Title:

Circulating insulin-like growth factor binding proteins in fish: their identities and physiological regulation

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Abstract

Insulin-like growth factor binding proteins (IGFBPs) play crucial roles in regulating the availability of IGFs to receptors and prolong the half-lives of IGFs. There are six IGFBPs present in the mammalian circulation with IGFBP-3 being most abundant. In mammals IGFBP-3 is the major carrier of circulating IGFs, facilitated by forming a ternary complex with IGF and an acid-labile subunit (ALS). IGFBP-1 is generally inhibitory to IGF action by preventing it from interacting with its receptors. In teleosts, the third-round of vertebrate whole genome duplication created paralogs of each IGFBP, except IGFBP-4. In the fish circulation, three major IGFBPs are typically detected at molecular ranges of 20-25, 28-32 and 40-50 kDa. However, their identities are not well established. Three major circulating IGFBPs in Chinook salmon have been identified through protein purification and cDNA cloning. Salmon 28- and 22-kDa IGFBPs are co-orthologs of IGFBP-1, termed IGFBP-1a and -1b, respectively. They are induced under catabolic conditions such as stress and fasting but their responses are somewhat different, with IGFBP-1b being the most sensitive of the two. Cortisol stimulates production and secretion of these IGFBP-1 subtypes while, unlike in mammals, insulin may not be a primary suppressor. Salmon 41-kDa IGFBP, a major carrier of IGF-I, is not IGFBP-3, as might be expected extrapolating from mammals, but is in fact IGFBP-2b. Salmon IGFBP-2b levels in plasma are high when fish are fed, and GH treatment increases its circulating levels similar to mammalian IGFBP-3. These findings suggest that salmon IGFBP-2b acquired the role and regulation similar to mammalian IGFBP-3. Multiple replication of fish IGFBPs offer a unique opportunity to investigate molecular evolution of IGFBPs.

Keywords: insulin-like growth factor binding protein, circulation, fish, identification, hormone, environment

1. Introduction

Insulin-like growth factor (IGF)-I plays a pivotal role in growth regulation in vertebrates and exerts its action through endocrine, paracrine and autocrine modes (Daughaday and Rotwein, 1989; LeRoith et al., 2001; Ohlsson et al., 2009). Endocrine IGF-I is produced mainly by the liver in response to stimulation by growth hormone (GH), and it mediates many of the GH actions in target tissues (Salmon and Daughaday, 1957; Daughaday and Rotwein, 1989). IGF-I is also expressed in virtually all tissues and acts as a local growth factor (D'Ercole et al., 1980; LeRoith et al., 2001). IGF-I is structurally related to insulin arising from a common ancestor, and the ancestral IGF molecule further diverged into IGF-I and -II by gene duplication (Collet et al., 1998; Reinecke and Collet, 1998; Wood et al., 2005a). In contrast to insulin, circulating levels of IGFs are relatively high for hormones averaging around 200 ng/ml for IGF-I and 500 ng/ml for IGF-II in humans (Daughaday and Rotwein, 1989). These high circulating levels are due to stabilization by IGF-binding proteins (IGFBPs). IGFBPs prolong the half-lives of IGFs by protecting them from glomerular filtration and enzymatic degradation (Guler et al., 1987, 1989). In the mammalian circulation, there are three pools of IGFs around 150-, 50- and 7.5-kDa (Fig. 1; Guler et al., 1987, 1989; Zapf, 1995; Rajaram et al., 1997). In humans, 75-80% of circulating IGFs are in the 150-kDa pool consisting of IGF, IGFBP-3/-5 and an acid-labile subunit (ALS) (Baxter and Martin, 1989). ALS does not bind IGFs but binds IGFBP-3 to form a ternary complex. As this complex is too large to cross the capillary barrier or to be filtered at the kidney, it constitutes a large pool of IGFs in the circulation (Binoux and Hossenlopp, 1988; Hasegawa et al., 1992). The 50-kDa pool consists of IGFBP and IGF and carries about 20% of circulating IGFs (Guler et al., 1987, 1989). Less than 1% of circulating IGFs is in the free form (Frystyk et al., 1994).

IGFBPs also regulate the availability of IGFs to target tissues. There are six IGFBPs in the mammalian circulation, and they modulate the activity of IGFs in various ways (Rajaram et al., 1997). IGFBP-1 is the first member of the IGFBP family to be identified and generally inhibits IGF action by preventing it from interacting with its receptor (Lee et al., 1993, 1997; Kajimura and Duan, 2007; Wheatcroft and Kearney, 2009). Unlike other IGFBPs, circulating levels of IGFBP-1 show dynamic fluctuations in response to meals and increase under catabolic conditions such as fasting (Yeoh and Baxter, 1988). In mammals insulin is the primary suppressor of IGFBP-1 whereas cortisol increases its production and secretion (Unterman et al., 1991; Katz et al., 1998). IGFBP-2 is also considered to be an inhibitor of IGF-I action and IGFBP-2 levels are generally high under catabolic conditions, although its levels are relatively stable and no clear

hormonal control has been reported (Rajaram et al., 1997; Wheatcroft and Kearney, 2009). IGFBP-3, as mentioned above, is a major carrier of circulating IGF-I in mammals by forming a ternary complex with IGF and ALS (Baxter and Martin, 1989; Martin and Baxter, 1992). The major site of production of IGFBP-3 and ALS is the liver, but different cell types within the liver produce them. IGFBP-3 is produced by Kupffer and endothelial cells, and ALS is produced by hepatocytes (Chin et al., 1994; Villafuerte et al., 1994). All components of the ternary complex are induced by GH. IGFBP-3 can be a stimulator of IGF-I action in terms of protecting IGF-I in the circulation and releasing it to target tissues when the binding protein is partly degraded by specific enzymes (Martin and Baxter, 1992; Rajaram et al., 1997; Firth and Baxter, 2002).

In fish blood, three major IGFBPs around 20-25, 28-32 and 40-50 kDa have been detected. They have molecular weights similar to mammalian IGFBP-4, -1/-2 and -3, respectively (Kelley et al., 1992, 2001). Their circulating levels are regulated by hormones, nutrition, stress and other factors as is the case for mammalian IGFBPs, suggesting that fish IGFBPs are also important for modulating activity of circulating IGFs (Duan, 1997; Kelley et al., 2000, 2001, 2002, 2006; Wood et al., 2005a). However, the identities of the three IGFBPs in fish are not well established, making comparisons with mammalian IGFBPs difficult. This review summarizes identities of the three major IGFBPs in the fish circulation with special reference to salmon and their physiological regulation. As this review focuses only on selected numbers of IGFBPs in the fish circulation, readers should refer to other excellent reviews/references dealing with molecular and functional aspects of fish IGFBPs (Wood et al., 2005a; Rodgers et al., 2008; Daza et al., 2011; Maqueen et al., 2013; Lappin et al., 2016).

2. Detection of IGFBPs in fish blood

IGFBPs in plasma/serum are usually detected by ligand blotting using ^{125}I-labeled human IGF-I developed by Hossenlopp et al. (1986). This method visualizes IGFBP bands using a labeled IGF-I instead of a primary antibody and is based on the ability of proteins to bind the ligand. Modified ligand blotting using non-radio labeled ligands such as biotinylated and digoxigenin-labled IGF-Is have been developed (Grulich-Henn et al., 1998; Shimizu et al., 2000). Normal human serum consistently exhibits five IGFBP bands at 41.5, 38.5, 34, 30 and 24 kDa, which correspond to IGFBP-3, -3, -2, -1 and -4, respectively (Fig. 2). The doublet bands at 41.5 and 38.5 kDa are of IGFBP-3 with different degrees of N-glycosylation (Firth and Baxter, 1999). IGFBP-5 and -6 are probably difficult to detect in normal human serum/plasma since their concentrations are low in the circulation, they are diffuse due to different degrees of glycosylation,

and/or they are unable to bind IGFs when electroblotted onto a nitrocellulose membrane (Rajaram et al., 1997). Since band intensity is a reflection of both relative abundance and affinity to IGF used as a ligand, care should be taken when comparing different types of IGFBPs.

The presence of fish IGFBPs was first reported by Kelley et al. (1992) in the circulation of four teleost species (coho salmon, *Oncorhynchus kisutch*; striped bass, *Morone saxatilis*; tilapia, *Oreochromis mossambicus*; longjawed mudsucker (goby), *Gillichthys mirabilis*) by ligand blotting using ^{125}I-labeled human IGF-I (Fig. 3). Niu and Le Bail (1993) also utilized this technique and detected a major IGFBP band at 41.5 kDa and minor bands at 47.7 kDa and 30-34 kDa in serum of rainbow trout (*O. mykiss*) (Fig. 3). The presence of fish IGFBP was also confirmed by IGF-binding assays (Anderson et al., 1993; Niu et al., 1993). The detection of IGFBPs both by ligand blotting and binding assay in plasma of the lamprey (*Geotria australis*) (Fig. 3) led researchers to hypothesize that IGFBP is an ancient protein family that emerged in the early history of vertebrates (Upton et al., 1993).

