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Title:

Circulating salmon 28- and 22-kDa insulin-like growth factor binding proteins (IGFBPs) are co-orthologs of IGFBP-1

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Abstract

Circulating insulin-like growth factor binding proteins (IGFBPs) play pivotal roles in stabilizing IGFs and regulating their availability to target tissues. In the teleost circulation, three major IGFBPs are typically detected by ligand blotting with molecular masses around 20-25, 28-32 and 40-45 kDa. However, their identity is poorly established and often confused. We previously identified salmon 22- and 41-kDa forms as IGFBP-1 and -2b, respectively. In the present study, we cloned the cDNA of 28-kDa IGFBP from Chinook salmon (*Oncorhynchus tshawytscha*) as well as rainbow trout (*O. mykiss*) based on the partial N-terminal amino acid sequence of purified protein and identified it as an ortholog of IGFBP-1. Structural and phylogenetic analyses revealed that the 28-kDa IGFBP is more closely related to human IGFBP-1 and zebrafish IGFBP-1a than the previously identified salmon IGFBP-1 (i.e. 22-kDa IGFBP). We thus named salmon 28- and 22-kDa forms as IGFBP-1a and -1b, respectively. Salmon IGFBP-1a contains a potential PEST region involved in rapid protein turnover and phosphorylation sites typically found in mammalian IGFBP-1, although the PEST and phosphorylation scores are not as high as those of human IGFBP-1. There was a striking difference in tissue distribution patterns between subtypes; Salmon *igfbp-1a* was expressed in a variety of tissues while *igfbp-1b* was almost exclusively expressed in the liver, suggesting that IGFBP-1a has more local actions. Direct seawater exposure (osmotic stress) of Chinook salmon parr caused increases in both IGFBP-1s in plasma, while IGFBP-1b appeared to be more sensitive. The presence of two co-orthologs of IGFBP-1 in the circulation in salmon, and most likely in other teleosts, provides a good opportunity to investigate subfunction partitioning of duplicated IGFBP-1 during postnatal growth.

Keywords

insulin-like growth factor binding protein; salmon; identification; gene duplication; subfunction partitioning

1. Introduction

Insulin and insulin-like growth factor (IGF)-I are peptide hormones derived from a common ancestral peptide in early metazoan evolution [4,24]. Although they are structurally similar and have overlapping effects, insulin and IGF-I distinctly regulate metabolism and growth through their own receptors. The functional distinction between the two hormones is accomplished in part by the presence of high-affinity IGF-binding proteins (IGFBPs). IGFBPs block IGF-I from interacting with insulin receptor and thus diminish the insulin-like effect of IGF-I. Six members of IGFBPs have been identified and characterized in mammals[11,14,15,37]. The IGFBPs play pivotal roles in regulating the stability of IGF-I in the circulation and its availability to target tissues. Depending on the type of IGFBP, physiological conditions and cellular environment, they can either inhibit or potentiate the activity of IGF-I and thus input an additional complexity to growth regulation. The six IGFBPs also share structural similarities and are believed to be derived from three rounds of gene duplication of a single protein prior to the emergence of the teleosts [4,6]. Supporting this, six different types of IGFBP genes can be found in teleosts [3,9,22,30]. An interesting phenomenon is that because the teleosts experienced an extra round of gene duplication, duplicate copies of *igfbps* have been reported in zebrafish (*Danio rerio*) [5,23,49,51], Atlantic salmon (*Salmo salar*) [2] and Chinook salmon (*Oncorhynchus tshawytscha*) [43]. A series of studies of zebrafish model by Duan and co-workers revealed important roles of these duplicated IGFBPs during embryogenesis [5,23,49,51].

Multiple IGFBPs are also detected in the circulation of adult fishes [27,32]. When assessed by ligand blotting using labeled-IGF-I, fish plasma typically exhibits three IGFBPs with molecular weights around 20-25 kDa, 28-32 kDa and 40-50 kDa [27]. The proportion of the three IGFBPs differs depending on the physiological conditions of fish examined. Two low-molecular-weight (LMW) IGFBPs (i.e. 20-25 and 28-32-kDa IGFBPs) are generally very low or undetectable under normal conditions but increase when fish are under catabolic conditions such as fasting and stress [25,34,46]. In contrast, the 40-50-kDa IGFBP is high when fish are fed or in unstressed states [38,42]. Many hormones are involved in the regulation of the IGFBPs [13,27]. Despite a significant body of work has been conducted on the physiological regulation of the three circulating IGFBPs in fishes, their identity is poorly established and often confused. Classification of fish IGFBPs is based mainly on the comparison of the molecular weight and physiological

responses with those of the six mammalian IGFBPs. For instance, the fish 40-50-kDa IGFBP was believed to be an ortholog of IGFBP-3 since it has a molecular weight similar to human IGFBP-3 and is increased by growth hormone treatment and under good nutritional status as is mammalian IGFBP-3 [27,42]. However, we have recently demonstrated through protein purification and molecular cloning that the 41-kDa IGFBP in salmon is actually an ortholog of IGFBP-2 (IGFBP-2b) [43]. On the other hand, fish 20-25-kDa IGFBP has a molecular weight similar to human IGFBP-4 but its physiological response corresponds to that of IGFBP-1 [25,34,46]. The 20-25-kDa IGFBP of salmon has been identified as IGFBP-1 [41]. These findings imply that the identity of the three fish IGFBPs in plasma may be different from what was originally assumed based on molecular size and function similarities to human plasma IGFBPs. Thus the characterization of fish IGFBPs in plasma should be done by using full-length amino acid sequence information and considering the possible presence of paralogs. As described above, three IGFBPs can be detected in fish circulation; two have been identified as IGFBP-1 and IGFBP-2b. The third IGFBP (i.e. 28-32-kDa form) has not been identified. This IGFBP is a candidate for IGFBP-1 or -2 based on the molecular weight and induction under catabolic states [25]. Identification of the three major IGFBPs in fish plasma is essential for investigating the functional diversity/conservation of the IGFBP family in vertebrates. The present study aimed to identify the circulating 28-32-kDa IGFBP in salmonids.

2. Materials and methods

2.1. Serum collection

Serum was collected from spawning male Chinook salmon (*Oncorhynchus tshawytscha*) in the adult return pond on the University of Washington campus, Seattle, WA, in late October and early November. 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.

One-year-old rainbow trout (*Oncorhynchus mykiss*) reared at Nanae Freshwater Experimental Station, Hokkaido University (Kameda, Hokkaido, Japan) were injected with cortisol (Sigma-Aldrich, St. Louis, MO) at a dose of 50 µg/g body weight and blood was withdrawn 8 h after injection. Serum was collected as described above and stored at -30°C until use. The

experiment was carried out in accordance with the guidelines of Hokkaido University Field Science Center Animal Care and Use Committee.

2.2. 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 [28]. 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, Hercules, CA). Molecular mass was estimated with Precision Marker (Bio-Rad).

Western ligand blotting with digoxigenin-labeled human IGF-I (DIG-hIGF-I) was carried out as described in Shimizu et al. [44]. After electroblotting, the nitrocellulose membrane was incubated with 10-50 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) at a dilution of 1:1500-2500 for 1 h at room temperature. IGFBP was visualized by use of the enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Life Science, Arlington Heights, IL).

