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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.

Keywords: insulin-like growth factor binding protein-1; recombinant protein; salmon; pituitary cell culture; growth hormone release

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 hr 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).

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 (*Oncorhynchus 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 sec, 60°C for 30 sec, 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 sec; 35 cycles of 98°C for 10 sec, 55°C for 5 sec, 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 sec; 35 cycles of

98°C for 10 sec, 50°C for 5 sec, 72°C for 15 sec. 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 1 g of bacterial pellet. Trx.His.rsIGFBP-1a and -1b appeared to be dominant in the insoluble fraction and were purified by His-Bind Resin (Novagen) chromatography. After binding of the recombinant protein to Ni-charged His-Bind Resin, the column was washed with a buffer containing 60 mM imidazole, and the samples were subsequently eluted with a buffer containing 1 M imidazole. The elute was dialyzed with ~1 L of 20 mM Tris-HCl (pH 8.0) containing 2% NaCl and 6 M urea and refolded by gradually decreasing the urea concentration during dialysis.

2.4. Enzymatic digestion and purification of recombinant proteins

Refolded Trx.His.rsIGFBP-1a and -1b were digested by an enterokinase using Tag.off™ High Activity rEK Kit (Novagen). Twenty micrograms of protein were mixed with 1 μl (1 unit) enterokinase and incubated at room temperature for 20 h. Digested rsIGFBP-1a or -1b were applied to reversed-phase high pressure liquid chromatography (HPLC) using a Vydac C-4 column (0.46 x 5 cm; Separation Group, Hesperia, CA). rsIGFBPs were separated from undigested Trx.His.rsIGFBPs and Trx.His by a linear gradient of 0-80% acetonitrile in 0.1% trifluoroacetic acid (TFA). Fractions were collected into low-adsorption 0.5 ml tubes (PGC Scientifics, Frederick, MD). Acetonitrile and TFA were removed by N_2 evaporation from the fractions and purified proteins were concentrated by semi-lyophilization. Protein concentrations of purified rsIGFBP-1s were determined using the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

174
175 *2.5. Electrophoresis and ligand blotting*
176 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel
177 and 12.5% separating gel was carried out. Samples were treated with an equal volume of the
178 sample buffer containing 2% SDS and 10% glycerol with or without 5% 2-mercaptoethanol at
179 85°C for 5 min. Gels were run in a solution of 50 mM Tris, 400 mM glycine and 0.1% SDS at 50
180 V in the stacking gel and at 100 V in the separating gel until the bromophenol blue dye front
181 reached the bottom of the gel. Gels were stained with 0.1% Coomassie Brilliant Blue (CBB) R250
182 (Bio-Rad, Hercules, CA). Molecular mass was estimated with Precision Marker (Bio-Rad).
183 Western ligand blotting with digoxigenin-labeled human IGF-I (DIG-hIGF-I) was
184 carried out as described in Shimizu et al. (2000). After electroblotting, the nitrocellulose
185 membrane was incubated with 10-50 ng/ml DIG-hIGF-I for 2 h at room temperature and then
186 incubated with an antibody against DIG, conjugated with horseradish peroxidase (Roche,
187 Indianapolis, IN) at a dilution of 1:1,500-2,500 for 1 h at room temperature. IGFBP was
188 visualized by use of enhanced Chemiluminescence (ECL) Prime Western blotting reagents
189 (Amersham Life Science, Arlington Heights, IL).
190
191 *2.6. Primary pituitary cell culture*
192 Primary pituitary cell culture was conducted according to methods of Ando et al. (2004) with
193 some modifications. Pituitaries were collected from 150 one-year-old masu salmon of both sexes
194 (fork length 21.0 ± 0.3 cm, body weight 88.8 ± 3.9 g; expressed as mean ± SE of 10 fish) at Toya
195 Lake Station, Field Science Center for Northern Biosphere, Hokkaido University (Abuta-gun,
196 Hokkaido, Japan) in late November 2016. The experiment was carried out in accordance with
197 guidelines of the Hokkaido University Animal Care and Use Committee. The fish were
198 anesthetized with 3.3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan), decapitated, and
199 pituitaries were collected. The pituitaries were immersed in ice-cold RPMI containing 20 mM
200 HEPES (Gibco Invitrogen, Carlsbad, CA), 9 mM sodium bicarbonate, penicillin-streptomycin
201 (100U/ml) (Gibco Invitrogen) and fungizone (0.25 mg/ml) (Gibco Invitrogen), and brought back
202 to the laboratory. They were minced with a surgical blade and transferred to the above medium
203 supplemented with 0.1% collagenase (Gibco Invitrogen) and 3% bovine serum albumin (BSA,
204 Gibco Invitrogen). After incubation at 12°C for 20 h, cells were mechanically dissociated by
205 aspiration several times in a 10-ml pipette. They were washed twice with the RPMI medium
206 supplemented with Serum Replacement 2 (Sigma-Aldrich, Tokyo, Japan), which does not contain