Multiple IGFBP bands have since been detected in several fish species and they are within a molecular range of 20-50 kDa. Since the molecular weights of these IGFBPs are close to each other, there is some inconsistency in reporting their sizes even in the same species. This inconsistency is probably due to differences in gel concentration for electrophoresis and molecular markers used. High-molecular-weight bands around 70 kDa are often detected in fish plasma on ligand blotting (Fig. 2; Fukuzawa et al., 1996; Shimizu et al., 2000), but these are unlikely to be specific IGFBP bands since the displacement of the binding by unlabelled IGF-I in ligand blotting is often difficult. However, a possibility that these bands are aggregates of IGFBP(s) cannot be ruled out.

3. Purification of fish IGFBPs

Bauchat et al. (2001) were the first to purify a 30-kDa IGFBP from conditioned medium of the rainbow trout hepatoma cell (RTH-149) culture. The medium was first fractionated by hydrophobic chromatography using a Phenyl-Sepharose column. Fractions containing IGF-binding activity were loaded onto an IGF-I affinity column, and IGFBP was eluted with 1 M acetic acid. The IGFBP fraction was further purified by reversed-phase HPLC using a Vydac C-4 column. Approximately 70 μg of purified IGFBP was obtained from 600 ml conditioned medium.

Chinook salmon serum contains three major IGFBPs at 41, 28 and 22 kDa (Fig. 2), and they were purified from serum of spawning males (Shimizu et al., 2003, 2005, 2011b). The purification procedures were based on those for mammalian and trout IGFBPs (Walton et al.,

1989; Bauchat et al., 2001) with some modifications. The 41- and 28-kDa IGFBPs were co-purified by the same procedure (Shimizu et al., 2003b, 2011a). Typically, one liter of salmon serum was mixed with proteinase inhibitors and acidified with 2 M acetic acid to dissociate endogenous IGFs from IGFBPs. IGFs were removed by adsorbing to a SP-Sephadex C-25 followed by centrifugation. The supernatant containing IGFBPs was neutralized and the precipitate was removed. The supernatant was loaded onto an IGF-I affinity column and IGFBPs were eluted with 1 M acetic acid. IGFBPs were purified by reversed-phase HPLC using a Vydac C-4 column as described in Bauchat et al. (2001). The 41- and 28-kDa IGFBPs were eluted at 32% and 33% acetonitrile, respectively (Shimizu et al., 2003b, 2011a). The recovery of purified proteins from 1 L serum was 70 µg and 11-24 µg for 41- and 28-kDa IGFBPs, respectively. It is worth noting that purified IGFBPs were "sticky" being easily adsorbed to tubes when lyophilized (Shimizu personal communication). They should be stored in a low adsorption tube and not be completely lyophilized. Since the 22-kDa IGFBP was found to be labile under acidic conditions and when unoccupied by endogenous IGFs, the acidification step was omitted from its purification (Shimizu et al., 2005). Instead, salmon serum was first fractioned by ammonium sulfate precipitation and loaded onto an IGF-I column. The 22-kDa IGFBP was further purified by reversed-phase HPLC and eluted at 37% acetonitrile. Final yield of the purified protein per 1 L serum was 45 µg (Shimizu et al., 2005).

When band patterns of purified salmon IGFBPs on SDS-PAGE were compared between non-reducing and reducing conditions, the molecular weight of the 41-kDa IGFBP decreased by 2 kDa while those of the 28- and 22-kDa IGFBPs increased by approximately 5 kDa (Shimizu personal communication). These changes should be attributed to differential conformational changes by the dissociation of intra-peptide disulfide bonds (Shimizu, personal communication). Purified 41-kDa IGFBP was shown to be N-glycosylated (Shimizu et al., 2003b). Purified fish IGFBPs were also analyzed for their partial N-terminal amino acid sequences (Bauchat et al., 2001; Shimizu et al., 2003b, 2005, 2011a). Four of five N-terminal amino acid sequences of rainbow trout and Chinook salmon 28-30-kDa IGFBPs were identical and similar to those of human IGFBP-1 and -4 (Shimizu et al., 2011a). Salmon 22-kDa IGFBP also showed relatively high sequence homologies to human IGFBP-1 and -4 (Shimizu et al., 2006). On the other hand, the partial N-terminal amino acid sequence of salmon 41-kDa, which had a molecular weight similar to human IGFBP-3, had the highest sequence homology with IGFBP-2 (Shimizu et al., 2003b). The discrepancy between its molecular weight and N-terminal amino acid sequence raised questions about the identity of salmon 41-kDa IGFBP.

4. cDNA cloning of circulating salmon IGFBPs

cDNAs for the three circulating salmon IGFBPs were cloned from the liver of Chinook salmon by reverse-transcribed (RT)-PCR followed by 5'- and 3'-rapid amplification of cDNA ends (RACE) (Shimizu et al., 2005, 2011a,b). IGFBPs were first amplified by RT-PCR using degenerate primers based on the partial N-terminal amino acid sequences of purified proteins. Forward primers were specific to each IGFBP and a reverse primer was universal to the IGFBP family designed based on a conserved C-terminal region. These combinations of primers successfully amplified different IGFBP cDNAs and partial cDNA sequences were used to further design gene specific primers for RACE. As results, full-length cDNAs for the 22-, 28- and 41-kDa IGFBPs were obtained (Shimizu et al., 2005, 2011a,b). An additional IGFBP sequence was obtained during the cloning of the 41-kDa IGFBP (Shimizu et al., 2011b). These cDNA sequences were compared with those of human and zebrafish IGFBPs. Sequence comparison and phylogenetic analysis revealed that salmon 22-, 28- and 41-kDa IGFBP were IGFBP-1b, -1a and -2b, respectively (Shimizu et al., 2011a,b). The additional IGFBP was identified as IGFBP-2a (Shimizu et al., 2011b). The presence of two subtypes of each IGFBP is common in teleosts except for IGFBP-4 (Daza et al., 2011). These two subtypes are due to the teleost-specific third round of whole genome duplication (Daza et al., 2011). In addition, since salmonids underwent a fourth round of whole genome duplication, they possess up to four copies of each IGFBP (Macqueen et al., 2013). The cloned circulating salmon IGFBPs were assigned to be IGFBP-1a1, -1b1, -2a and -2b1, respectively (Macqueen et al., 2013). However, in this review, they are simply called "a" or "b" to avoid confusion about exact correspondence to IGFBP subtypes of other fishes.

The finding that salmon 41-kDa IGFBP was IGFBP-2b was controversial to what was understood for this protein. Salmon 41-kDa IGFBP had a molecular weight similar to that of mammalian IGFBP-3. Salmon IGFBPs often appeared as doublet bands of 41- and 43-kDa and they were N-glycosylated as was the case of mammalian IGFBP-3 (Shimizu et al., 2003b). Salmon 41-kDa IGFBP was also similar to mammalian IGFBP-3 in terms of its physiological regulation; it was up-regulated under anabolic states and induced by GH treatment (Shimizu et al., 1999, 2003a). These biochemical and physiological data suggested that the salmon 41-kDa IGFBP corresponded functionally to mammalian IGFBP-3 (Shimizu et al., 2003b). However, when a cDNA for salmon IGFBP-3 was cloned, its N-terminal amino acid sequence did not match that of purified 41-kDa IGFBP (Shimizu et al., 2011b). Moreover, amino acids obtained after

digesting purified 41-kDa IGFBP by cyanogen bromide were assigned to the internal regions of the deduced IGFBP-2b sequence. These results indicated that circulating salmon 41-kDa IGFBP was not IGFBP-3 but was instead IGFBP-2b (Shimizu et al., 2011b). Fish 40-50 kDa IGFBP had been assumed to be an ortholog of mammalian IGFBP-3, and the structure and function of IGFBP-3 were assumed to be "conserved". However, it was IGFBP-2b that physiologically corresponded to mammalian IGFBP-3. Our findings suggested that there is a functional convergence between mammalian IGFBP-3 and salmon IGFBP-2b. On the other hand, despite a relatively high sequence homology between human and salmon IGFBP-3, their functions appeared to have diverged.

5. Structural features of circulating salmon IGFBPs

IGFBPs are a single chain polypeptide consisting of three functional domains: N-, C- and L-domains (Fig. 4; Shimasaki and Ling, 1991; Hwa et al., 1999; Firth and Baxter, 2002; Forbes et al., 2012). The N-terminal domains of IGFBP-1-5 have 12 cysteine residues to form six disulfide bonds within the domain, which is necessary for IGF binding. A GCGCCXXC motif is well conserved in the IGFBP family except for IGFBP-6. IGFBP-6 lacks two cysteine residues resulting in five disulfide bonds. There are several IGFBP-related proteins (IGFBPrPs) having sequence homology to the IGFBP N-terminus but not to other domains (Kim et al., 1997; Hwa et al., 1999). It is of note that IGFBPrPs have the ability to bind IGFs and insulin but with much lower affinity compared to IGFBPs due to lack of the IGFBP C-terminus (Hwa et al., 1999). The C-terminal domain of IGFBP has 6 cysteine residues with a conserved CWCV motif, which are also required for high affinity binding to IGFs (Forbes et al., 2012). There are some motifs in the C-terminus important for IGFBPs to associate with the cell surface or translocate into the cell. Such motifs include an Arg-Gly-Asp (RGD) sequence found in IGFBP-1 and -2 (Firth and Baxter, 2002) and a nuclear localization signal (NLS) found in IGFBP-3, -5 and -6 (Forbes et al., 2012). The middle linker (L) domain of IGFBP is less conserved among IGFBPs and contains several motifs specific to each type such as sites for N-glycosylation, proteolytic cleavage, phosphorylation, nuclear localization and heparin binding (Shimazaki and Ling, 1991; Hwa et al., 1999; Firth and Baxter, 2002; Forbes et al., 2012).