2.3. Purification of salmon 28-kDa IGFBP

Salmon 28-kDa IGFBP was purified from serum using the same procedure for 41-kDa IGFBP [45]. Briefly, seven hundred milliliters of salmon serum was first acidified to dissociate the IGF/IGFBP complex. Endogenous IGF-I was removed using SP-Sephadex C-25 (Amersham Pharmacia Biotech, Uppsala, Sweden) and supernatant was collected and neutralized. The neutralized supernatant was subjected to IGF-I-affinity chromatography. IGFBPs were eluted from the column by adding 0.5 M acetic acid and applied to reversed-phase high pressure liquid chromatography (HPLC) using a Vydac C-4 column. IGFBPs were separated by a linear gradient of 18-41% acetonitrile in 0.1% trifluoroacetic acid (TFA).

2.4. Amino acid analysis

Partial N-terminal amino acid sequence of purified 28-kDa IGFBP was determined using the procedure described in Shimizu et al. [45]. In addition, internal amino acid sequence was analyzed by digesting the purified protein with trypsin. Purified protein (2.2 µg/16.6 µl 100 mM Tris-HCl, pH 8.5) was mixed with a sequence grade trypsin (Roche Applied Science, Mannheim, Germany) (40 ng/0.4 µl 1 mM HCl) and incubated at 37°C for 10 min. Digested protein was immediately mixed with the sample buffer containing 2-mercaptoethanol and separated by 15% SDS-PAGE. After electrophoresis, it was electroblotted onto a PVDF membrane and stained using CBB R-250. A major fragment band around 17 kDa was sequenced by the Edman degradation method at Instrumental Analysis Division, Equipment Management Center, Hokkaido University (Sapporo, Hokkaido, Japan).

2.5. cDNA cloning of trout and salmon 28-kDa IGFBP

Liver cDNA was prepared from a 1-year-old rainbow trout injected with cortisol. A degenerate forward primer for trout 30-kDa IGFBP was designed from the N-terminal amino acid sequence of purified protein [1] (5' ATCAGGTGYGCHCCHTGYWSNCC 3', where Y = C or T; H = A, C or T; W = A or T; S = G or C; N = any base), and a degenerate reverse primer was designed from the C-terminal region conserved for IGFBP-1 in several teleosts (5' GCATTCCAGGAGGANACRCACCARCA 3', where R = A or G). Reverse transcriptase (RT)-PCR was performed with a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA) and components from Promega (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 cloned into the pGEM-T Easy Vector Systems (Promega, Madison, WI) and positive clones were sequenced as described in Shimizu et al. [43]. Gene specific primers were designed from the sequence of the partial cDNA (Forward: 5' GAGCCCGAGAGCAGCTCTGTGTC 3'; Reverse: 5' GGCTGGAGCCGGAGTCTGATACCAT 3'). A cDNA containing the open reading frame (ORF) of trout 30-kDa IGFBP was obtained by 3'- and 5'-rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA).

Liver cDNA was prepared from 1-year-old Chinook salmon that had been transferred from freshwater to seawater as direct seawater transfer was shown to increase expression of IGFBP-1 [41]. Gene-specific primers flanking the ORF of trout 30-kDa IGFBP were designed for

cloning of salmon 28-kDa IGFBP (Forward: 5' CTCCTTATTCCAGTCTCTCCTTATAC 3'; Reverse: 5' TGCATGCGTGCGTGGTCT 3'). RT-PCR and cDNA cloning were performed as described above.

2.6. Analyses of 28-kDa IGFBP sequences

Deduced amino acid sequences of salmon IGFBPs were aligned with human and fish IGFBPs using the ClustalW method in the DNA Data Bank of Japan website (<http://www.ddbj.nig.ac.jp>). Molecular weight of mature protein was calculated by using the Compute pI/MW tool (http://us.expasy.org/tools/pi_tool.html). Regions rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST domain) were detected by using EMBOSS epestfind (<http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind>). Potential N-glycosylation sites were detected by using the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc>). Potential phosphorylation sites were detected by using NetPhos 2.0 server (<http://www.cbs.dtu.dk/services/NetPhos>). Regions with NetPhos score >0.9 were considered as potential phosphorylation site. Amino acid sequences of full-length IGFBPs were subjected to the ClustalW analysis to create a phylogenetic tree using a neighbor-joining method (based on uncorrected p-distance). The reliability of the tree topology was assessed by the bootstrap method with 1000 replications. NJplot software was used to prepare a graphical view of the phylogenetic tree [35].

2.7. Tissue distribution of salmon 28-kDa IGFBP

Various tissues were collected from male (immature and maturing) and female (immature) adult Chinook salmon. Sampling was carried out in accordance with the guidelines of the University of Washington Institutional Animal Care and Use Committee. Expression of salmon IGFBPs in various tissues was analyzed by RT-PCR. Sequences of primer pairs for IGFBP-1a are as described above and those for IGFBP-1b are as follows: Forward, 5' GAGATGCTTGGATTATATAAGAAGTTG 3'; Reverse, 5' TTAGAGCTCCAGCTGGCACT 3'. Elongation factor-1 α (EF-1 α) was used as a reference gene (Forward: 5' GAATACCCTCCTCTTGGTCGTTT 3'; Reverse: 5' TCGACGGCCTTGATGACA 3'). PCR cycle was adjusted for each gene (35, 35 and 30 cycles for IGFBP-1a, IGFBP-1b and EF-1 α).

respectively).

2.8. Seawater challenge test

Under-yearling Chinook salmon were reared in fresh water at Northwest Fisheries Science Center, NOAA Fisheries in Seattle, WA, USA. They were maintained in recirculated freshwater 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). In October, fish were sorted into two groups by size (above or below fork length of 15 cm). The large (17.8 ± 0.5 cm and 69.2 ± 4.5 g, Ave $\pm$ SE) and small (12.6 ± 0.4 cm and 23.1 ± 2.0 g) groups were considered to be smolt and parr, respectively, which are two phenotypes with differing seawater adaptability. The both groups were directly transferred to either full seawater (SW) (30 ppt) or freshwater (FW) tanks. Blood was collected 0 h, 6 h, 12 h, 24 h, 3 days and 7 days after transfer. Fish were not fed during the transfer experiment. The experiment was carried out in accordance with the guidelines of the University of Washington Institutional Animal Care and Use Committee.

2.9. Measurement of plasma IGFBPs and gill Na^+, K^+ -ATPase activity

Plasma 22-kDa IGFBP and 41-kDa IGFBP levels were measured by radioimmunoassays (RIAs) as described in Shimizu et al. [40] and Shimizu et al. [42], respectively. Plasma levels of the 28-kDa IGFBP were semi-quantified by measuring the band intensity on ligand blotting by using ImageJ program. The intensities of the bands were normalized to that of the human IGFBP-4 band and expressed as arbitrary density units (ADU). Gill Na^+, K^+ -ATPase activity was measured by the method of McCormick et al. [31].

2.10. Statistical analysis

The values of IGFBP-1a, -1b and -2b from the seawater transfer experiment were first natural-log transformed to obtain normal distribution. Results of the seawater transfer experiment were analyzed by three-way ANOVA by including fish size (large and small), salinity (FW and SW) and time after transfer as factors. When significant effects were found, differences were identified by the Fisher's protected least significant difference (PLSD) test using the JMP program (SAS Institute Inc. Cary, NC). Differences among groups were considered to be significant at $P < 0.05$.