steroid hormones, glucocorticoids or growth factors (Sigma-Aldrich). The dispersed cells were immediately plated on 12-well culture plates (BD Biosciences, Franklin Lakes, NJ), pre-coated with 50 μg/ml poly-D-lysine hydrobromide (Sigma, St. Louis, MO), at a density of 2.3 x 10^5 cells/1.3 ml medium/well (n = 3) and allowed to attach to the wells at 12°C for 48 h. After the wells were washed with the medium, human IGF-I (GroPep Bioreagents, Thebarton, Australia) was added to the medium and cells were further incubated for 24 h. After incubation, media were collected and frozen at -80°C until assayed for GH. The cells were harvested by scraping them twice in 0.7 ml of medium, collected by centrifugation at 5,000 rpm for 10 min, and frozen at -80°C until use. Our preliminary experiments on the effect of incubation time (6, 12, 24, 48 and 72 h) after adding human IGF-I (hIGF-I; GroPep, Adelaide, Australia) suggested that incubation for 24 or 48 h was optimal to capture the release of GH in response to hIGF-I treatment (data not shown).

2.7. Immunoblotting

GH levels in the culture media were semi-quantified by immunoblotting using an antiserum against GH. Purified chum salmon GH and antiserum against this GH were gifts from Dr. Shunsuke Moriyama, Kitasato University, and Dr. Akihiko Hara, Hokkaido University, respectively. The culture media (20 μl each) were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane as described in Shimizu et al. (2000). The membrane was incubated with anti-GH serum at a dilution of 1:50,000 for 2 h at room temperature and then with goat anti-rabbit IgG-HRP conjugate (Bio-Rad) at a dilution of 1:20,000 for 1 h at room temperature. Immunoreactive bands were visualized using ECL Prime Western blotting reagent. Intensities of the GH bands were quantified using ImageJ program and normalized between blots using purified GH as a reference.

2.8. Real-time quantitative PCR (qPCR)

Primers for qPCR of *gh* (Forward: 5' AATGGTCAGAAACGCCAACCAG 3'; Reverse: 5' CAGCTGCTGAGAGTCATTGTCATC 3') were designed from a masu salmon *gh* cDNA sequence (GenBank Accession No. X59762). Primer sequences for *ef-1a* (Forward: 5' GAATCGGCCATGCCCGGTGAC 3'; Reverse: 5' GGATGATGACCTGAGCGGTG 3') were from Shimomura et al. (2012). Both primer sets were designed to span an exon/exon boundary to avoid amplification of genomic DNA. Reverse transcribed-PCR using these primer sets was performed to prepare assay standards. PCR products run on 1.5% agarose gels were excised and

purified using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). Copy numbers of the purified amplicons were calculated from the molecular weight of the amplicon and concentration. The standard cDNAs were serially diluted from 1 x 10^7 to 3 x 10^2 copies. qPCR assays were prepared using Power SYBR Green PCR Master Mix (Applied Biosystems) in a reaction volume of 20 µl with primer concentration of 100 nM and run on a 7300 Sequence Detector (Applied Biosystems) using the manufacturer's recommended cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Measured values for *gh* were normalized to those of *ef-1α*. Specificity of each primer pair was confirmed by the appearance of a single peak in the dissociation curve and the lack of amplification for no template controls (data not shown). The performance of qPCR was evaluated by calculating the amplification efficiencies of the standard curves, which were within the range of 97-100%. The coefficients of determination for the standard curves were between 0.99-1.00.

2.9. Statistical analysis

Results of the cell culture experiments were analyzed first by two-way ANOVA (IGF x IGFBP) using the JMP program (SAS Institute Inc., Cary, NC). When significant effects were found, differences were further identified by one-way ANOVA followed by Fisher's protected least significant difference (PLSD) test. Differences among groups were considered significant at $P < 0.05$.

3. Results

3.1. cDNA cloning of masu salmon igfbp-1a and -1b ORFs

cDNAs for masu salmon *igfbp-1a* and *-1b* ORFs were cloned and their sequences deposited in GenBank (Submission ID# 1983152). Masu salmon IGFBP-1a and -1b consist of 268 and 247 amino acid residues with estimated molecular weights of 25,988 and 24,127 Da, respectively.

3.2. Expression and purification of rsIGFBP-1a and -1b

Trx.His.rsIGFBP-1a and -1b were induced by adding IPTG to the culture medium and appeared as 49- and 39-kDa bands, respectively, on SDS-PAGE under reducing conditions (Fig. 1). Both Trx.His.rsIGFBP-1a and -1b were mainly found in the insoluble fraction, and were extracted using 0.2% lysonate and solubilized in a buffer containing 6 M urea. They were separated from bacterial proteins by Ni-affinity chromatography (data not shown) and refolded by gradually reducing the urea concentration by dialysis. The refolded Trx.His.rsIGFBP-1a showed the ability

to bind DIG-IGF-I on ligand blotting (Fig. 2). The fusion partners (i.e. Trx and His-tag) were cleaved from rsIGFBP-1a by enzymatic digestion using enterokinase (Fig. 2). rsIGFBP-1a was further purified by reversed-phase HPLC using a C4 column (Fig. 3). Similarly, Trs.His.rsIGFBP-1b was enzymatically cleaved (Fig. 4) and subjected to HPLC (Fig. 5). Purified rsIGFBP-1a was detected as a primary band at 27 kDa and minor band at 29 kDa, while rsIGFBP-1b appeared as broad bands around 22 kDa on SDS-PAGE under non-reducing conditions (Fig. 6). By ligand blot, rsIGFBP-1a and -1b appeared at 28 and 22 kDa, respectively, which corresponded to respective molecular weights of the intact proteins in the serum (Fig. 6).