The deduced amino acid sequences of cloned salmon IGFBPs have relatively high sequence homologies to mammalian counterparts, especially for the N- and C-terminal domains, but some motifs were absent (Fig. 4). The RGD motif is an integrin recognition site important for IGFBP interaction with cell surface $\alpha5\beta1$ integrin, and is present in mammalian IGFBP-1 and -2

(Jones et al., 1993). This motif is conserved in fish IGFBP-2s but substituted in fish IGFBP-1 (Fig. 4; Maures and Duan, 2002; Kamei et al., 2008; Shimizu et al., 2011a). NLS is an important motif for IGFBP-3 to express IGF-independent action by translocating to the nucleus and acting as a transcription factor (Schedlich et al., 2000). Zebrafish (*Danio rerio*) IGFBP-3 possesses NLS and has been shown to exert its ligand-independent action by antagonizing bone morphogenetic protein (BMP) (Zhong et al., 2011). This motif is conserved in salmon IGFBP-3 but its functionality has not been confirmed (Shimizu et al., 2011b).

Post-translational modification is an important step for proteins to exert their functions. Phosphorylation and N-glycosylation are two major modifications for IGFBPs. Mammalian IGFBP-1 is highly phosphorylated, which may be important for high IGF binding since a non-phosphorylated form had a lower IGF binding ability and failed to suppress IGF action (Jones et al., 1991). A Pro-Glu-Ser-Thr (PEST)-rich domain is responsible for phosphorylation of mammalian IGFBP-1 (Julkunen et al., 1988) and also present in salmon IGFBP-1s (Shimizu et al., 2005; 2011a), suggesting they are phosphorylated although it has not been directly confirmed by phosphoprotein staining. N-glycosylation is characteristic of mammalian IGFBP-3 (Firth and Baxter, 1999). There are three N-glycosylation sites in human IGFBP-3 whereas two sites are found in salmon IGFBP-3 (Fig. 4; Shimizu et al., 2011b). It is of note that human IGFBP-2 has no N-glycosylation sites while salmon IGFBP-2b has three (Fig. 4; Shimizu et al., 2011b). These multiple glycosylations are probably why salmon IGFBP-2b appears as doublet bands at 41- and 43-kDa similar to the size of mammalian IGFBP-3 on electrophoresis gels. Glycosylation of these two bands was confirmed by digestion with endoglycopeptidase of purified salmon IGFBP-2b (Shimizu et al., 2003b). The exact role of N-glycosylation of mammalian IGFBP-3 and salmon IGFBP-2b is unknown, but it may affect interaction with the cell surface (Firth and Baxter, 1999). No N-glycosylation sites are found in salmon IGFBP-1s, but zebrafish IGFBP-1a has one site (Maures and Duan, 2002; Kamei et al., 2008; Shimizu et al., 2011a).

6. Molecular distribution of circulating IGF-I in fish

As mentioned earlier, the ternary complex of IGFBP-3, IGF and ALS is critical for maintaining high concentrations of IGFs in the mammalian circulation. However, there is so far no evidence of the presence of the ternary complex in the fish circulation. When lamprey plasma was separated by size-exclusion chromatography, IGF-binding activity was detected around 50 kDa (Upton et al., 1993), which was much smaller than the mammalian 150-kDa complex. A similar result was obtained with barramundi (*Lates calcarifer*; Degger et al., 2000). However, it should be noted that

under unequilibrated conditions the IGF-binding assay detects mainly unoccupied IGFBPs but may not do so with IGFBPs saturated with endogenous IGFs. Shimizu et al. (1999) fractionated coho salmon serum by size-exclusion chromatography under neutral conditions and measured IGF-I in the eluted fractions. As a result, IGF-I immunoreactivity was detected at approximately 50-kDa (binary complex) and 7.5 kDa (free form), but not at 150 kDa (ternary complex) (Fig. 5). These attempts were unable to detect a high-molecular-weight pool of IGF-I in the fish circulation. However, sequences of ALS are present in fish genome databases and appear to be similar to those of mammalian counterparts (Shimizu, personal communication). The apparent lack of the ternary complex is probably due to the extremely low hepatic expression of *igfbp-3* at least in salmon (Shimizu et al., 2011b). The liver is the major site of production of IGFBP-3 in mammals. Shimizu et al. (2011b) cloned a cDNA of salmon *igfbp-3* from the heart since its expression was very low in the liver. Macqeen et al. (2013) comprehensively analyzed tissue expression levels of 19 salmon *igfbp*s and found four *igfbp-3* paralogs had low expression in the liver and also other tissues examined. Such low hepatic expression makes it unlikely that IGFBP-3 is a major circulating IGFBP in salmon. It is not known if ALS circulates in fish blood, but the extremely low levels of production of IGFBP-3 by the liver may be a major reason for the lack of ternary complex in salmon circulation.

The significance and/or reason of the apparent lack of ternary complex in the salmon circulation are not known at present, but the following is a possible explanation why the ternary complex is not formed. An advantage of forming the high-molecular-weight complex of 150 kDa is to prevent IGF from being filtered out by the kidney and from leaving the capillary barrier (Zapf, 1995; Rajaram et al., 1997). The capillary barrier in mammals has a molecular cutoff around 60 kDa, which does not allow the 150 kDa ternary complex to pass through, although the 50 kDa binary complex may be filtered (Hasegawa et al., 1992). For this reason, the ternary complex is critical to form large pools of IGFs in mammalian circulation. In contrast, fish capillaries are relatively "leaky" lacking a clear molecular cutoff (Hargens et al., 1974), so that even if the ternary complex is formed, IGFs may not be sequestered in the circulation. Furthermore, renal glomerular filtration in fish is at least an order of magnitude lower than in mammals, so renal loss of IGF may not be as great in fish as in mammals (Hickman and Trump, 1969). In addition, mammalian IGFBP-3 is produced by the Kupffer and endothelial cells in the liver. Fish apparently lack Kupffer cells (Robertson and Bradley, 1992; Bruslé and Anadon, 1995), which may be a reason for the low *igfbp-3* expression in the liver. Supporting this, cultured striped bass liver pieces did not secrete a 35-39-kDa IGFBP, but did produce 28-30-kDa and 23-24-kDa

IGFBPs (Fukazawa et al., 1995; Siharath et al., 1996). Based on this evidence, it is hypothesized that the apparent lack of the ternary complex in fish may be due to the leaky nature of the vascular system and low renal filtration in fish, which does not provide a selective advantage for the formation of a ternary complex along with the apparent lack of the Kupffer cells in the liver, all of which might prevent IGFBP-3 from being a major circulating IGFBP. The significance of the lack of the ternary complex needs to be addressed by taking account of the difference in the vascular system between mammals and fishes.

7. Hormonal regulation of circulating fish IGFBPs

Kelley et al. (1992) detected IGFBPs in fish blood and demonstrated that they were under control of hormones. Fukazawa et al. (1995) examined in vitro effects of several hormones (GH, insulin, prolactin, glucagon, triiodothyronine, thyroxine, testosterone and estradiol) and growth factors (epidermal growth factor and IGF-I) on the secretion of the two low-molecular-weight IGFBPs from liver pieces of striped bass. These results indicate that many hormones are involved in the regulation of fish IGFBPs.

7.1. Insulin

In mammals, insulin is a potent inhibitor of IGFBP-1 (Unterman et al., 1991). Circulating levels of IGFBP-1 are generally inversely related to those of insulin after meals, and insulin treatment inhibits IGFBP-1 both at the mRNA and protein levels (Lee et al., 1993, 1997). In the goby, an experimental model to create insulin-dependent diabetes mellitus (IDDM) by surgical removal of pancreatic islets (isletectomy) was established (Kelley et al., 1993). The isletoctomized goby showed induction of 24- and 30-kDa IGFBPs in plasma, and insulin treatment restored levels of these IGFBPs to basal conditions (Kelley et al., 2001), suggesting that negative regulation by insulin of putative fish IGFBP-1 is conserved at least in this species. In contrast, an in vitro experiment using primary cultured salmon hepatocytes found that insulin had no effect on reducing *igfbp-1b* mRNA and protein (Pierce et al., 2006). On the other hand, there was a weak negative correlation between circulating IGFBP-1b and insulin levels in coho salmon (Shimizu et al., personal communication). Thus, the inhibitory effect of insulin may be species-specific or an indirect effect in fish.

