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Research paper

Production of recombinant salmon insulin-like growth factor binding protein-1 subtypes

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ABSTRACT

Insulin-like growth factor (IGF)-I is a growth promoting hormone that exerts its actions through endocrine, paracrine and autocrine modes. Local IGF-I is essential for normal growth, whereas circulating IGF-I plays a crucial role in regulating the production and secretion of growth hormone (GH) by the pituitary gland. These actions of IGF-I are modulated by six insulin-like growth factor binding proteins (IGFBPs). In teleosts, two subtypes of each IGFBP are present due to an extra round of whole-genome duplication. IGFBP-1 is generally inhibitory to IGF-I action under catabolic conditions such as fasting and stress. In salmon, IGFBP-1a and -1b are two of three major circulating IGFBPs and assumed to affect growth through modulating IGF-I action. However, exact functions of salmon IGFBP-1 subtypes on growth regulation are not known due to the lack of purified or recombinant protein. We expressed recombinant salmon (rs) IGFBP-1a and -1b with a fusion protein (thioredoxin, Trx) and a His-tag using the pET-32a(+) vector expression system in *Escherichia coli*. Trx.His.rsIGFBP-1s were isolated by Ni-affinity chromatography, enzymatically cleaved by enterokinase to remove the fusion partners and further purified by reversed-phase HPLC. We next examined effects of rsIGFBP-1a and -1b in combination with human IGF-I on GH release from cultured masu salmon (*Oncorhynchus masou*) pituitary cells. Unexpectedly, IGF-I increased GH release and an addition of rsIGFBP-1a, but not rsIGFBP-1b, restored GH levels. The results suggest that IGFBP-1a can inhibit IGF-I action on the pituitary in masu salmon. Availability of recombinant salmon IGFBP-1s should facilitate further functional analyses and assay development.

1. Introduction

Insulin-like growth factor (IGF)-I is a 7.5 kDa peptide hormone produced mainly by the liver in in response to stimulation by growth hormone (GH) (Daughaday and Rotwein, 1989). Hepatic IGF-I is secreted into the bloodstream and mediates many actions of GH. IGF-I is also expressed in virtually all tissues and regulates cell proliferation, differentiation, growth and apoptosis in paracrine and autocrine modes (Le Roith et al., 2001). Local IGF-I is essential for postnatal growth, whereas endocrine IGF-I is important for regulating circulating GH by inhibiting its synthesis and secretion at the pituitary level, as well as the hypothalamic level (Ohlsson et al., 2009).

Although the contribution of endocrine IGF-I in postnatal growth of mammals may not be as significant as local IGF-I, it forms a relatively large pool in the circulation and affects many tissues. This can be achieved by the presence of six IGF-binding proteins (IGFBPs). IGFBPs are not structurally related to the IGF-receptor, but are single chain peptides 23–31 kDa in size, consisting of three domains (Firth and Baxter, 2002; Forbes et al., 2012). The cysteine-rich N- and C-terminal domains are required for high-affinity IGF-binding and the mid linker (L)-domain contains sites for phosphorylation, glycosylation and enzymatic cleavage that are specific to each IGFBP (Firth and Baxter, 2002; Forbes et al., 2012). IGFBPs prolong the half-life of IGF-I from 5–10 min up to 12 h by forming a high-molecular weight complex which prevents IGF-I from being ultrafiltered by the kidney and protects it from enzymatic degradation (Rajaram et al., 1997). IGFBPs can either inhibit or promote IGF-I action through regulating the availability of IGF-I to its receptor in target tissues. In addition, some IGFBPs translocate into the nucleus and regulate gene transcription independent of IGF-I (Forbes et al., 2012).

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Multiple whole-genome duplication events in combination with local modifications shaped the number of IGFBP genes. Phylogenetic studies suggest that six vertebrate IGFBPs were created first by a local duplication of an ancestral protein (two genes) followed by two whole-genome duplications (eight genes) and subsequent loss of two genes (six genes) (Daza et al., 2011). Since teleosts experienced an extra round of whole-genome duplication, they usually have two copies of each member of the six IGFBPs, except IGFBP-4 (Daza et al., 2011). Moreover, a recent study by Macqueen et al. (2013) demonstrated that salmonids such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) have 19 IGFBP subtypes due to their tetraploid origin. These studies highlight the presence of multiple IGFBP subtypes in fish and suggest their functional partitioning.

IGFBP-1 is one of the major IGFBPs in the circulation and generally inhibitory to IGF action by preventing it from interacting with its receptor (Lee et al., 1993, 1997; Wheatcroft and Kearney, 2009). Unlike other IGFBPs, IGFBP-1 shows dramatic daily changes in response to meals. Insulin is the major inhibitor of IGFBP-1 production, whereas cortisol stimulates its production (Lee et al., 1993, 1997; Wheatcroft and Kearney, 2009). These findings suggest that IGFBP-1 is important for glucose regulation under catabolic conditions.