3. Results

Plasma from spawning Chinook salmon and rainbow trout injected with cortisol were analyzed for IGFBPs by Western ligand blotting using digoxigenin-labeled human IGF-I (Fig. 1). Three IGFBP bands at 41, 28 and 22 kDa are consistently detected in plasma during spawning season whereas the 28-kDa IGFBP band was more intense during the later stages of spawning (Fig. 1a). Cortisol injection of rainbow trout induced both 30- and 22-kDa IGFBP in plasma, whereas injection of vehicle only also had a moderate effect on the IGFBP induction (Fig. 1b).

The 28-kDa IGFBP was purified from serum of spawning male Chinook salmon. The same purification procedure used for the 41-kDa IGFBP was applied to the 28-kDa IGFBP. Reversed-phase HPLC of an affinity purified IGFBP mixture yielded peaks of 41- and 28-kDa IGFBP at 32 and 33% acetonitrile, respectively (Fig. 2). Final yields of purified 28-kDa from 1000 ml serum were 11 - 24 µg depending on the serum batches. A single band at 28 kDa was stained by CBB on SDS-PAGE under non-reducing conditions, whereas ligand blotting visualized an additional minor band at 23 kDa besides the 28-kDa band (Fig. 2). The partial amino-acid sequences of purified and digested 28-kDa IGFBP were compared to other IGFBPs (Fig. 3). The sequences were almost identical to that of trout 30-kDa IGFBP.

A cDNA for rainbow trout 30-kDa IGFBP was first cloned using degenerate primers and liver template was prepared from a cortisol-treated fish. Degenerate PCR amplified a partial cDNA of trout 30-kDa IGFBP (data not shown). The partial cDNA was extended for both ends by 5'- and 3'-RACE, and cDNA sequence containing the ORF of the 30-kDa IGFBP was obtained (Accession No. JF920119). This sequence was used to clone cDNA of Chinook salmon 28-kDa IGFBP. A cDNA containing the ORF of salmon 28-kDa IGFBP (Accession No. JF920120) was obtained and its N-terminal amino acid sequence matched that from the purified protein (Fig. 4). Cloned Chinook salmon IGFBP had a higher sequence homology with human IGFBP-1 and zebrafish IGFBP-1a than did salmon 22-kDa IGFBP (Table 1). Phylogenetic analysis revealed that these IGFBPs are placed in a clade with zebrafish IGFBP-1a, and carp and catfish IGFBP-1, whereas previously reported salmon and trout IGFBP-1s were placed in another clade with zebrafish IGFBP-1b (Fig. 5). Based on these results, we named salmon 28- and 22-kDa IGFBP as IGFBP-1a and IGFBP-1b, respectively. Salmon IGFBP-1a had an estimated molecular weight of

25.9 kDa and a weak PEST region but no N-glycosylation site or Arg-Gly-Ser (RGD) sequence (Table 2).

Tissue distribution of salmon *igfbp-1a* and *igfbp-1b* mRNA was examined by RT-PCR (Fig. 6). *igfbp-1a* was expressed in a variety of tissues such as pituitary, gill, heart, stomach, intestine, spleen and muscle. *igfbp-1a* is also detected in the liver but its expression was relatively low. However, one of the three individuals examined showed a clear band in the liver (data not shown). Expression patterns of *igfbp-1a* between male and female were similar and ovary was a site of the expression (data not shown). In contrast, *igfbp-1b* was expressed predominantly in the liver and weakly in the kidney, intestine and spleen.

Effects of direct seawater transfer of under-yearling Chinook salmon with two size categories (large and small) on the three major circulating IGFBPs were assessed by Western ligand blotting and radioimmunoassay (Fig. 7). Overall, direct seawater transfer strongly induced both IGFBP-1a and -1b in small fish but weakly in large fish (Fig. 7a). Plasma IGFBP-1a (28-kDa form) levels significantly increased in fish in SW within 6 h but became not significant 12 h after transfer (Fig. 7b). In SW large fish, IGFBP-1a level stayed relatively low thereafter. In contrast, IGFBP-1a in SW small fish increased again from 24 h and reached 42-fold higher level than that in FW small fish 72 h after transfer. It returned to the basal levels 7 days after transfer, which was accompanied with a significantly high gill Na^+/K^+ -ATPase activity (Fig. 7e). Plasma IGFBP-1b (22-kDa form) levels were significantly increased in SW in both sizes within 6 h (Fig. 7c). IGFBP-1b in SW small fish maintained its increased levels for 24 h and dramatically increased 72 h after transfer. IGFBP-1b returned to the basal levels at the end of the experiment (7 days after transfer). Large fish also showed an induction of IGFBP-1b after SW transfer but its degree was much lower than that of small fish. IGFBP-2b (41-kDa form) showed no change in SW when compared to FW controls (Fig. 7d). There was a positive size effect on the circulating IGFBP-2b levels throughout the experiment.

4. Discussion

The presence of three IGFBPs in fish plasma with the molecular masses of 20-25 kDa, 28-32 kDa and 40-50 kDa are widely recognized; however, the identity of these IGFBPs has been a matter of debate since they were discovered [26,27,50]. Chinook salmon serum/plasma exhibits three

IGFBPs at 22, 28 and 41 kDa [44]. We previously identified Chinook salmon 22- and 41-kDa IGFBP as IGFBP-1 and -2b, respectively [41,43]. In the present study, we demonstrated that salmon 28-kDa IGFBP is a co-ortholog of mammalian IGFBP-1 and 22-kDa IGFBP. Structural and phylogenic analyses revealed that salmon 28-kDa IGFBP is more closely related to mammalian IGFBP-1 and zebrafish IGFBP-1a, leading us to name the 28- and 22-kDa forms as IGFBP-1a and -1b, respectively. The present study completes the identification of the three major circulating IGFBPs in Chinook salmon and provides a reference for considering the identities of other fish IGFBPs in plasma.

We first purified the 28-kDa IGFBP from Chinook salmon serum to obtain the partial N-terminal amino acid sequence. The same purification procedure used for the 41-kDa IGFBP [45] could be applied for the 28-kDa IGFBP. However, recovery of purified protein varied among serum pools. Sera were collected from spawning fish during late October and early November. Even with the relatively short sampling period, the level of the 28-kDa IGFBP appeared to be higher in fish sampled at later dates presumably due to prolonged starvation. Purification of fish 28-32 kDa IGFBP was first achieved by Bauchat et al. [1] from conditioned medium of trout hepatoma cells. This trout 30-kDa IGFBP can be also detected in plasma [1,32]. The partial N-terminal amino acid sequence of this protein was most similar to human IGFBP-1 (60% identity) and IGFBP-4 (56% identity) [1]. The authors suggested that trout 30-kDa IGFBP is an ortholog of IGFBP-1 while leaving open the possibility of it being IGFBP-4 [1]. As the partial sequence of purified salmon 28-kDa IGFBP was identical to that of trout except for the first residue, the salmon and trout versions should be equivalent proteins.

