically IGFBP-1.

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

CBB staining of purified 22 kDa IGFBP on SDS-PAGE revealed a single band at 22 kDa, whereas some other minor bands were also detected on Western ligand blotting. Some bands migrated at the same position as 41 kDa IGFBP. They are presumably aggregates of the 22 kDa form rather than the contamination of the 41 kDa form, since those bands were not immunoreactive to antiserum against the salmon 41 kDa form (M Shimizu, A Hara & WW Dickhoff, unpublished data). Although there may be slight contamination with other IGFbps, the 22 kDa IGFBP fraction was pure enough to obtain a partial N-terminal amino-acid sequence.

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

Despite the limited numbers of fish IGFBP sequence available at present, it appears that fish IGFbps are structurally similar to mammalian forms. This is true for

salmon IGFBP-1; it has the 18 cysteines and its sequence homology with human IGFBP-1 is high in N- and C-termini (46%) and low in the midregion (28%). However, some dissimilarity is seen. For instance, salmon IGFBP-1 does not have the Arg-Gly-Asp (RGD) sequence in the C-terminus, which is present in all mammalian counterparts. The RGD sequence is important for localization of proteins on the cell surface by interacting with the  $\alpha 5 \beta 1$  integrin (Ruoslahti & Pierschbacher 1987). The lack of RGD sequence has been reported for zebrafish IGFBP-1 (Maures & Duan 2002). As discussed by Maures and Duan (2002), acquisition of the RGD sequence to IGFBP-1 might have occurred after the divergence of teleosts from other vertebrates.

Unlike other IGFbps, circulating human IGFBP-1 rapidly changes in response to nutritional and physiologic status. The rapid change can be explained, at least in part, by its structure. Human IGFBP-1 mRNA contains AT-rich regions in 3'-UTR characterized by repeats of the nucleotide sequence ATTTA (Luthman *et al.* 1989). This sequence is involved in selective mRNA degradation (Shaw & Kamen 1986). Salmon IGFBP-1 mRNA has four repeats of the sequence, suggesting that its mRNA is unstable, like mammalian IGFBP-1. Another factor 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 midregion (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 midregion corresponding to the PEST region. In view of these structural differences, salmon IGFBP-1 mRNA is presumably as unstable as its 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 biologic significance of the phosphorylation may differ among species, it is a common feature of mammalian IGFBP-1. In mammals, the main sites of phosphorylation are serines in the midregion, 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 in 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 1-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 & 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 physiologic condition. Expression of fish IGFBP-1 is influenced by fasting and hypoxia (Gracey *et al.* 2001, Maures & Duan 2002). In the present study, we found that expression of 22 kDa IGFBP in the liver was influenced by possible stress during direct seawater transfer. A notable finding is that the expression level of the 22 kDa form appears to correlate well with plasma level in each individual. This implies that circulating 22 kDa IGFBP levels may be regulated primarily at the level of transcription.

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

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

## Acknowledgements

We thank Piper Schwenke and Dr Krista M Nichols, Northwest Fisheries Science Center, NOAA-Fisheries, Seattle, WA, USA, for their technical help in nucleotide sequencing. This work was supported by grants from the US Department of Agriculture, NRICGP, Animal Growth and Nutrient Utilization Program (Projects 2001–03320 and 2003–03314). This publication was also funded by the Joint Institute for the Study of the Atmosphere and Ocean (JISAO) under NOAA Cooperative Agreement no. NA17RJ11232, Contribution no. 1079. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 16 September 2004

Accepted 20 September 2004