3.3. Effects of rsIGFBP-1a and -1b on GH release from pituitary cells

Effects of hIGF-I and rsIGFBP-1a and -1b on GH release from cultured, adult masu salmon pituitary cells were examined (Fig. 7a). hIGF-I alone increased GH release by 24 h. The addition of rsIGFBP-1a diminished the IGF-induced GH release, whereas the addition of rsIGFBP-1b had no significant effect. Neither rsIGFBP-1a nor -1b affected the GH release. Pituitary *gh* mRNA levels were not affected by hIGF-I or rsIGFBP-1a (Fig. 7b). On the other hand, a signficant overall effect of IGFBP-1b on *gh* mRNA ($P = 0.011$) was apparent by two-way ANOVA (Fig. 7b).

4. Discussion

Production of recombinant protein is useful for functional analysis. Expression of recombinant IGFBPs has been primarily conducted with zebrafish (Duan et al., 1999; Kamei et al., 2008; Zhou et al., 2008; Dai et al., 2010). For example, Kamei et al. (2008) expressed and purified recombinant zebrafish IGFBP-1a and -1b with fusion partners Myc and His-tag and showed that both IGFBPs inhibited the IGF-I-induced cell proliferation of cultured zebrafish embryonic cells. Also, recombinant zebrafish IGFBP-2 and -5 were shown to inhibit IGF actions (Duan et al., 1999; Zhou et al., 2008; Dai et al., 2010). However, there are only a few studies in other fish species that have characterized IGFBPs (Bauchat et al., 2001; Shimizu et al., 2003; Zhou et al., 2013). This is mainly due to the lack of purified/recombinant protein. The present study produced recombinant salmon (rs) IGFBP-1 subtypes using a bacterial expression system and examined their effects on GH release from salmon pituitary cells.

We expressed rsIGFBP-1 with fusion partners, thioredoxin (Trx) and His-tag, using the pET-32a(+) expression vector. Trx is often used as a fusion partner of recombinant protein since it increases translation efficiency and solubility of recombinant protein (LaVallie et al., 1993). In addition, it acts as a molecular chaperon to help the protein fold correctly and form

disulfide bonds (Kern et al., 2003). Expressing Trx as a fusion partner was effective to produce a soluble, correctly folded rat IGFBP-4 (Chelius et al. 2001). In the present study, however, Trx was not effective to retain he recombinant protein in the soluble fraction. On the other hand, the Trx.His.IGFBP-1s showed an ability to bind the labeled IGF-I, as confirmed by ligand blotting, indicating that correct protein folding was achieved with the presence of Trx. Protein folding is one of the critical steps for producing functional recombinant protein, especially for proteins with disulfide bonds. Since Trx.His.IGFBP-1s were found in the insoluble fraction, addition of 6 M urea was necessary to solubilize them. With urea present, the disulfide bonds of the IGFBP-1s were dissociated. IGFBP-1 has 12 and 6 cysteine residues in the N- and C-terminal domains, respectively, and forms intra-domain disulfide bonds (Neumann and Bach, 1999). Our attempts to produce rsIGFBP-1s with only His-tags using the pET-16b vector were unsuccessful due to the formation of a precipitate during dialysis, which was probably due to incorrect refolding without Trx (data not shown). Thus, addition of Trx was indeed necessary to achieve correct protein refolding. Indeed, Wilkinson et al. (2004) reported that the strategy to express rsIGF-II using the pET32a first in the insoluble fraction and then refold it with the aid of Trx was effective to obtain a high yield of functional protein. Thus, the procedure established in this study is one good way to obtain these functional recombinant proteins.

We prepared recombinant fish IGFBP for the first time without a fusion partner. The fusion partners Trx and His-tag were enzymatically cleaved from rsIGFBP-1 using an enterokinase and rsIGFBP-1 was purified by reversed-phase HPLC. Although Trx.His.IGFBP-1s had the ability to bind IGF-I, it is preferable not to have fusion partners for functional analyses. An enterokinase cleavage site in the vector was used to separate rsIGFBP-1s from the fusion partners. However, an apparent non-specific digestion of rsIGFBP-1b occurred when the recommended amount of enterokinase was used (data not shown). Applying half the amount of enterokinase protected rsIGFBP-1s but resulted in lower cleavage efficiencies. The recovery rate of rsIGFBP-1b from Trx.His.IGFBP-1b after HPLC was 10% while it was 22% for rsIGFBP-1a. Further optimization of the production of rsIGFBP-1s, such as using another enzyme recognition site, would increase the yield.