An initial attempt to clone cDNA for Chinook salmon 28-kDa IGFBP was unsuccessful due to the limited amino acid sequence available for designing degenerate primers and possibly a low expression under normal physiological conditions. We thus sought to first clone cDNA of rainbow trout 30-kDa IGFBP where more continuous amino acid sequence was available. We also injected trout with cortisol to increase expression of the 30-kDa IGFBP in the liver since cortisol has been shown to increase 28-32-kDa IGFBPs in plasma of tilapia (*Oreochromis mossambicus*) [21]. Induction of trout 30-kDa IGFBP in plasma by cortisol was confirmed in the present study. These adjustments enabled us to clone trout IGFBP, and its sequence information was used to clone Chinook salmon IGFBP cDNA. A cloned cDNA was confirmed to code salmon 28-kDa IGFBP,

since its N-terminal amino acid sequence matched that of the purified protein. Salmon 28-kDa IGFBP had the highest sequence identity with human IGFBP-1 and it is placed in the IGFBP-1 clade in the phylogenetic analysis. These results indicate that salmon 28-kDa IGFBP is an ortholog of mammalian IGFBP-1.

Since we previously identified Chinook salmon 22-kDa IGFBP as IGFBP-1 [41], the 28- and 22-kDa IGFBP are IGFBP-1 paralogs in this species. The presence of IGFBP-1 paralogs was reported in zebrafish [23]. They named the two zebrafish paralogs as IGFBP-1a and -1b based on the sequence homology with human IGFBP-1 [23]. Salmon 28-kDa IGFBP is more closely related to human IGFBP-1 in terms of sequence homology and molecular weight. In addition, salmon 28-kDa IGFBP has a weak PEST domain in the mid region. The Pro (P), Glu (E), Ser (S) and Thr (T) rich segment is typically found in rapidly metabolized proteins and mammalian IGFBP-1 contains this domain in the midregion [17,29]. In contrast, salmon 22-kDa IGFBP lacks this domain resulting in a smaller molecular weight [41]. Phylogenetic analysis also revealed that salmon 28-kDa IGFBP was grouped with zebrafish IGFBP-1a whereas salmon 22-kDa IGFBP with zebrafish IGFBP-1b. Based on these characters, we named the 28- and 22-kDa IGFBP as IGFBP-1a and -1b, respectively. However, it is worth noting that zebrafish IGFBP-1b has a high-score PEST domain in the mid region whereas it is weak in salmonid IGFBP-1a [23]. The presence of the PEST domain may be more species-specific than subtype-specific.

Glycosylation and phosphorylation are two modifications found in certain types of IGFBPs [37]. In mammals, IGFBP-1 is not glycosylated but phosphorylated. An exception is zebrafish IGFBP-1a, which has a potential N-glycosylation site [23]. When the purified 28-kDa IGFBP was visualized by ligand blotting, an additional minor band at 23 kDa was detected. The 23-kDa band is unlikely to be a non-glycosylated form of 28-kDa IGFBP as it has no glycosylation sites in the sequence. Rather, the appearance of the multiple bands may reflect a different degree of phosphorylation based on the prediction that the 28-kDa IGFBP has potential phosphorylation sites. Phosphorylation is involved in proteolysis as well as affecting the binding affinity for IGFs [7,16]. However, this is speculation solely based on the amino acid sequence and needs to be confirmed experimentally whether salmon IGFBP-1a is phosphorylated.

In humans, liver, uterine decidua and placenta are the major sites of *IGFBP-1* expression [29]. In fetal rats, *igfbp-1* mRNA is also detected in stomach, kidney, lung, heart, skin and

intestine along with the highest expression in the liver [33]. The present study showed that Chinook salmon *igfbp-1a* was atypically expressed in several tissues and the liver had a relatively weak expression. This pattern clearly contrasts with that of *igfbp-1b*, which was almost exclusively expressed in the liver. Siharath et al. [47] examined *in vitro* secretion of IGFBP from various organs of striped bass and found that the 28-30-kDa IGFBP was produced by several tissues such as liver, pituitary, trunk kidney and bulbus arteriosus. Production of IGFBP-1a in peripheral tissues suggests that it modulates autocrine/paracrine actions of IGFs. In contrast, zebrafish *igfbp-1a* is expressed only in the liver [23,30]. It needs to be clarified whether multiplicity of *igfbp-1a* expression is general in teleosts or restricted in certain species.

The LMW IGFBPs in fish plasma are known to increase under catabolic conditions such as fasting and stress [25,26,34,46]. In the present study, under-yearling Chinook salmon were transferred to SW to create a catabolic state and assessed for responses of circulating IGFBPs. Juvenile salmon undergo a parr-smolt transformation prior to seaward migration to acquire hyperosmoregulatory ability. Pre-mature transfer of juvenile salmon to seawater causes a stress and retardation of growth involving the GH-IGF-I system [8,10]. In the present study, we had large and small size groups, which were considered as smolt and parr, respectively, as judged by gill Na^+ , K^+ -ATP activity. Our assumption was that direct seawater transfer should cause a more severe catabolic state in small fish (parr) than in large fish (smolt) and indeed, some small fish died during the experiment indicating lethal stress (data not shown). Under such stressful conditions, IGFBP-1a and -1b were dramatically induced in small fish in SW. This induction was presumably through cortisol, although no cortisol measurement was performed in the present study. It needs to be noted that the levels of IGFBP-1a were semi-quantified by measuring the band intensity on the blots. Care should be taken that the increase in the IGFBP-1a intensity might not be due to *de novo* synthesis but chemical modification such as phosphorylation, which is known to affect the IGF-binding activity of IGFBP-1 [16]. Shepherd et al. [39] assessed effects of gradual salinity change on trout plasma IGFBPs and found that a 21-kDa IGFBP moderately increased in SW fish 5 days after transfer whereas a 32-kDa IGFBP levels were not significantly different between fish in FW and SW. The difference in the LMW IGFBP responses between our study and theirs could mainly be due to procedures employed for SW transfer: direct transfer or gradual acclimation.

It is worth noting that Chinook salmon IGFBP-1b may be more sensitive than IGFBP-1a

to catabolic states. Both IGFBP-1a and -1b increased after SW transfer, but the degree of increase in small fish was greater in IGFBP-1b than IGFBP-1a. Moreover, fish were not fed during the experiment (7 days). Seven days of fasting resulted in the higher basal IGFBP-1b levels compared to the initial control, whereas the IGFBP-1a band was invisible at the end of experiment. Similarly, prolonged fasting where under-yearling Chinook salmon fasted for 6 weeks exhibited increased levels of IGFBP-1b but not IGFBP-1a [41]. The mechanism by which two salmon IGFBP-1s are differently regulated is not known at present. A series of studies using zebrafish embryos under hypoxic conditions revealed that *igfbp-1* mRNA increased in response to the reduction of oxygen and this response was mediated by the hypoxia-inducible factor 1 (HIF-1) pathway [18-20,30]. In addition, there was a difference in the responses between zebrafish *igfbp-1a* and *-1b* to hypoxia; Hypoxia induced *igfbp-1a* expression in early embryogenesis whereas *igfbp-1b* induction was seen later in embryogenesis [23]. This may be attributed to the differences in the numbers and locations of hypoxia response elements (HREs) and HIF-1 ancillary sequences (HAS) in the promotor regions of the genes. Kamei et al. [23] also found that in adult zebrafish *igfbp-1a* is more sensitive to hypoxia and fasting than *igfbp-1b*, which contrasts to our finding that IGFBP-1a may be more sensitive to catabolic conditions. The species difference in sensitivity of duplicated IGFBP-1s invites future comparative studies as discussed below.