The effect of insulin on the 40-50-kDa IGFBP is not clear. A study using isletectomized goby showed that a high dose of insulin (1U/kg) increased the intensity of the

339 40-50-kDa IGFBP band (Kelley et al., 1992), although no statistical analysis was conducted.
340
341 *7.2. Glucocorticoids*
342 Cortisol is known to induce IGFBP-1 in mammals although its positive effect is secondary to the
343 inhibitory effect of insulin (Katz et al., 1998). In fish, the involvement of cortisol in inducing the
344 low-molecular-weight IGFBPs in blood has been suggested based on the findings that IGFPB
345 levels were increased under catabolic conditions such as fasting, isletectomy, or handling stress
346 when cortisol is also elevated (Kelley et al., 2001; 2002; Peterson and Small, 2004; Davis and
347 Peterson, 2005, 2006). Several studies also provided evidence of a direct induction by cortisol of
348 these smaller IGFBPs in fish (Kajimura et al., 2003; Peterson and Small, 2005; Pierce et al., 2006;
349 Shimizu et al., 2011a). A single cortisol injection of tilapia resulted in a clear induction of 24- and
350 30-kDa IGFBPs within two hours and its effect lasted for eight hours, but levels returned to
351 baseline by 24 hours (Kajimura et al., 2003). A long-term (four weeks) treatment of channel
352 catfish with dietary cortisol also induced a 20-kDa IGFBP in plasma (Peterson and Small, 2005).
353 A glucocorticoid agonist, dexamethasone, directly stimulated *igfbp-1b* mRNA in salmon
354 hepatocytes in vitro (Pierce et al., 2006). In rainbow trout, IGFBP-1a as well as IGFBP-1b were
355 inducible by exogenous cortisol (Shimizu et al., 2011a). These findings well support the notion
356 that cortisol induces salmon IGFBP-1s and the low-molecular-weight IGFBPs of other fishes.
357 However, Kelley et al. (2000) reported that a 30-kDa IGFBP in the goby was induced by
358 isletectomy but cortisol treatment reduced it in a dose dependent manner. This result suggests an
359 interactive regulation by cortisol and pancreatic hormone(s) of the 30-kDa IGFBP in the goby.
360 Thus, cortisol may have divergent effects on multiple IGFBPs in various tissues.
361 Cortisol has been found to reduce the plasma 40-50-kDa IGFBP in tilapia (Kajimura et
362 al., 2003). However, it is not known if the same regulation exists in other fish species.
363
364 *7.3. Growth hormone (GH)*
365 In mammals GH is the major regulator of the components of the ternary complex (i.e. IGF-I,
366 IGFBP-3 and ALS) (Martin and Baxter, 1992). The liver is the primary site of production of all
367 three components, and GH treatment induces their production, although cellular localization of
368 the components in the liver is different as mentioned earlier. Induction by GH of fish IGFBPs was
369 first reported in coho salmon (Kelley et al., 1992). In striped bass, hypophysectomy was effective
370 in reducing a 35-kDa IGFBP and injecting ovine GH restored its levels (Siharath et al., 1995b).
371 The same approach was used to assess the effect of GH on tilapia IGFBPs (Park et al., 2000). In

that study, fish were first hypophysectomized and given homologous GH. Exogenous GH increased a 40-kDa IGFBP, which had been reduced by hypophysectomy (Park et al., 2000). However, the GH effect in pituitary-intact fish was less clear. In a study of tilapia by Breves et al. (2014) injection of GH increased hepatic expression of *igfbp-2b*, suggesting that GH induces hepatic production of the 40-kDa IGFBP in intact tilapia. GH treatment of intact coho salmon increased 41-kDa IGFBP (IGFBP-2b) levels as measured by radioimmunoassay (RIA) (Shimizu et al., 2003a). In channel catfish (*Ictalurus punctatus*), GH injection reduced plasma levels of 44- and 47-kDa IGFBPs, which goes along with other atypical responses to GH treatment in channel catfish such as increased body fat and reduced body protein (Johnson et al., 2003). However, long-term GH treatment of the same species had no significant effect on a 45-kDa IGFBP (Peterson et al., 2004). It should be noted that in none of these fish species has 40-50-kDa IGFBPs been proven to be IGFBP-3 orthologs. On the contrary, salmon 41-kDa IGFBP was identified as IGFBP-2b (Shimizu et al., 2011b). In humans, GH is weakly inhibitory to IGFBP-2 (Blum et al., 1993), which contrasts to the GH effect on salmon IGFBP-2b. In this respect, a major target of GH has diversified between mammals and salmon.

The effect of GH on other IGFBPs in fish is not consistent. GH injection had no effect on the striped bass 23-24-kDa IGFBP, reduced the tilapia 20-kDa IGFBP and the catfish 35-kDa IGFBP, or increased the tilapia 29- and 32-kDa IGFBPs (Siharath et al., 1995b; Park et al., 2000; Johnson et al., 2003). These results suggest that GH does not act directly on the lower-molecular-weight IGFBPs, and its effect is secondary through modulating other hormones such as insulin and/or cortisol. In contrast, in zebrafish GH injection decreased whole body mRNA levels of *igfpb-2*, which corresponds to IGFBP-2a with molecular weight of 31 kDa when expressed in CHO-K1 cells (Duan et al., 1999).

7.4. Sex steroids

Only a few studies examined effects of sex steroids on circulating IGFBPs in fish (Fukuzawa et al., 1995; Larsen et al., 2004), but the IGFBP responses at the mRNA level have been comprehensively evaluated in rainbow trout (Cleveland and Weber, 2015). Fukuzawa et al. (1995) examined in vitro effects of testosterone and estradiol-17β on the secretion of the low-molecular-weight IGFBPs from the liver pieces of striped bass and found that estradiol-17β at 5 ng/ml significantly increased the secretion of two IGFBPs, whereas testosterone at the same concentration had no effect. The striped bass 35-39 kDa IGFBP was undetectable in the cultured media (Fukazawa et al., 1995; Siharath et al., 1995a). On the other hand, in vivo injection of

testosterone or 11-ketotestosterone significantly increased circulating IGFBP-2b levels in postsmolt coho salmon (Larsen et al., 2004), although responses of IGFBP-1a or -1b were not examined in that study.

8. Environmental and developmental regulation of circulating fish IGFBPs

Circulating fish IGFBPs are presumably important for adjusting growth under changing environments through regulating the availability of IGFs to target tissues. Accordingly, circulating levels of IGFBPs are controlled by environmental factors (Picha et al., 2008a).

8.1. Feeding and nutrition

Circulating levels of fish IGFBPs are affected by nutritional status, but their responses differ among the IGFBP types in terms of direction and/or magnitude of change. The 20-25 kDa IGFBP including salmon IGFBP-1b is relatively sensitive to food deprivation or feeding ration. Fasting of striped bass for 30 days induced a 25-kDa IGFBP in plasma, and refeeding for two weeks reduced it to undetectable levels (Siharath et al., 1996). Similar responses of the 20-25-kDa IGFBP have been reported using ligand blotting in coho salmon, goby, channel catfish and Atlantic salmon (*Salmo salar*) (Shimizu et al., 1999; Kelley et al., 2001; Peterson and Small, 2004; Hevrøy et al., 2011). The availability of an RIA for salmon 22-kDa IGFBP (IGFBP-1b) made it possible to process a large number of samples with greater measurement precision (Shimizu et al., 2006). When postsmolt coho salmon were reared under different feeding rations (1.75, 1.0, 0.5 and 0% body weight/day), IGFBP-1b levels as measured by RIA were graded by the ration being highest in the lowest ration (Shimizu et al., 2006). Changes in feeding ration influenced circulating IGFBP-1b levels within two weeks (Shimizu et al., 2006). Moreover, a significant reduction in IGFBP-1b was observed by four hours after a meal in postsmolt coho salmon (Shimizu et al., 2009). In mammals, IGFBP-1 shows a dynamic change within a day in response to a meal (Yeoh and Baxter, 1988). Salmon IGFBP-1b is similar to the mammalian counterpart by showing a significant fluctuation within a day (Shimizu et al., 2009).

The 28-32-kDa IGFBP, including salmon IGFBP-1a, is also inducible by fasting in some species, but its response is not as sensitive as the 20-25-kDa form. In the goby, a 30-kDa IGFBP was induced by fasting and reduced by re-feeding, which was in parallel with a 24-kDa form (Kelley et al., 2001). On the other hand, fasting of channel catfish up to 45 days had no effect on a 35-kDa IGFBP (Peterson and Small, 2004). In Chinook salmon, IGFBP-1a was not induced after six weeks of fasting (Shimizu et al., 2011a), whereas fasting of masu salmon (*O. masou*) for

four weeks resulted in increases of both IGFBP-1a and -1b (Fig. 6; Kawaguchi et al., 2013). These findings suggest that IGFBP-1 sensitivity to fasting may be species-specific.

The response of the 40-50-kDa IGFBP including salmon IGFBP-2b appears to be opposite to what is seen for the 20-24-kDa IGFBP; the 40-50-kDa levels are high when fish are fed, and fasting decreases its levels. However, its response to fasting is sometimes unclear partly because this IGFBP often appears as diffused bands on ligand blotting, making it difficult to quantify. For example, fasting of striped bass for 60 days tended to decrease a 35-39-kDa IGFBP, but the response was not significant (Siharath et al., 1996). On the other hand, in a hybrid striped bass (*M. chrysops* X *M. saxatilis*) a 40-kDa IGFBP, presumably corresponding to the 35-39 kDa form in striped bass, significantly decreased in fasted fish in 20 days; it recovered to fed control levels after re-feeding for 20 days (Picha et al., 2008b). With the development of an RIA for salmon 41-kDa IGFBP (IGFBP-2b), it became clear in salmon that IGFBP-2b is sensitive to nutritional conditions including fasting and feeding ration (Shimizu et al., 2003a, 2009; Beckman et al., 2004a,b). IGFBP-2b responded to fasting as early as four days in Chinook salmon and a single meal increased it within several hours in fasted coho salmon (Pierce et al., 2005; Shimizu et al., 2009). In addition, the trout 47-kDa IGFBP increased when fish were fed diets with increasing percentage of plant proteins (0, 50, 75 or 100%) (Gomez-Requeni et al., 2005). In juvenile olive flounder (*Paralichthys olivaceus*), dietary supplementation of glycoprotein extracts from the sea mustard (*Hizikin fusiformis*) increased a plasma 43-kDa IGFBP while decreasing a 34-kDa IGFBP (Choi et al., 2014). It is of note that in some fish species the 40-50-kDa IGFBP is undetectable, which could partly account for considerably lower IGF-I levels in fish compared to mammals (Kelley et al., 2000, 2001, 2002, 2006).

