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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 (*Oncorhynchus 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.

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 embryogeneis [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, 28–32 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

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(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 (*O. 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) R-250 (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 one-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 s, 50 °C for 45 s, 72 °C for 1 min 30 s; 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 (Foward: 5′ GAGCCCGAGAGCAGCTCTGTGTC 3′; Reverse: 5′ GGCTGGAGCCGGAGTCTGATACCAT 3′). A cDNA containing