Inhibition of pituitary GH production and secretion by circulating IGF-I is an important negative feedback loop of the somatotropic system (Ohlsson et al., 2009). In vitro experiments in mammals and fishes have demonstrated that IGF-I directly acts on the pituitary cells (Pérez-Sánchez et al., 1992; Fruchtman et al., 2000, 2002; Luckenbach et al., 2010). In the present study, IGF-I alone unexpectedly increased GH release from the pituitary cells in the

present study. This may be due to the season and/or stage of the masu salmon used in the experiment. Pituitaries were collected from one-year-old male and female masu salmon in late November. These fish were likely under a transition from immature to early maturing stage (Furukuma et al., 2008). Previous studies showing the inhibitory effects of IGF on GH release used pituitary cells from immature fish (Pérez-Sánchez et al., 1992; Fruchtman et al., 2000, 2002; Luckenbach et al., 2010). In addition, fish were sampled in early winter when growth was reduced. Thus, during the early reproductive stage and/or non-growing season, the negative feedback loop between GH and IGF-I could be diminished. This of course is speculative and future studies are necessary to examine the stage/season dependent IGF-I action on GH release.

The present study examined for the first time, combined effects of IGF-I and rsIGFBP-1a or -1b on GH release in vitro in fish. Whether or not IGF-I can modulate GH production and secretion in vivo may depend on the type of IGFBP carrying the IGF-I. Circulating IGFBP-1 could inhibit the interaction of IGF-I with IGF-receptors on the pituitary cells. Results of the present study suggest that rsIGFBP-1a is capable of modulating IGF-I action at the pituitary level. When rsIGFBP-1a was added in combination with IGF-I to the culture medium, increased GH release was restored to the basal level, suggesting that effects of IGF-I were inhibited by IGFBP-1a. Our finding is in accord with the suggestion by Fruchtman et al. (2002) that IGFBPs play a significant role in modulating the action of IGF-I on GH release. On the other hand, IGFBP-1b had no significant inhibitory effect on IGF-I action. These disparate effects may be due to differences in IGF-binding affinity between rsIGFBP-1a and -1b. In zebrafish, IGFBP-1a had a higher affinity for IGFs than did IGFBP-1b (Kamei et al., 2008). We did not specifically measure the affinity of rsIGFBP-1a and -1b for IGFs in the present study. However, when the same amount of rsIGFBP-1a and -1b was loaded on the gel and analyzed by ligand blotting, the band intensity of IGFBP-1a was stronger than that of IGFBP-1b, suggesting IGFBP-1a has a higher affinity for IGF-I. Kamei et al. (2008) proposed that zebrafish IGFBP-1a and -1b partitioned IGF-inhibitory actions temporally and physiologically. In agreement with this, our results support subfunctionalization of the two salmon IGFBP-1 subtypes, in terms of their degree of IGF-inhibitory action.

Post-translational modification of proteins, such as phosphorylation and glycosylation, occur in IGFBPs and may modulate their actions (Firth and Baxter, 2002; Forbes et al., 2012). It is of note that rsIGFBP-1a and -1b produced in the bacterial system are unlikely to be phosphorylated. Mammalian IGFBP-1 is usually highly phosphorylated and the degree of phosphorylation affects its affinity for IGFs, being higher for the phosphorylated form in humans

(Jones et al., 1991). Conversely, dephosphorylation of rat IGFBP-1 did not reduce its IGF-binding affinity (Peterkofsky et al., 1998). Since the deduced amino acid sequences of salmon IGFBP-1a and -1b possess potential phosphorylation sites in the L-domain (Shimizu et al., 2005, 2011a), results of the present study using non-phosphorylated rsIGFBP-1s need to be interpreted with caution.

IGFBPs can exhibit IGF-independent actions on cell growth through interacting with the cell surface and/or translocating into the nucleus (Firth and Baxter, 2002; Forbes et al., 2012). Mammalian IGFBP-1 has an Arg-Gly-Asp (RGD) motif that is a cell surface α5β1 integrin binding site. In contrast, there is no RGD motif in salmon or other fish IGFBP-1s (Kamei et al., 2008; Shimizu et al., 2011a). In addition, neither mammalian nor fish IGFBP-1s have a nuclear localization sequence. These structural features suggest that fish IGFBP-1s function through modulation of IGF-I actions. Supporting this, when rsIGFBP-1a or -1b was added to the culture medium in the absence of IGF-I, they had no effect on GH release. However, there was an overall effect of rsIGFBP-1b on *gh* mRNA, which may suggest its direct action. More experiments are needed to address whether or not salmon IGFBP-1s have IGF-independent actions.