There are a number of studies looking at responses of multiple *igfbp* mRNAs in the liver and/or white muscle to fasting and refeeding in fish (Gabillard et al., 2006; Bower et al., 2008; Pedroso et al., 2009; Bower and Johnston, 2010; Safian et al., 2012; Cleveland and Weber, 2014; Garcia de la serrana et al., 2017). Fasting of adult zebrafish increases whole body mRNA levels of an *igfbp-2a* (Duan et al., 1999). Fasting and refeeding of rainbow trout showed significant changes in *igfbp* expression, including in liver increases in *igfbp-2, -4, and -6* after refeeding, and in muscle increases in *igfbp-2, -4, and -5* (Gabillard et al. 2006). An in vitro approach to a fasting and refeeding experiment is removal of amino acids and subsequent addition in cultured Atlantic salmon myotubes (Bower and Johnston, 2010). This approach showed an increase in *igfbp-5* expression in myotube cells due to amino acid addition alone and an increase in *igfbp-4* expression when amino acid addition was combined with IGF or insulin addition

(Bower and Johnston, 2010). These studies indicate that hepatic and muscle IGFBPs are regulated by nutrition and emphasize that local IGFBPs play significant roles in regulating muscle growth in response to changes in nutritional status.

8.2. Stress

Stress is a strong inducer of the two low-molecular-weight IGFBPs in the fish circulation (Kelley et al., 2000, 2001, 2002, 2006). Hypophysectomy of tilapia induced a 20-kDa IGFBP in the circulation, and levels of a 29-kDa IGFBP were increased (Park et al., 2000). These increases should be due, at least in part, to the stress associated with surgery. Kelley et al. (2001) pointed out that although both 24- and 30-kDa IGFBPs of jack mackerel (*Trachurus symmetricus*) were induced by handling stress, the 30-kDa form was more sensitive than the 24-kDa form. In contrast, a direct transfer of Chinook salmon parr, which had low hypoosmoregulatory capacity, to full-strength seawater resulted in a strong induction of IGFBP-1b, but not IGFBP-1a, at six hours after transfer (Shimizu et al., 2011a). In the same experiment, IGFBP-1a was induced at 12 h after transfer (Shimizu et al., 2011a). A 15-min low-water stress at 25°C caused increases in both 24- and 30-kDa IGFBPs in sunshine bass, a hybrid between female white bass (*M. chrysops*) and male striped bass (Davis and Peterson, 2006). These studies suggest that the relative sensitivity of the two low-molecular-weight IGFBPs vary among species or type of stress employed. Supporting this, in rainbow trout an acute handling stress (five min handling) had no effect on 21- and 32-kDa IGFBPs (Shepherd et al., 2011).

The 40-50-kDa IGFBP appears to be less sensitive to stress. An acute handling stress tended to increase circulating 42- and 50-kDa IGFBPs in rainbow trout (Shepherd et al., 2011). An osmotic stress to Chinook salmon parr had no effect on circulating IGFBP-2b levels when assessed by RIA (Shimizu et al., 2011a). More work need to be done to reveal stress effects on the 40-50-kDa IGFBP.

8.3. Temperature

Temperature affects all biological processes as well as specific components of the GH/IGF/IGFBP system in poikilothermic fish (Gabillard et al., 2005). Several studies examined effects of decreased or increased water temperature on IGFBPs and suggested that responses were different among IGFBP types. When postsmolt coho salmon were reared under two water temperatures (11°C or 7°C) in combination with feeding ration (1.8% or 1.0%/body weight), a drop in water temperature from 11°C to 7°C decreased plasma IGFBP-1b levels in postsmolt coho

504 salmon regardless of feeding ration in the first two weeks (Shimizu et al., 2006). However, four
505 weeks after a water temperature change, IGFBP-1b levels in fish at 11°C became lower than those
506 at 7°C and the effect of the water temperature change disappeared by the ninth week (Shimizu et
507 al., 2006). In the same experiment, however, the change in water temperature alone had no effect
508 on IGFBP-2b levels but masked the effect of decreased feeding ration (Beckman et al., 2004a).
509 These findings suggest that IGFBP-1b is more sensitive than IGFBP-2b to decreasing water
510 temperature. Hevrøy et al. (2013) examined effects of elevated water temperatures in Atlantic
511 salmon in seawater and found that a 32-kDa IGFBP showed the highest levels at 17°C but further
512 increase in water temperature to 19°C restored it to the basal levels. Another study by the same
513 group showed that an elevated water temperature at 19°C decreased plasma IGFBP-1b in Atlantic
514 salmon whereas higher levels of IGFBP-1b were seen in rainbow trout, suggesting these two
515 species handled elevated temperature differently (Hevrøy et al., 2015). In channel catfish, a
516 higher water temperature (26°C versus 20°C) increased a 19-kDa IGFBP (Johnson et al., 2003).
517 In sunshine bass a decrease in water temperature from 23°C to 5°C suppressed plasma 33-kDa
518 IGFBP levels whereas 24- and 28-kDa IGFBPs were unchanged (Davis and Peterson, 2006).
519 These studies indicate contrasting regulation by water temperature change for different types of
520 IGFBPs in fish, although pathways by which temperature affects IGFBPs are unknown. Since
521 fish growth is largely affected by water temperature, its direct and indirect effects on circulating
522 IGFBPs need to be comprehensively explored.
523
524 *8.4. Salinity*
525 Salinity influences the GH-IGF-I system in euryhaline fishes such as salmon. However, relatively
526 little is known about the effect of salinity on fish IGFBPs. Shepherd et al. (2005) were the first to
527 examine salinity effects on IGFBPs. A gradual acclimation of rainbow trout from freshwater to
528 66% seawater (22 ppt) resulted in elevated IGFBPs at 21, 32, 42 and 50 kDa sizes (Shepherd et al.,
529 2005). When postsmolt coho salmon were transferred from freshwater to 50% seawater (15 ppt),
530 IGFBP-2b transiently increased one day after transfer and then returned to levels similar to
531 freshwater controls (Shimizu et al., 2007). In the same study, GH treatment was found to be more
532 effective in fish in 50% seawater presumably due to a lowered glomerular filtration rate so that
533 exogenous GH was retained longer in the circulation and stimulated IGFBP-2b to a greater extent
534 (Shimizu et al., 2007).
535
536 *8.5. Developmental/seasonal effects (smoltification)*

Smoltification is a transitional process for juvenile salmon by which river-dwelling parr become ocean-type smolt (Hoar, 1988; Stefansson et al., 2008; Björnsson et al., 2011; McCormick, 2013). Smolt acquire hypoosmoregulatory ability during smoltification through activating gill Na^+,K^+-ATPase (NKA) prior to the seawater entry. Smoltification is a seasonal event generally occurring in spring, although some strains undergo smoltification in autumn. Many endocrine axes including the GH-IGF-I system are activated during smoltification (Dickhoff et al., 1997; Stefansson et al., 2008; Björnsson et al., 2011; McCormick, 2013). It is thus not surprising that IGFBPs change during this period. However, a limited number of studies examined changes in circulating IGFBPs in salmon. In coho salmon, plasma IGFBP-1b levels showed a peak at the end of April when condition factor was decreasing (Shimizu et al., 2006). A negative relationship between plasma IGFBP-1b and condition factor is generally found in salmon, suggesting that this form is involved in catabolism (Shimizu et al., 2006). In addition, serum IGFBP-1b levels in smolting masu salmon were positively correlated with gill NKA activity (Fukuda et al., 2015). During smoltification, circulating IGF-I in smolting salmon are generally high and both growth and hypo-osmoregulatory ability are promoted (Dickhoff et al., 1997; Stefansson et al., 2008; Björnsson et al., 2011; McCormick, 2013). Thus, circulating IGF-I is most likely adequately distributed to different organs involved in growth or osmoregulation. Circulating IGFBP-1b may be involved in delivering IGF-I to the gills (Fukuda et al., 2015). Activity of the endocrine IGF-I at the gills in turn may be regulated by local IGFBPs. A recent work by Breves et al. (2017) reported changes in local *igfbp-6b*s in the gills and suggested their importance in the development of hypoosmoregulatory ability.

Plasma IGFBP-2b levels also changed during smoltification of coho salmon reaching a peak at the end of March, one month earlier than the peak of IGFBP-1b (Shimizu et al., 2003a, 2006). In the same fish, plasma IGF-I showed two peaks in late March and late April corresponding to peaks of IGFBP-2b and -1b, respectively (Shimizu et al., 2003a, 2006). These results suggest that IGFBPs play different roles during smoltification, although unraveling the role of each IGFBP is subject to future study.