Since duplicated *igfbp-1* genes as well as other *igfbps* are commonly found in the genomes of several teleosts, it has been suggested that duplication of each of six IGFBPs occurred during the teleost-specific third round of gene duplication [6]. Given that the two LMW IGFBPs can be detected in a wide range of teleosts [25], the presence of two functional IGFBP-1s may be a common feature of this group. Gene duplication provides an opportunity for a protein to acquire a new function or/and regulation. The fate of duplicated genes falls into three categories: nonfunctionalization, neofunctionalization and subfunctionalization [12]. In most cases, one of duplicated gene pairs would be lost while the other retains the original function (nonfunctionalization or divergent resolution) whereas acquisition of a new function (neofunctionalization) is a rare case [48]. In some cases, however, duplicated gene pairs share the original function of the ancestral gene by partitioning temporal, spatial and quantitative expression [36], which may create a variety of regulatory mechanisms to adapt to different environments or niches. It is hypothesized that such lineage-specific subfunction partitioning might be a force for

the teleost radiation [36]. The finding of the present study that two salmon *igfbp-1s* were expressed in different tissue types suggests that they have undergone spatial subfunction partitioning. In addition, Kamei et al. [23] proposed inducible or regulation subfunction partitioning for the differential responses of zebrafish *igfbp-1s* to hypoxia, making it an attractive model to gain insight into functional gene evolution. As mentioned earlier, the two LMW IGFBPs in a wide range of teleosts are most likely to be IGFBP-1 co-orthologs, which provides a good opportunity to investigate species-specific subfunction partitioning of IGFBP-1 to understand regulatory mechanism and functional divergence. As salmon underwent an extra round of genome duplication, a possibility is that this group may have four copies of IGFBP-1 [6]. However, we only found two copies so far and the analysis of the salmon genome would address this issue.

In conclusion, we showed that two of the three major circulating IGFBPs in salmon are co-orthologs of IGFBP-1 and suggested that they appeared to have undergone subfunction partitioning in terms of spatial distribution and response to catabolic states. The possible presence of the two IGFBP-1s in a wide range of teleosts provides a good opportunity to investigate evolution of regulatory mechanism of IGFBP-1 in vertebrates.

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Figure legends

Fig. 1 Western ligand blotting with digoxigenin-labeled human IGF-I (DIG-hIGF-I) of sera from Chinook salmon (a) and rainbow trout (b). (a) Spawning Chinook salmon were collected during late October and early November. (b) One-year-old rainbow trout were injected with cortisol (Cort) and blood was taken 8 h after injection. Normal human serum (NHS; 1 ul) and salmon and trout plasma (2 ul) were separated by 12.5% SDS-PAGE under non-reducing conditions and blotted onto nitrocellulose membranes. The membranes were incubated with DIG-hIGF-I for 2 h and then with antibody against DIG for 1 h. Bands were visualized on radiographic film (a) or measured by an image analyzer (b) with the Enhanced Chemiluminescence Reagents. Arrows indicate migration positions of IGFBPs or non-specific (NS) bands.

Fig. 2 Elution profiles of salmon 28-kDa IGFBP on reversed-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. Arrow indicates a peak containing 28-kDa IGFBP. Inset: SDS-PAGE (1) and Western ligand blotting (2) of purified 28-kDa IGFBP. (1) Two micrograms of purified protein 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 in the inset indicates the migration position of purified 28-kDa IGFBP.

Fig. 3 Comparison of the partial N-terminal amino-acid sequence of salmon 28-kDa IGFBP with those of human and fish IGFBPs. Xs denote amino acid residues that were not sequenced.

Fig. 4. Comparison of deduced amino-acid sequences of cloned salmon and trout IGFBPs with those of human and fish IGFBP-1 and -4. The amino-acid sequences of fish and human IGFBP-1 and -4 were obtained from GenBank or Fugu genome database. They are aligned by the ClustalW method. The cysteine residues conserved in the IGFBP family are asterisked. The N-terminal amino acid sequences of purified salmon 28-kDa IGFBP and trout 30-kDa IGFBP, and the sequence obtained from a trypsin-digested salmon 28-kDa IGFBP are circled and assigned to corresponding positions. Potential PEST region of human IGFBP-1 is in dotted-lined box and

phosphorylated serines of human IGFBP-1 are in solid-line boxes. The position of Arg-Gly-Asp (RGD) integrin recognition sequence is underlined.

Fig. 5 Phylogenetic analysis of IGFBP-1 and -4 amino-acid sequences. Full-length sequences of human, mouse, rat, chicken, frog and several teleost species obtained from Genbank were analyzed by ClustalW using a neighbor-joining method based on uncorrected p-distance. The reliability of tree topology was assessed by the bootstrap method with 1000 replications. Numbers on branches are percentage of times that the two clades branched as sisters. Scale bar shows amino acid substitution per site.

Fig. 6 Tissue distribution of salmon *igfbp-1* expressions. Various tissues were collected from male Chinook salmon and expression was analyzed by RT-PCR. EF-1 α was used as a reference gene. A representative result from one of three individuals is shown.

Fig. 7 Effects of direct seawater transfer of under-yearling Chinook salmon smolt and parr on plasma IGFBP profiles (a) and levels of IGFBPs (b-d) and gill Na⁺, K⁺-ATPase activity (e). Under-yearling Chinook salmon smolt and parr were sorted by size and transferred to full seawater. Plasma was collected 0 h, 6 h, 12 h, 24 h, 48 h, 3 days and 7 days after transfer. (a) Representative Western ligand blots of plasma. NHS: normal human serum. (b) Plasma IGFBP-1a levels semi-quantified by scanning band intensities, normalized to human IGFBP-4 band intensity and expressed as ADU (n = 4-5). (c) Plasma IGFBP-1b levels quantified by radioimmunoassay (n = 6-7). (d) Plasma IGFBP-2b levels quantified by radioimmunoassay (n = 6-7). (f) Gill Na⁺,K⁺-ATPase activity (n = 6-7). Asterisks indicate significant difference ($P < 0.05$) between fish in freshwater (FW) and seawater (SW) for a given size category and time point.