In summary, this study is the first to produce and purify rsIGFBP-1a and -1b without fusion partners in a bacterial expression system. The rsIGFBP-1s demonstrated an ability to bind IGF-I and rsIGFBP-1a specifically exhibited IGF-inhibitory action in our salmon pituitary cell culture system. The availability of rsIGFBP-1a and -1b enable us to further analyze their physiological functions. In addition, these recombinant proteins should be useful as antigens for raising specific antisera and as standards for immunoassays.

Acknowledgement

We thank Akihiko Hara, Faculty of Fisheries Sciences, Hokkaido University, and Shunsuke Moriyama, Kitasato University School of Marine Biosciences, for providing antiserum against chum salmon GH and purified chum salmon GH, respectively. We also thank Nanae Freshwater Experimental Station and Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University, Japan, for providing experimental fish. We extend our thanks to J. Adam Luckenbach, Northwest Fisheries Science Center, NOAA Fisheries, for correcting the English in the manuscript. This work was supported by JSPS KAKENIHI Grant Numbers JP24580256 and JP16H04966.

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540

Figure legends

Fig. 1. SDS-PAGE of extracts of bacterial cells transformed with pET32a-Trx.His.IGFBP-1a (left panel) and -1b (right panel) before (-) and after (+) addition of IPTG to the culture medium. Bacterial proteins induced by IPTG were fractionated into soluble and insoluble fractions via the Bugbuster Protein Extraction Kit. SDS-PAGE was run under reducing conditions. Migration positions of the molecular weight marker are indicated by arrowheads on the left. Arrowheads on the right indicate proteins induced by IPTG and their approximate molecular weights.

Fig. 2. SDS-PAGE and ligand blotting of recombinant IGFBP-1a before (-) and after (+) digestion by enterokinase (EK). Twenty micrograms of Trx.His.rsIGFBP-1a was incubated with 1 unit of EK at room temperature for 20 h. They were separated by 12.5% SDS-PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue (CBB; left panel). The same samples were electroblotted onto a nitrocellulose membrane after SDS-PAGE and subjected to ligand blotting (LB; right panel) using digoxigenin-labeled human IGF-I (50 ng) and antiserum against digoxigenin (1:20,000). Arrowheads indicate migration positions and molecular weights of undigested Trx.His.rsIGFBP-1a and digested rsIGFBP-1a, respectively.

Fig. 3. Elution profiles of rsIGFBP-1a on reversed-phase HPLC. The digested sample was applied to a Vydac c-4 column and eluted by a linear gradient of 0-80% acetonitrile in 0.1% TFA (indicated by the dashed line). Numbers indicate fractions collected for analysis by ligand blotting (inset). Arrowheads on the ligand blotting indicate migration positions of undigested (42 kDa) and digested (28 kDa) recombinant IGFBP-1a.

Fig. 4. SDS-PAGE and ligand blotting of recombinant IGFBP-1b before (-) and after (+) digestion by enterokinase (EK). Twenty micrograms of Trx.His.rsIGFBP-1b was incubated with 1 unit of EK at room temperature for 20 h. They were separated by 12.5% SDS-PAGE under non-reducing conditions and stained with Coomassie Brilliant Blue (CBB; left panel). The same samples were electroblotted onto a nitrocellulose membrane after SDS-PAGE and subjected to ligand blotting (LB; right panel) using digoxigenin-labeled human IGF-I (50 ng) and antiserum against digoxigenin (1:20,000). Arrowheads indicate migration positions and molecular weights of undigested Trx.His.rsIGFBP-1b and digested rsIGFBP-1b, respectively.

Fig. 5. Elution profiles of rsIGFBP-1b on reversed-phase HPLC. The digested sample was

applied to a Vydac c-4 column and eluted by a linear gradient of 0-80% acetonitrile in 0.1% TFA (indicated by the dashed line). Numbers indicate fractions collected for analysis by ligand blotting (inset). Arrowheads on the ligand blotting indicate migration positions of undigested (35 kDa) and digested (22 kDa) recombinant IGFBP-1b.

Fig. 6. SDS-PAGE and ligand blotting of purified rsIGFBP-1a and -b. Eight micrograms of purified protein was loaded onto 12.5% gels and electrophoresed under non-reducing conditions. One gel was stained with Coomassie Brilliant Blue (CBB; left panel) and the other was subjected to ligand blotting (LB; right panel) using digoxigenin-labeled human IGF-I (50 ng) and antiserum against digoxigenin (1:20,000). Arrowheads indicate migration positions and molecular weights of purified proteins. Serum: masu salmon serum.

Fig. 7. Effects of rsIGFBP-1a and -1b on GH release by cultured masu salmon pituitary cells into the media (a) and *gh* mRNA in the pituitary cells (b). Pituitary cells from one-year-old male and female masu salmon were pre-cultured for 2 days and incubated for 24 h without (-) or with (+) human IGF-I and rsIGFBP-1a or rsIGFBP-1b. Asterisks indicate overall effects. Symbols sharing the same letters are not significantly different from each other.