9. Perspective

Our better understanding of the fish IGFBPs invites speculation on how this system evolved and how it compares functionally to the well-characterized system in mammals. The evolution of IGFBP genes has been elegantly described in a number of studies proposing that an ancestral IGFBP gene was duplicated in chordates and was followed by a number of whole

genome duplications; two whole-genome duplications in ancestral vertebrates, followed by a third whole-genome duplication leading to teleosts, and a fourth whole-genome duplication in some fish species, e.g., salmonids (Daza et al., 2011, Macqueen et al., 2013). Thus, the Atlantic salmon has 19 IGFBP genes, which are 13 more than found in humans (Macqueen et al., 2013). Therefore we should be cautious in extrapolating the functions of IGFBPs in mammals to those in fish because there is an approximately 500 million year span involving a lot of genetic change since their evolutionary divergence. However, this complexity in IGFBP evolution offers a great opportunity for future study of the range of functions (subfunctional partitioning) of IGFBPs in fish and how they compare with mammals. Subfunction partitioning is one of the fates of duplicated copies of a gene where ancestral regulatory and structural subfunctions are preserved by gene duplicates (Postlethwait et al., 2004). Subfunction partitioning in turn provides gene duplicates opportunities to acquire new function and/or regulation. Interestingly, the earliest IGFBP function may have been independent of binding IGF (Zhou et al., 2013; Zhong and Duan, 2017).

Studies of IGFBP functions in fish have made good use of the zebrafish model. Zebrafish IGFBPs are generally inhibitory to IGF-induced cell proliferation (Duan et al., 1999). Knocking down IGFBPs resulted in abnormal organ formation (Li et al., 2005; Wood et al., 2005b). In addition, while IGFBP-1 delayed the speed of embryonic development under hypoxic conditions (Kajimura et al., 2005), such a response limits oxygen consumption due to IGF-induced anabolism and may be adaptive to increase embryo survival. Functions of duplicated zebrafish IGFBPs are overlapping, but there are certain differences such as affinity for IGFs, site of production, developmental changes and responses to fasting (Kamei et al., 2008; Zhou et al., 2008; Wang et al., 2009; Dai et al., 2010). The fish circulation appears to contain duplicated IGFBP-1s where they may play overlapping yet distinct roles in regulating postnatal growth. However, there are few attempts examining functions of circulating fish IGFBPs in the context of gene duplication. This is most likely due to the lack of purified proteins. Plasma/serum is a source for protein purification, however levels of circulating IGFBPs are low, e.g., < 300 ng/ml (Shimizu et al., 2003a, 2006). Thus, producing recombinant IGFBPs is desirable for functional analyses of fish IGFBPs. Recombinant salmon IGFBP-1a, -1b, -2a and -2b are currently being produced by using bacterial expression systems (Tanaka et al., in press). Although recombinant proteins produced in bacteria are not glycosylated or phosphorylated, the availability of recombinant fish IGFBPs should promote functional analyses.

A series of studies of circulating salmon IGFBPs suggest that IGFBP-3 plays little role

in regulating endocrine IGFs (Shimizu et al., 1999, 2003a, 2009, 2011b). However, whether or not the finding on salmon IGFBP-3 applies to other fishes is unknown. In zebrafish, since IGFBP-3 plays a crucial role in embryonic development and exhibits an IGF-independent action (Li et al., 2005; Zhong et al., 2011), it is important for normal development at least in this species. In tilapia and yellowtail (*Seriola quinqueradiata*), GH treatment increased *igfbp-3* mRNA in the liver (Cheng et al., 2002; Pedroso et al., 2009), suggesting it is secreted into the bloodstream and modulates IGF action. Local action of IGFBP-3 is also suggested in fine flounder (*Paralichthys adspersus*; Safian, et al., 2012). The stage-, tissue- or species-specific roles of fish IGFBP-3 need to be examined in future studies.

In view of the large number of IGFBP genes that have been retained in fish, the fish circulation probably contains more than three IGFBPs. There is some evidence for this to be the case. For example, in coho salmon there are two additional bands often detected at 37 and 31 kDa, and the 31-kDa IGFBP appeared to be decreased by fasting (Fig. 7). In order to compare regulation and function of fish IGFBPs with mammalian counterparts, the fish proteins need to be identified. One strategy to clone cDNAs of unidentified IGFBPs is to use degenerate primer(s) designed from partial amino acid sequences of purified proteins. However, as mentioned above, purifying each IGFBP from serum/plasma is not practical. Instead, partial N-terminal amino acid sequence could be obtained by IGF-affinity chromatography of serum/plasma followed by electrophoresis and MALDI-TOF MS/MS (Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry/Mass Spectrometry). Once a cDNA is cloned, identification and recombinant production of an IGFBP are possible. Moreover, IGFBPs can be identified from genome sequences when they are available, thus avoiding the need to clone cDNAs. In the case of salmonids, protein-coding sequences of all 19 *igfbp* paralogs in 10 species are now available (Macqueen et al., 2013; Lappin et al., 2016), which provides a very useful reference to assign unidentified circulating salmon IGFBPs and accelerate functional studies on IGFBP paralogs.

The evidence for salmon IGFBPs suggests that salmon IGFBP-2 diverged in its function from that of its mammalian counterpart, and convergently acquired characters similar to mammalian IGFBP-3 (Fig. 8). Roch et al. (2009) stated that studies on duplicate hormones and receptors were vulnerable to misidentification if only structural similarity was used. Salmon IGFBP-2b is a good example for such case and highlights the importance of actually testing the function to unravel the evolutionary fate of duplicated IGFBPs. Moreover, both salmon IGFBP-1a and -1b are increased under catabolic conditions but may have different sensitivity, suggesting they underwent subfunction partitioning (Fig. 8). Thus, salmon as well as other fish

636 species provide a unique opportunity to investigate how functional divergence, convergence and
637 subfunction partitioning of IGFBPs occurred during vertebrate evolution.
638
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Figure legends

Fig. 1. Three forms of circulating IGFs in humans. The majority of circulating IGF is complexed with IGFBP-3 and an acid-labile subunit (ALS). IGFBP-5 also participates in the formation of the ternary complex. This ternary complex is too large to leave the vascular system so that a large circulating pool of IGF is formed. IGF is also bound to other IGFBPs to form binary complexes. The binary complexes can cross the capillary barrier, but the availability of IGF to the receptor is limited by IGFBP. Less than 1% of IGF is in the free form and biologically active.

Fig. 2. Ligand blotting using labeled IGF-I of human and Chinook salmon sera. IGFBP bands were visualized by using digoxigenin (DIG)-labeled human IGF-I and anti-DIG conjugated with horseradish peroxidase. Migration positions of human and Chinook salmon IGFBPs are indicated by arrows on the left and right, respectively. Molecular weights (kDa) of Chinook salmon IGFBPs are indicated. NS: non-specific bands.

Fig. 3. Schematic patterns of IGFBP bands in human and fish sera/plasma on ligand blotting using labeled IGF-I. Migration positions of circulating IGFBPs in human, chicken, Chinook salmon, rainbow trout, catfish, striped bass, tilapia, goby and lamprey are reproduced from Hossenlopp et al. (1986), Schoen et al. (1992), Niu et al. (1993), Johnson et al. (2003), Siharath et al. (1995), Park et al. (2000), Kelley et al. (1992) and Upton et al. (1993), respectively. Types of IGFBP are indicated by numbers and alphabets when identity is known.

Fig. 4. Alignment of deduced amino acid sequences of Chinook salmon IGFBP-1a, -1b, -2a, -2b and -3 with those of human counterparts. Sequences are from NP_000587, NP_000588, NP_000589, AEO18300.1, AAV83995.1, AEC33109.1, AEC33110.1 and AEC33113.1. Asterisks indicate cysteine residues conserved among the IGFBP family. Underlines and boxes indicate a Arg-Gly-Asp (RGD) integrin recognition motif and potential N-glycosylation sites, respectively. The starting positions of the N-, L- and C-domains of human IGFBPs are shown by triangles.

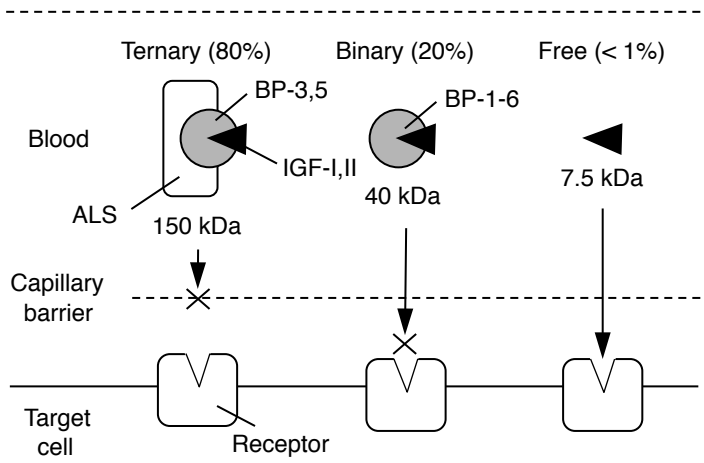
Fig. 5. Elution profiles of serum IGF-I in coho salmon on gel filtration under neutral conditions. Arrows indicate expected elution positions of ternary, binary and free forms of IGF-I. Modified from Shimizu et al. (1999) with permission.