Fish likely possess two IGFBP-1s in their circulation. In the fish circulation, three IGFBPs are consistently detected around 20–25, 28–32 and 40–50 kDa (Kelley et al., 2001). The two low-molecular-weight IGFBPs were assumed to be IGFBP-1 or -2 since they increased in response to fasting, stress and cortisol injection (Siharath et al., 1996; Park et al., 2000; Kajimura et al., 2003; Kelley et al., 2006; Kajimura and Duan, 2007). In salmon plasma/serum, three IGFBPs are detected at 41, 28 and 22 kDa, respectively (Shimizu et al., 2000). We demonstrated, through protein purification and cDNA cloning, that the 28- and 22-kDa IGFBPs were co-orthologs of mammalian IGFBP-1 and named them IGFBP-1a and -1b, respectively (Shimizu et al., 2005, 2011a). However, based on nomenclature proposed by Macqueen et al. (2013), circulating salmon 28- and 22-kDa IGFBPs correspond to IGFBP-1a1 and -1b1, respectively. Given their similar molecular weights and physiological regulation, the two circulating low-molecular-weight IGFBPs in other fishes are likely also IGFBP-1 subtypes.

The presence of two subtypes of fish IGFBP-1 and their function were first shown in zebrafish (*Danio rerio*; Kamei et al., 2008). Zebrafish IGFBP-1a and -1b are capable of inhibiting proliferation of embryonic cells, demonstrating their inhibitory actions, consistent with mammalian IGFBP-1. Kamei et al. (2008) proposed that although their IGF-inhibitory action overlapped, they underwent subfunctional partitioning in terms of IGF-binding affinity, temporal expression, and physiological response. We showed that salmon *igfbp-1* subtypes were differentially expressed: *igfbp-1a* was widely distributed in many tissues while *igfbp-1b* was almost exclusively expressed in the liver, suggesting spatially partitioned functions (Shimizu et al., 2011a). Together this suggests that IGFBP-1 subtypes play pivotal roles in inhibiting circulating IGF-I actions in fish.

Functional studies on fish IGFBP-1 have been done in zebrafish and carp (*Cyprinus carpio*) using a morpholino knockdown approach (Kajimura et al., 2005; Kamei et al., 2008; Sun et al., 2011). However, such analysis is restricted to developing fish embryos, and no studies have examined roles of IGFBPs in postnatal growth in fish. This is mainly due to the lack of enough purified IGFBPs. Purification of IGFBP-1 from serum is not practical since its circulating levels are not high (Shimizu et al., 2005, 2011a). The aims of the present study were to produce recombinant salmon IGFBP-1 subtypes using a bacterial expression system and test their effects on IGF-regulated GH secretion from salmon pituitary cells in vitro.

2. Materials and methods

2.1. Cloning of open reading frames (ORFs) of masu salmon igfbp-1a and -1b

Liver was collected from yearling masu salmon (*O. masou*) that had been fasted for 1 month at Nanae Freshwater Experimental Station, Field Science Center for Northern Biosphere, Hokkaido University, Japan (Kameda-gun, Hokkaido, Japan). Total RNA was extracted from the liver using Isogen (Nippon gene; Tokyo, Japan) and single-strand cDNA was reverse-transcribed using SuperScript III (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions.

A primer set flanking the ORF of *igfbp-1a* (Table 1) was designed based on the cDNA sequences of Chinook salmon (*O. tshawytscha*) and rainbow trout (*O. mykiss*) as reported in Shimizu et al. (2011a). Reverse transcriptase (RT)-PCR was performed using AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Foster City, CA) and a Veriti Thermal Cycler (Applied Biosystems). PCRs consisted of 1 cycle of 95 °C for 10 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; 1 cycle of 72 °C for 7 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. (2011b).

Part of the ORF of *igfbp-1b* was amplified using a primer set designed similarly as described above (Table 1). The complete ORF sequence of *igfbp-1b* was obtained by 5′- and 3′-RACE (rapid amplification of cDNA ends) using SMARTer™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) with gene-specific primers (Table 1).

2.2. Subcloning into the expression vector

Subcloning of cDNAs encoding the ORFs of *igfbp-1a* and *-1b* into the pET-32a(+) expression vector (Novagen, Madison, WI) was performed by use of In-Fusion™ Advantage PCR Cloning Kit (Clontech) according to the manufacturer's instructions and the methods described in Mizuta et al. (2013). Briefly, the PCR reaction was performed with primers that contained adaptor sequences corresponding to the terminal 15 bp sequences of the expression vector (Table 1). PCRs consisted of 1 cycle of 98 °C for 10 s; 35 cycles of 98 °C for 10 s, 55 °C for 5 s, 72 °C for 2 min. The pET-32a (+) vector (1 ng) was linearized by an inverse PCR using PrimeSTAR® Max Premix (Takara Bio, Shiga, Japan) and a primer set (10 μM each) complementary to the vector (Table 1) in a 50-μl reaction. PCRs consisted of 1 cycle of 95 °C for 10 s; 35 cycles of 98 °C for 10 s, 50 °C for 5 s, 72 °C for 15 s. The PCR products were separated by 1.5% agarose gel, purified and cloned into the pET32a(+) expression vector which provides thioredoxin (Trx) and a His-tag (6 x His) at the N-terminal region of the resulting recombinant protein as fusion partners. This construct was confirmed by sequencing.

2.3. Expression of recombinant proteins

The constructed plasmids (pET-Trx.His.rsIGFBP-1a and -1b) were transformed into the *Escherichia coli* strain Rosetta-gami™ B(DE3)pLysS (Novagen). Transformants were cultured overnight in 3 ml Luria-Bertani (LB) medium containing ampicillin (50 μg/ml), tetracycline (12.5 μg/ml), kanamycin (15 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C and expression of Trx.His.rsIGFBP-1a and -1b was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The cell pellet collected after centrifugation was resuspended with 5 ml of Bugbuster Protein Extraction Reagent (Novagen) containing 0.2% lysonase and 1% cocktail protease inhibitor (Novagen) per

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