Table 1

Table 1

Primer sequences used for cDNA cloning and subcloing

Primer Name	Primer sequence (5'-3')	Direction	Use
IGFBP-1a ORF F	CTTCCTTATTCCAGTCTCTCCTTAT	Foward	RT PCR
IGFBP-1a ORF R	TGCATGCGTGCGTGTTCT	Reverse	RT PCR
IGFBP-1b ORF F	GAGATGCTTGGATTATATAAGAAGTTG	Forward	RT PCR
IGFBP-1b ORF R	TTAGAGCTCCAGCTGGCACT	Reverse	RT PCR
IGFBP-1b GSP F	GACCTCAGCTCCCTGCCCTACCTCCT	Forward	3'-RACE
IGFBP-1b GSP R	CAGCTCAATGTGGCAGGGCCCCTG	Reverse	5'-RACE
pET32a F	GCCATGGCTGATATCGGATC	Foward	Inverse PCR
pET32a F	CTTGTCGTCGTCGTCGGTAC	Reverse	Inverse PCR
pET32a-IGFBP-1a F	GACGACGACGACAAGTCCCCGGTGTTAGCGCAA	Forward	InFusion PCR
pET32a-IGFBP-1a R	GATATCAGCCATGGCTCAGTGGTTGATCTCATAAGCGCA	Reverse	InFusion PCR
pET32a-IGFBP-1b F	GACGACGACGACAAGTCCCCCGTGGTAGGTCCA	Forward	InFusion PCR
pET32a-IGFBP-1b R	GATATCAGCCATGGCTTAGTGAGTGAGCTCCTGCTGACA	Reverse	InFusion PCR

ORF: open reading frame; GSP: gene-specific primer. Underlines are sequences complement to pET-32a