Fig. 6. IGFBP patterns in sera of fed, fasted and refed masu salmon. Serum samples were the same as used in Kawaguchi et al. (2013). Postsmolt masu salmon were fed or fasted for 6 weeks. An additional group (Re-fed) was fasted for 4 weeks followed by 2 weeks of refeeding. Arrows indicate migration positions of salmon IGFBPs.

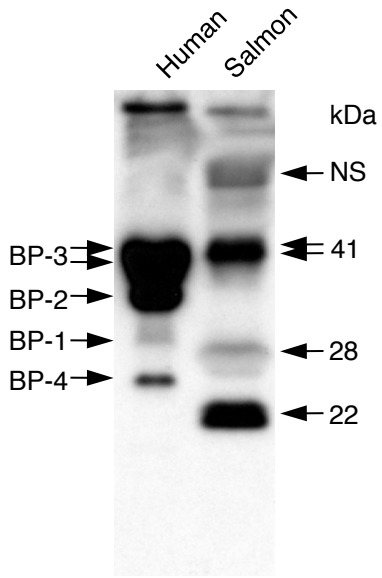
Fig. 7. IGFBP patterns in plasma of fed and fasted coho salmon. Postsmolt coho salmon were fed or fasted for 3 weeks. Arrows indicate migration positions of identified and unidentified (New?) IGFBPs. NS: non-specific bands.

Fig. 8. Hypothetical functional relationships between human and salmon IGFBPs in the circulation.