Table 1. Amino acid sequence homologies (%) between human and fish IGFBP-1s

	human BP-1	zebrafish BP-1a	zebrafish BP-1b	salmon 28K BP	salmon 22K BP
human BP-1	-	40	35	41	40
zebrafish BP-1a	40	-	50	69	58
zebrafish BP-1b	35	50	-	56	63
salmon 28K BP	41	69	56	-	61
salmon 22K BP	40	58	63	61	-

Table 2. Molecular weights and structural characters of human and fish IGFBP-1s

	MW	PEST score	Phospholylation	N-glycosylation	RGD sequene
human BP-1		11.63	7 (5)	-	+
zebrafish BP-1a	25.5	3.05	7	+	-
zebrafish BP-1b	23.8	17.87	2	-	-
salmon BP-1a	25.9	2.44	2	-	-
salmon BP-1b	23.8	-7.11	1	-	-

Parence indicates actual number of phospholylation sites in human IGFBP-1.
MW: molecular weight (kDa); PEST: Pro (P), Glu (E), Ser (S) and Thr (T) rich segment
RGD: Arg-Gly-Asp

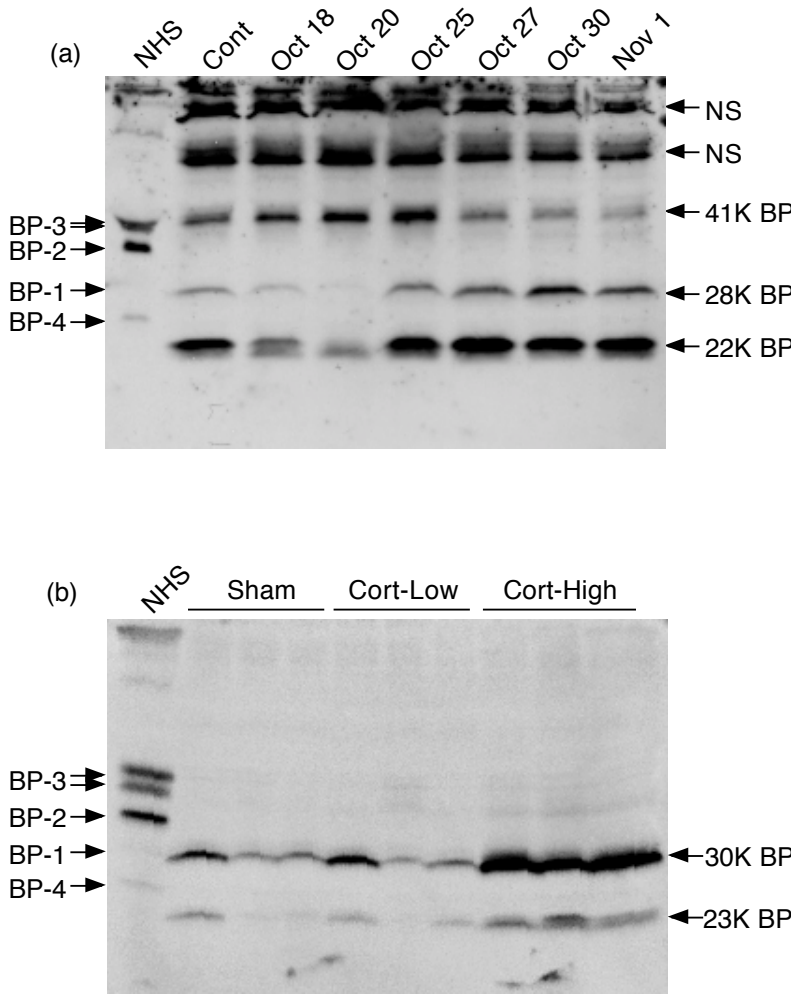
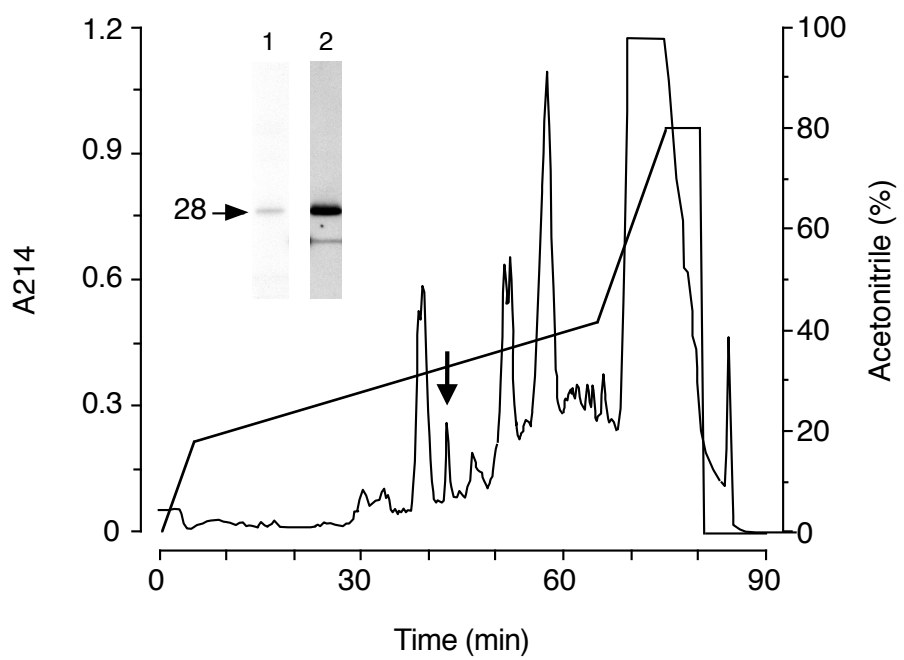