Figure 1

Tanaka et al., Fig. 1

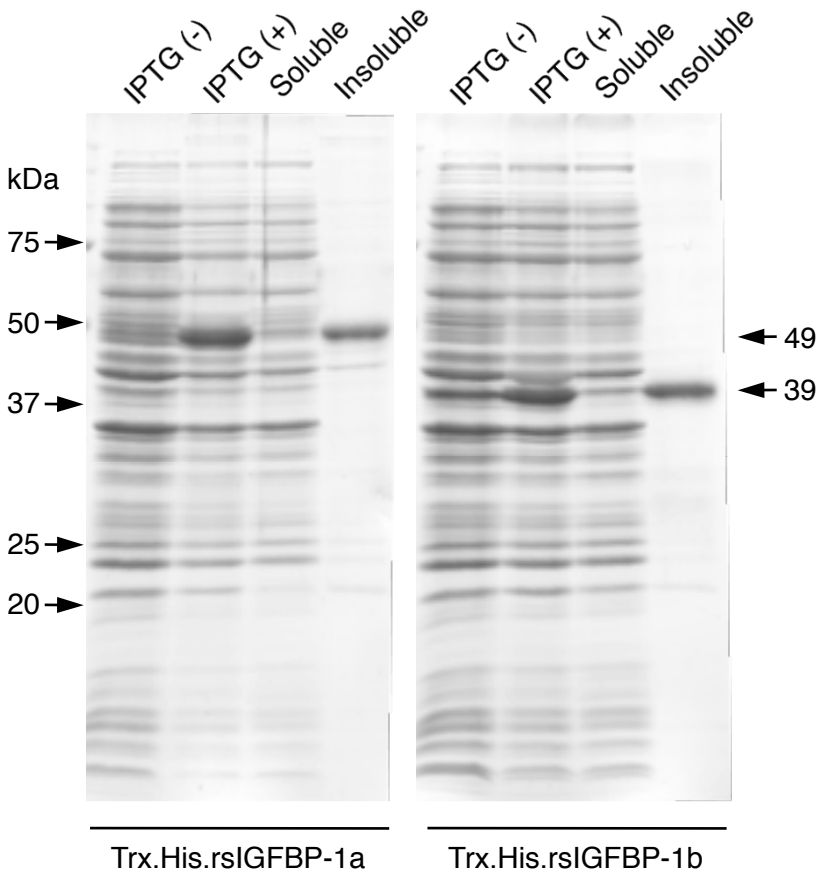

Figure 2

Tanaka et al., Fig. 2

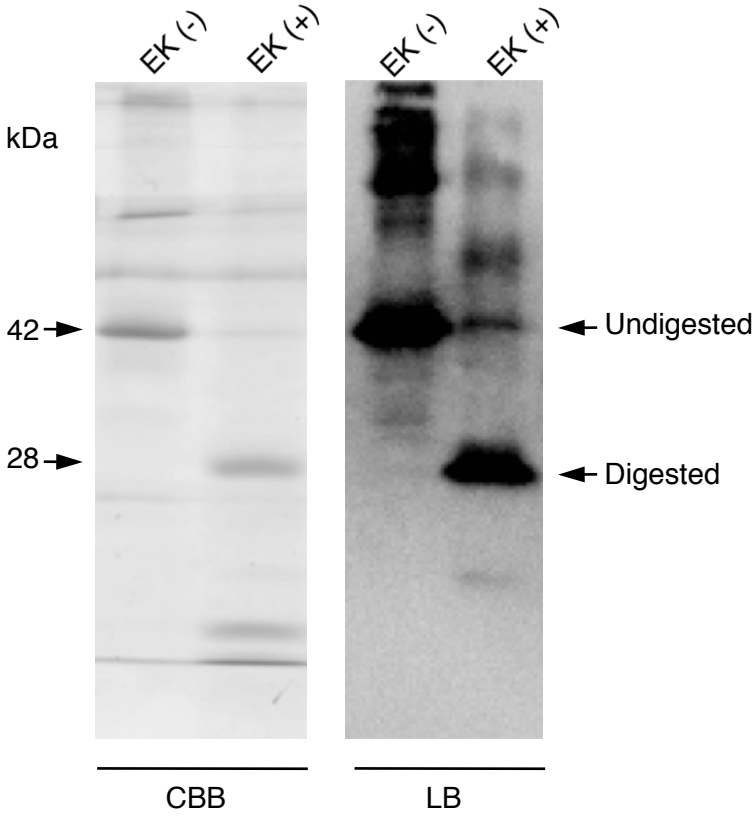

Figure 3

Tanaka et al., Fig. 3

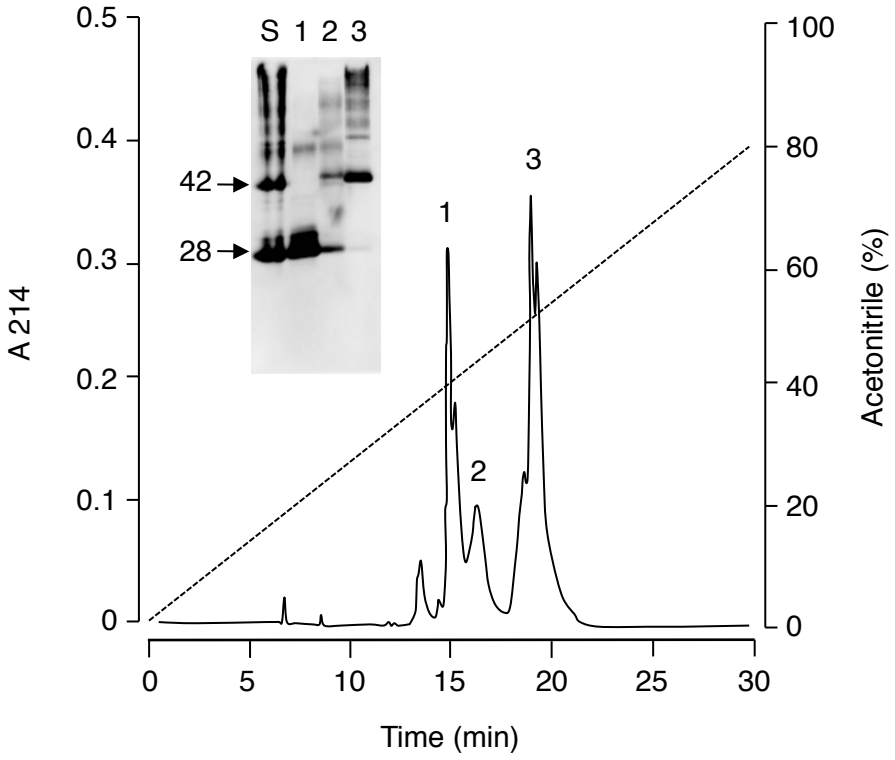

Figure 4