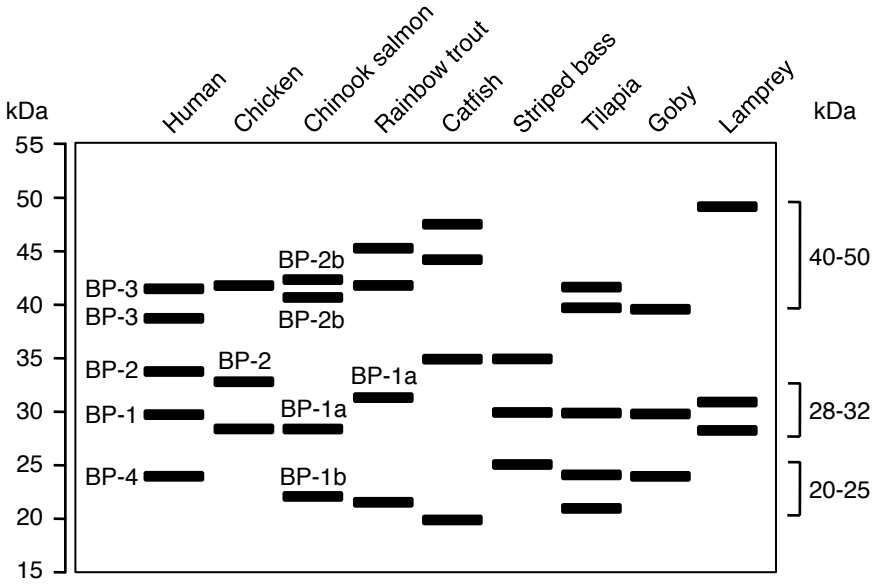
Shimizu, Fig. 1

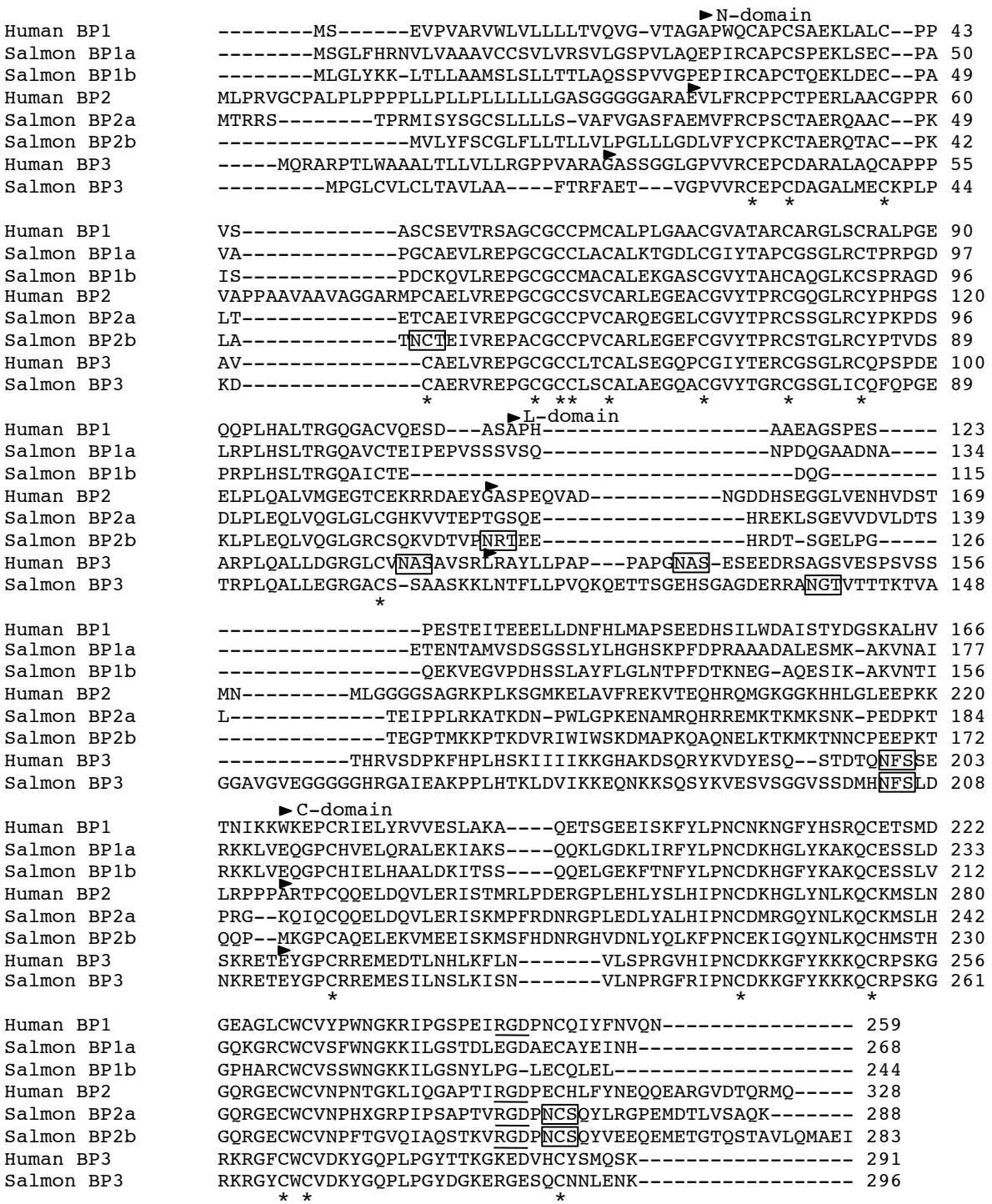


Shimizu, Fig. 2

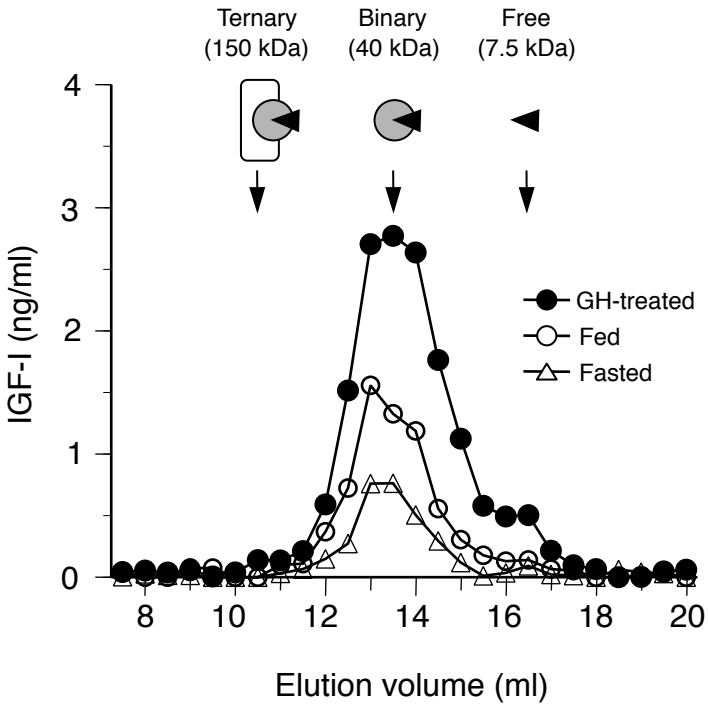


Shimizu, Fig. 3

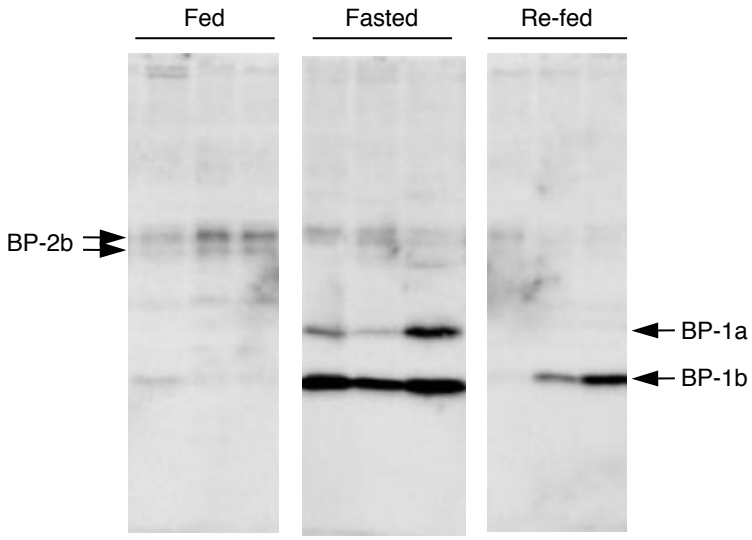



►N-domain
Human BP1 --------MS------EVPVARVWLVLLLLTVQVG-VTAGAPWQCAPCSAEKLALC--PP 43
Salmon BP1a --------MSGLFHRNVLVAAAVCCSVLVRSVLGSPVLAQEPIRCAPCSPEKLSEC--PA 50
Salmon BP1b --------MLGLYKK-LTLLAAMSLSLLTTLAQSSPVVGPEPIRCAPCTQEKLDEC--PA 49
Human BP2 MLPRVGCPALPLPPPPLLPLLPLLLLLLGASGGGGGARAEVLFRCPPCTPERLAACGPPR 60
Salmon BP2a MTRRS--------TPRMISYSGCSLLLLS-VAFVGASFAEMVFRCPSCTAERQAAC--PK 49
Salmon BP2b ----------------MVLYFSCGLFLLTLLVLPGLLLGDLVFYCPKCTAERQTAC--PK 42
Human BP3 -----MQRARPTLWAAALTLLVLLRGPPVARAGASSGGLGPVVRCEPCDARALAQCAPPP 55
Salmon BP3 ---------MPGLCVLCLTAVLAA----FTRFAET---VGPVVRCEPCDAGALMECKPLP 44
Human BP1 VS-------------ASCSEVTRSAGCGCCPMCALPLGAACGVATARCARGLSCRALPGE 90
Salmon BP1a VA-------------PGCAEVLREPGCGCCLACALKTGDLCGIYTAPCGSGLRCTPRPGD 97
Salmon BP1b IS-------------PDCKQVLREPGCGCCMACALEKGASCGVYTAHCAQGLKCSPRAGD 96
Human BP2 VAPPAAVAAVAGGARMPCAELVREPGCGCCSVCARLEGEACGVYTPRCGQGLRCYPHPGS 120
Salmon BP2a LT-------------ETCAEIVREPGCGCCPVCARQEGELCGVYTPRCSSGLRCYPKPDS 96
Salmon BP2b LA-------------TNCTEIVREPACGCCPVCARLEGEFCGVYTPRCSTGLRCYPTVDS 89
Human BP3 AV---------------CAELVREPGCGCCLTCALSEGQPCGIYTERCGSGLRCQPSPDE 100
Salmon BP3 KD---------------CAERVREPGCGCCLSCALAEGQACGVYTGRCGSGLICQFQPGE 89
►L-domain
Human BP1 QQPLHALTRGQGACVQESD---ASAPH-----------------AAEAGSPES----- 123
Salmon BP1a LRPLHSLTRGQAVCTEIPEPVSSSVSQ-----------------NPDQGAADNA---- 134
Salmon BP1b PRPLHSLTRGQAICTE-------------------------------DQG--------- 115
Human BP2 ELPLQALVMGEGTCEKRRDAEYGASPEQVAD----------NGDDHSEGGLVENHVDST 169
Salmon BP2a DLPLEQLVQGLGLCGHKVVTEPTGSQE----------------HREKLSGEVVDVLDTS 139
Salmon BP2b KLPLEQLVQGLGRCSQKVDTVPNRTEE----------------HRDT-SGELPG----- 126
Human BP3 ARPLQALLDGRGLCVNASAVSRLRAYLLPAP---PAPGNAS-ESEEDRSAGSVESPSVSS 156
Salmon BP3 TRPLQALLEGRGACS-SAASKKLNTFLLPVQKQETTSGEHSGAGDERRANGTVTTTKTVA 148
Human BP1 ----------------PESTEITEEELLDNFHLMAPSEEDHSILWDAISTYDGSKALHV 166
Salmon BP1a ----------------ETENTAMVSDSGSSLYLHGHSKPFDPRAAADALESMK-AKVNAI 177
Salmon BP1b ----------------QEKVEGVPDHSSLAYFLGLNTPFDTKNEG-AQESIK-AKVNTI 156
Human BP2 MN---------MLGGGGSAGRKPLKSGMKELAVFREKVTEQHRQMGKGGKHHLGLEEPKK 220
Salmon BP2a L-------------TEIPPLRKATKDN-PWLGPKENAMRQHRREMKTKMKSNK-PEDPKT 184
Salmon BP2b -------------TEGPTMKKPTKDVRIWIWSKDMAPKQAQNELKTKMKTNNCPEEPKT 172
Human BP3 -----------THRVSDPKFHPLHSKIIIIKKGHAKDSQRYKVDYESQ--STDTQNFSSE 203
Salmon BP3 GGAVGVEGGGGGHRGAIEAKPPLHTKLDVIKKEQNKKSQSYKVESVSGGVSSDMHNFSLD 208
►C-domain
Human BP1 TNIKKWKEPCRIELYRVVESLAKA----QETSGEEISKFYLPNCNKNGFYHSRQCETSMD 222
Salmon BP1a RKKLVEQGPCHVELQRALEKIAKS----QQKLGDKLIRFYLPNCDKHGLYKAKQCESSLD 233
Salmon BP1b RKKLVEQGPCHIELHAALDKITSS----QQELGEKFTNFYLPNCDKHGFYKAKQCESSLV 212
Human BP2 LRPPPARTPCQQELDQVLERISTMRLPDERGPLEHLYSLHIPNCDKHGLYNLKQCKMSLN 280
Salmon BP2a PRG--KQIQCQQELDQVLERISKMPFRDNRGPLEDLYALHIPNCDMRGQYNLKQCKMSLH 242
Salmon BP2b QQP--MKGPCAQELEKVMEEISKMSFHDNRGHVDNLYQLKFPNCEKIGQYNLKQCHMSTH 230
Human BP3 SKRETEYGPCRREMEDTLNHLKFLN-------VLSPRGVHIPNCDKKGFYKKKQCRPSKG 256
Salmon BP3 NKRETEYGPCRREMESILNSLKISN-------VLNPRGFRIPNCDKKGFYKKKQCRPSKG 261
Human BP1 GEAGLCWCVYPWNGKRIPGSPEIRGDPNCQIYFNVQN---------------- 259
Salmon BP1a GQKGRCWCVSFWNGKKILGSTDLEGDAECAYEINH----------------- 268
Salmon BP1b GPHARCWCVSSWNGKKILGSNYLPG-LECQLEL------------------- 244
Human BP2 GQRGECWCVNPNTGKLIQGAPTIRGDPECHLFYNEQQEARGVDTQRMQ----- 328
Salmon BP2a GQRGECWCVNPHXGRPIPSAPTVRGDPNCSQYLRGPEMDTLVSAQK------- 288
Salmon BP2b GQRGECWCVNPFTGVQIAQSTKVRGDPNCSQYVEEQEMETGTQSTAVLQMAEI 283
Human BP3 RKRGFCWCVDKYGQPLPGYTTKGKEDVHCYSMQSK----------------- 291
Salmon BP3 RKRGYCWCVDKYGQPLPGYDGKERGESQCNNLENK----------------- 296

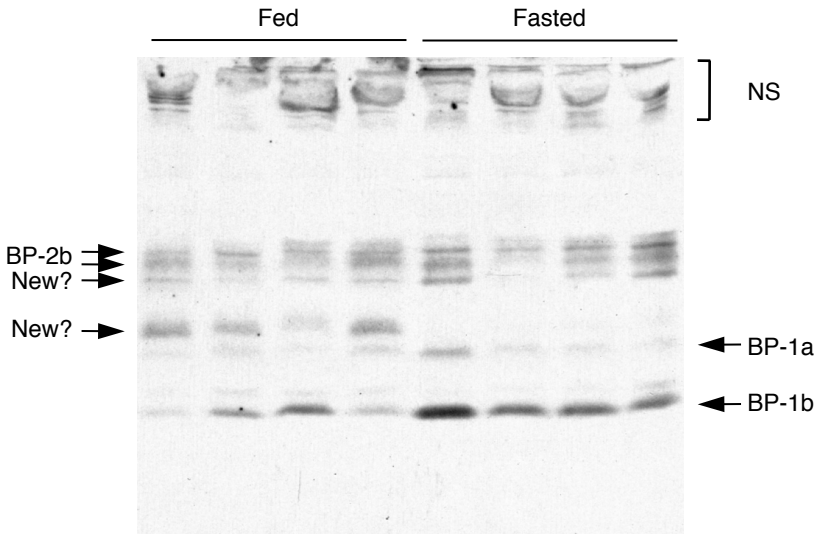
Shimizu, Fig. 5



Shimizu, Fig. 6



Shimizu, Fig. 7



Shimizu and Dickhoff, Fig. 8