Figure2

Shimizu et al., Fig. 2



Shimizu et al., Fig. 3

human BP-1										A	P	W	Q	C	A	P	C	S	A	E	K	L	A	L	C	P	P	V		
trout 30K BP	K	P	V	L	A	O	E	P	I	R	C	A	P	C	S	P	E	K	L	S										
salmon 28K BP	S	P	V	L	A	X	X	X	X	X	C	A	P	C	S	P	E	K	L	S	E	C	P	A	V					
salmon BP-1	S	P	V	V	G	P	E	P	I	R	C	A	P	C	T	Q	E	K	L	D	E	C	P	A	I					
zebrafish BP-1a	S	P	V	V	G	O	E	P	I	R	C	A	P	C	S	P	E	R	L	A	E	C	P	A	V					
zebrafish BP-1b	S	P	V	P	A	P	E	P	I	R	C	A	P	C	S	A	D	Q	L	S	A	C	P	A	V					
human BP-4																														
											D	E	A	I	H	C	P	P	C	S	E	E	K	L	A	R	C	R	P	P

```

humanBP-1      M S - - - - - E V P V A R V L V L L L L T V Q V G - - - V T A G A P W Q C A P C S A E K L A L C P P V S A S C S E V 51
zebrafishBP-1a M N R L L L - N F F W V A A F S A L L A A P G L R A S P - - V V G Q E P I R C A P C S P E R L A E C P A V D A G C E E V 57
salmon28KBP    M S G L F H R N I L V A A A V C C S V L V R S V L G S P - - V V G Q E P I R C A P C S P E R L A E C P A V D A G C E E V 58
trout30KBP     M S G L F H R N I L V A A A V C C S V L V R S V L G S P - - V V G Q E P I R C A P C S P E R L A E C P A V D A G C E E V 58
zebrafishBP-1b M R - - - - - A V C V C V L L V C V L V C V L D A S P - - V P A P E P I R C A P C S A D Q L S A C P A V S P G C A E V 52
salmonBP-1     M L G L Y K - K L T L L A A M S L S L L T T L A Q S S P - - V V G P E P I R C A P C T Q E K L D E C P A I S P D C K Q V 57
troutBP-1      M L G L Y K - K L T L L A A M S L S L L T T L A Q S S P - - V V G P E P I R C A P C T Q E K L D E C P A I S P D C K Q V 57
A. salmonBP-4  M V R G G G A A I A P P C W G L W A V G A L S L A T - - - L C L T E E A I R C P V C S E E R L A S C Q L P D G S C E E T 57
humanBP-4      - - - - - M L P L C L - - - V A - A L L L A A G P G P S L G D E A I H C P P C S E E K L A R C R P P V G - C E E L 47

humanBP-1      T R S A G C G C C P M C A L P L G A A C G V A T A R C A R G L S C R A L P G E Q Q P L H A L T R G Q G A C V Q E S D A S 111
zebrafishBP-1a L R E P G C G C C L A C A L K R G D P C G I Y T A P C G S G L R C L P K P G E A R P L H A L T R G Q A V C T E S P E P N 117
salmon28KBP    L R E P G C G C C L A C A L K T G D L C G I Y T A P C G S G L R C T P R P G D L R P L H S L T R G Q A V C T E I P E P V 118
trout30KBP     L R E P G C G C C L A C A L K T G D L C G I Y T A P C G S G L R C T P R P G D L R P L H S L T R G Q A V C T E I P E P E 118
zebrafishBP-1b V R D P G C G C C S C A L R S G D S C G V H T A N C A G A L R C V P R A G D P R P L H S L T R G H A V C V E H - H P T 111
salmonBP-1     L R E P G C G C C M A C A L E K G A S C G V Y T A H C A Q G L K C S P R A G D P R P L H S L T R G Q A I C T E - - D Q G 115
troutBP-1      L R E P G C G C C M A C A L E K G A S C G V Y T A H C A Q G L K C S P R A G D P R P L H S L T R G Q A I C T E - - D Q G 115
A. salmonBP-4  V R E A G C G C C P T C A L A K G V H C G V Y S P R C G T G L R C Y P P R N V E R P L H S L M H G Q G V C T D E R D V E 117
humanBP-4      V R E P G C G C C A T C A L G L G M P C G V Y T P R C G S G L R C Y P P R G V E K P L H T L M H G Q G V C M E L A E I E 107

humanBP-1      A - P H A A E A G S P - - - - - E S P E S T E I T E E E L L D N F H L M A P S E E D H S I L W D A I S T Y D G S K A L 164