Tanaka et al., Fig. 4

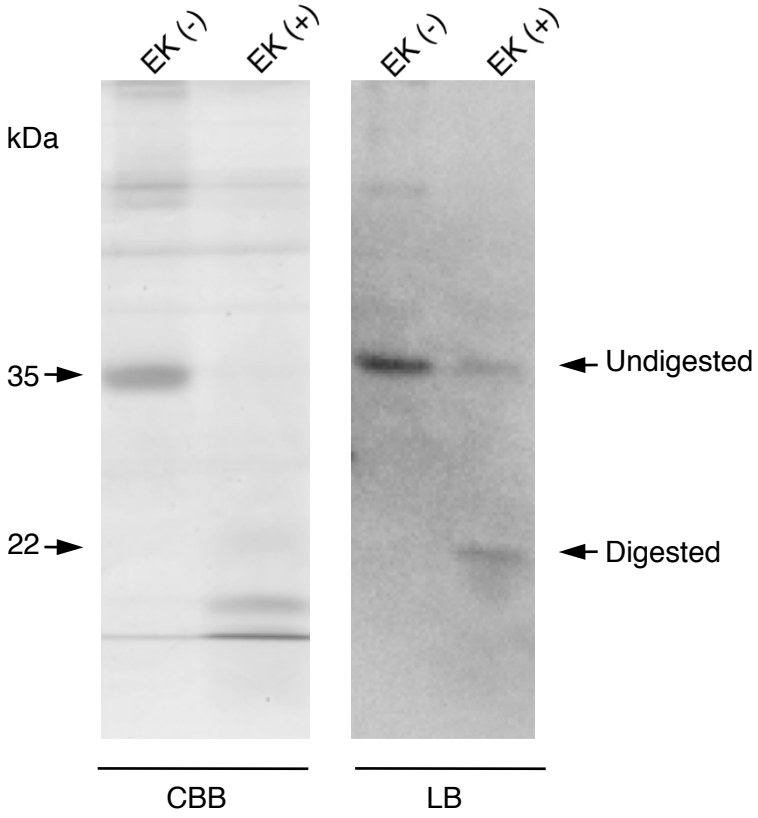

Figure 5

Tanaka et al., Fig. 5

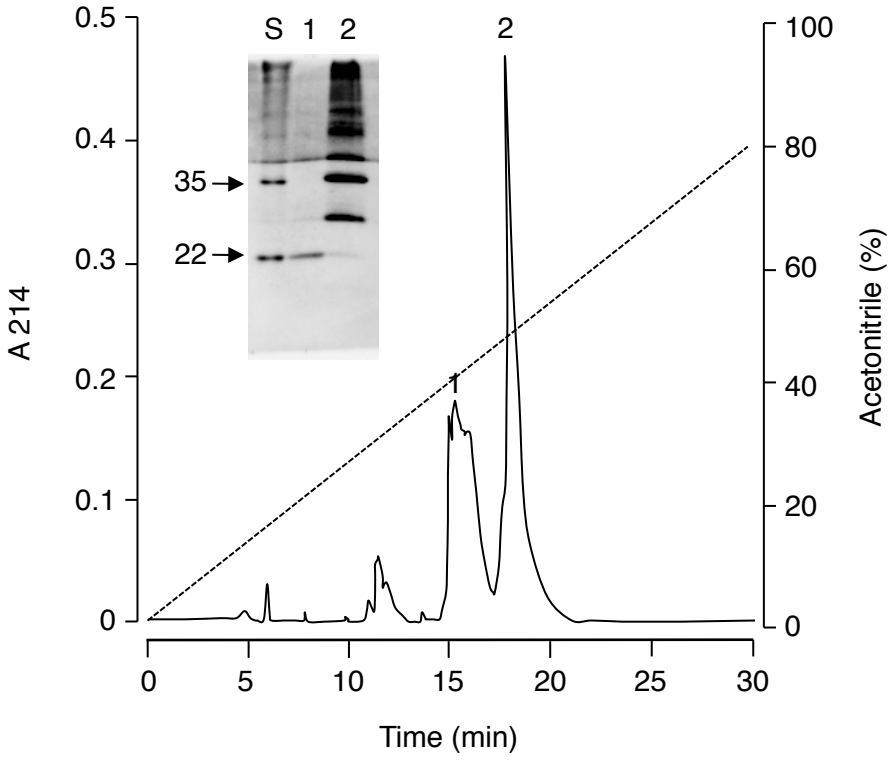

Figure 6

Tanaka et al., Fig. 6

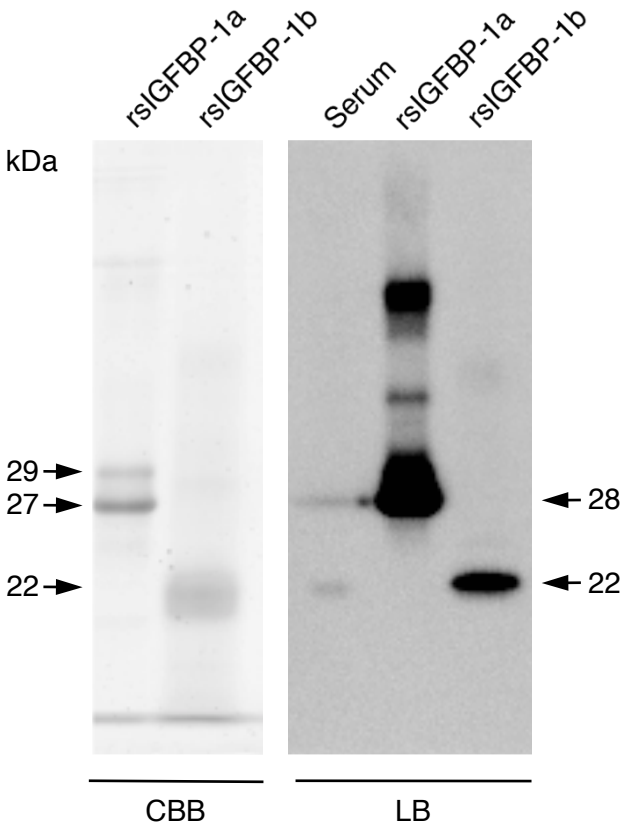

Figure 7

Tanaka et al., Fig. 7