zebrafishBP-1a H - S Q S E T A P D H P E S H N G A T A - - - V N E G S S T V F V T G H G K P F D P R I I T N K - E S M K A K V N A I 171
salmon28KBP    S - S S V S Q N P D Q G A A D N A E T E N T A M V S D S G S S L Y L H G H S K P F D P R A A A D A L E S M K A K V N A I 177
trout30KBP     S - S S V S Q N P E Q G A A D N A E T E N T A M V S D S G S S L Y L H G H S K P F D P R A A A D A L E S M K A K V N A I 177
zebrafishBP-1b E - E D T E L T S E P - - - - - G S - - - - - L H H L L S L N R A L D P R D T A E A Q E S I K S Q L N A I 153
salmonBP-1     Q - E K V E G V P D H - - - - - S S - - - - - L A Y F L G L N T P F D T K N E G A Q - E S I K A K V N T I 156
troutBP-1      Q - E K V E G V P D H - - - - - S S - - - - - L A Y F L G L N T P F D T K N E G A Q - E S I K A K V N T I 156
A. salmonBP-4  E - N S A M D R Q D E I I P E H P N N S - - - - - N I R C S P Q D K R C I Q K T L A R H P A K S T N - - - Q R S N N A - 167
humanBP-4      A I Q E S L Q P S D K D E G D H P N N S - - - - - F S P C S A H D R R C L Q K H F A K I R D R S T S G G K M K V N G A P 162

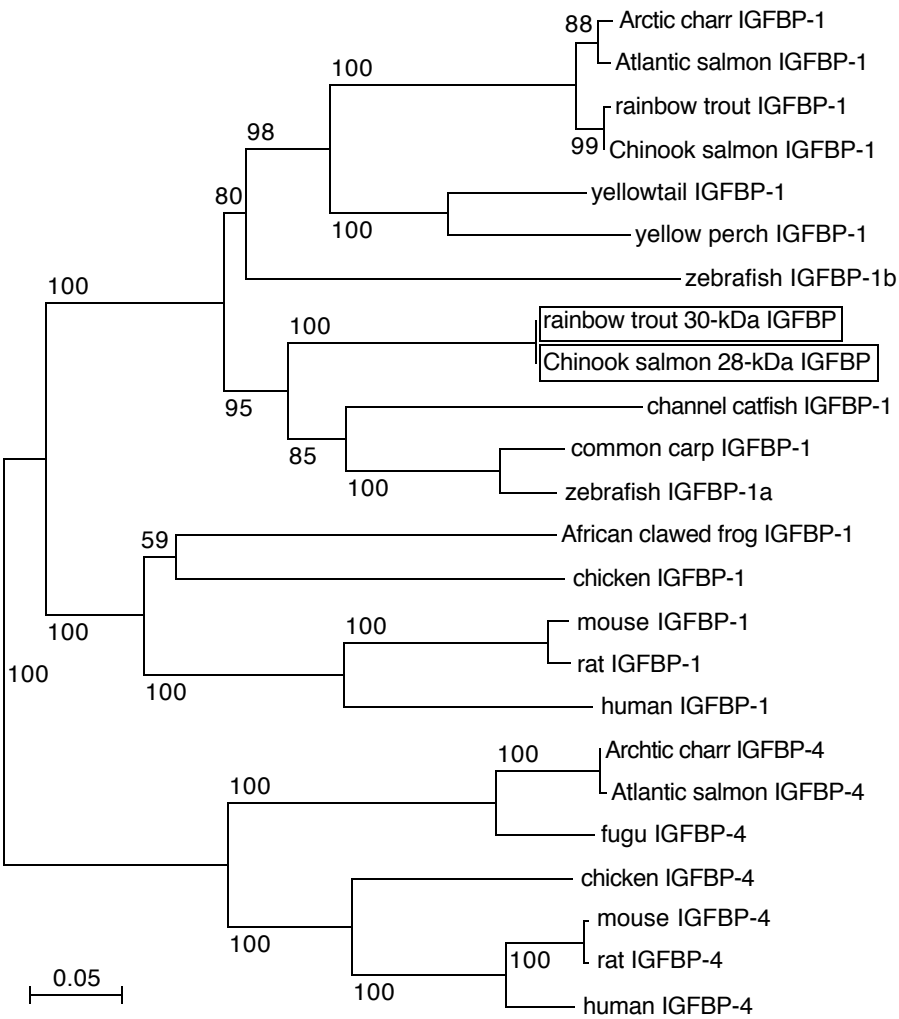
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zebrafishBP-1a R K K L V E - - Q G P C H I E L Q T A L D K I T K S Q Q K L G D K L I R F Y L P N C D K H G L Y K I K Q C E S S L D G Q 229
salmon28KBP    R K K L V E - - Q G P C H V E L Q R A L E K I A K S Q Q K L G D K L I R F Y L P N C D K H G L Y K A K Q C E S S L D G Q 235
trout30KBP     R K K L V E - - Q G P C H V E L Q R A L E K I A K S Q Q K L G D K L I R F Y L P N C D K H G L Y K A K Q C E S S L D G Q 235
zebrafishBP-1b R Q K L I Q - - Q G P C H T E L L A S L D V I T E S Q Q A L G E K F T S F Y L P N C D K H G F Y K A K Q C V S S L V A Q 211
salmonBP-1     R K K L V E - - Q G P C H I E L H A A L D K I T S S Q Q E L G E K F T N F Y L P N C D K H G F Y K A K Q C E S S L V G P 214
troutBP-1      R K K L E E - - Q G P C H I E L H A A L D K I T S S Q Q E L G E K F T N F Y L P N C D K H G F Y K A K Q C E S S L V G P 214
A. salmonBP-4  R E D P K - A A L A P C R A E L Q R A L D R L V S N - T R T H E D L Y S I P I P N C D K N G D F H A K Q C Q P A R D G Q 225
humanBP-4      R E D A R P V P Q G S C Q S E L H R A L E R L A A S Q S R T H E D L Y I I P I P N C D R N G N F H P K Q C H P A L D G Q 222

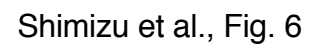
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zebrafishBP-1a R G K C W C V S S W N G K K I P G S S D L P A D A E C P E E L N H 262
salmon28KBP    K G R C W C V S F W N G K K I L G S T D L E G D A E C A Y E I N H 268
trout30KBP     K G R C W C V S F W N G K K I L G S T D L E G D A E C A Y E I N H 268
zebrafishBP-1b A P R C W C V S S W N G K R L A G T A D V E A D T P C P Q E L T H 244
salmonBP-1     H A R C W C V S S W N G K K I L G S N Y L P G - L E C Q L E L 244
troutBP-1      H A R C W C V S S W N G K K I L G S N Y L P G - L E C Q L E L 244
A. salmonBP-4  R G K C W C V D Q K T G M R L P G P L E L R G E L D C H Q L M T A T M R E 262
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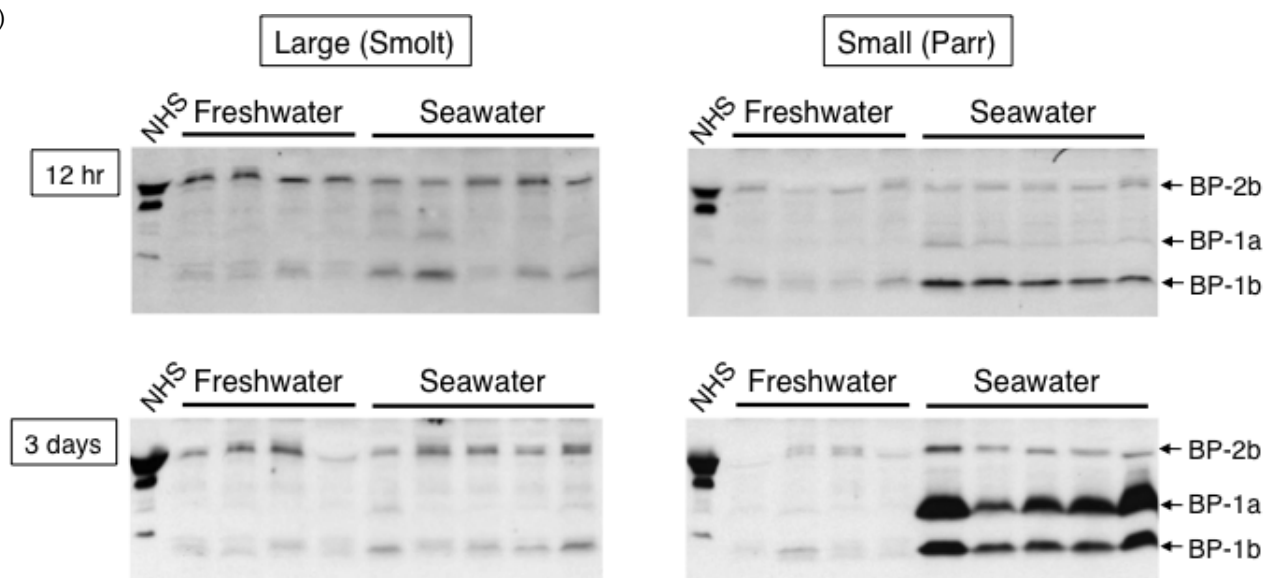
Figure5

Shimizu et al., Fig. 5

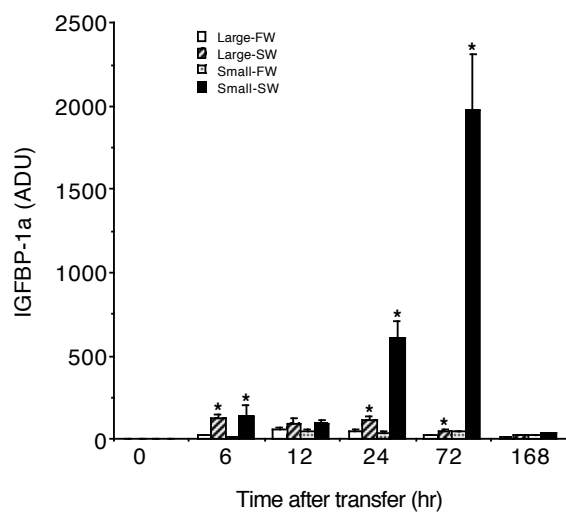




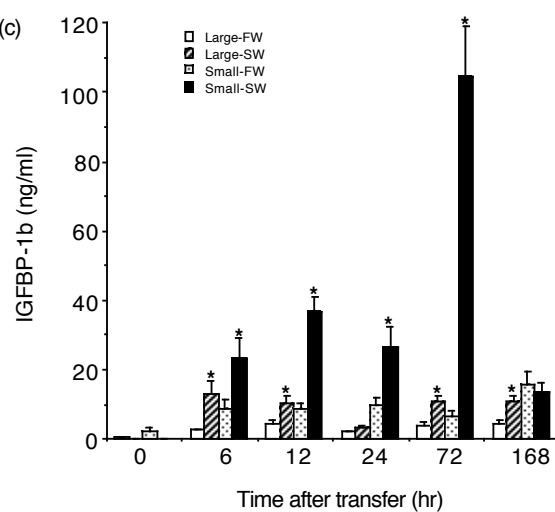
(a)



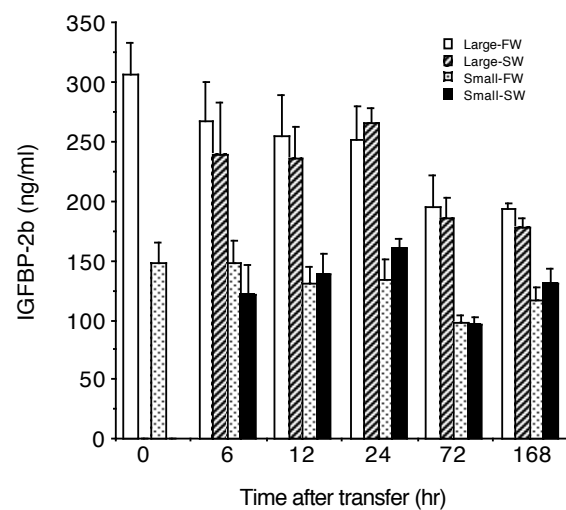
(b)



(c)



(d)



(e)

