Structural, gene expression, and functional analysis of the fugu (Takifugu rubripes) insulin-like growth factor binding protein-4 gene

Mingyu Li, Yun Li, Ling Lu, Xianlei Wang, Qingli Gong, and Cunning Duan

1Laboratory of Molecular Medicine, School of Medicine and Pharmacy; 2Department of Aquaculture, School of Fisheries, Ocean University of China, Qingdao, Shandong, China; and 3Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan

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Li M, Li Y, Lu L, Wang X, Gong Q, Duan C. Structural, gene expression, and functional analysis of the fugu (Takifugu rubripes) insulin-like growth factor binding protein-4 gene. Am J Physiol Regul Integr Comp Physiol 296: R558–R566, 2009. First published December 17, 2008; doi:10.1152/ajpregu.90439.2008.—The insulin-like growth factor (IGF) signaling pathway is a conserved pathway that regulates animal development, growth, metabolism, reproduction, and aging. The biological actions of IGFs are modulated by IGF-binding proteins (IGFBPs). Although the structure and function of fish IGFBP-1, -2, -3, and -5 have been elucidated, there is currently no report on the full-length structure of a fish IGFBP-4 nor its biological action. In this study, we cloned and characterized the IGFBP-4 gene from fugu. Sequence comparison, phylogenetic, and syntenic analyses indicate that its chromosomal location, gene, and protein structure are similar to its mammalian orthologs. Fugu IGFBP-4 mRNA was easily detectable in all adult tissues examined with the exception of spleen. Older animals tended to have higher levels of IGFBP-4 mRNA in the muscle and eyes compared with younger animals. Starvation resulted in significant increases in IGFBP-4 mRNA abundance in the muscle, liver, gallbladder, and brain. Overexpression of fugu and human IGFBP-4 in zebrafish embryos caused a significant decrease in body size and somite number, suggesting that fugu IGFBP-4 inhibits growth and development, possibly by binding to IGFs and inhibiting their binding to the IGF receptors. These results provide new information about the structural and functional conservation, expression patterns, and physiological regulation of the IGFBP-4 gene in a teleost fish.

teleost; gene expression; growth
MATERIALS AND METHODS

Animal and tissue collection. Juvenile and adult Fugu (Takifugu rubripes) fish were obtained from a local fish farm (Rushan, Weihai, China). Juvenile fish were 6 mo old (weighed 0.1–0.15 kg) and adult fish were 1.5 yr old (weighed 0.7–0.8 kg) and 2.5 yr old (1.4–1.5 kg). Wild-type zebrafish (Danio rerio) were maintained on a 14:10-h light-dark cycle at 28°C and were fed twice daily. Embryos were generated from natural crosses, reared in embryo medium (37), and staged as previously described (19). All experiments were conducted in Ocean University of China. The protocols used met the guidelines established by the University of Michigan Committee on the Use and Care of Animals.

Molecular cloning, structural, and phylogenetic analysis. Using the human IGFBP-4 amino acid sequence as a query, we searched the public database (http://www.ensembl.org) and found a putative IGFBP-4 like gene (Ensembl ID: SINFRUT00000169066). Next, RT-PCR was performed to amplify a 640-bp DNA fragment of this gene product (forward primer, 5'-TTTGCTCCAGGAGAGGCTG-3'; reverse primer, 5'-TGAGCATGCCGAGCTCCTGCTCG-3') using RNA isolated from adult fugu liver. The 5'- and 3'-rapid amplifications of cDNA ends (RACE) experiments were carried out using the SMART RACE cDNA amplification kit (Clontech Laboratories, Mountain View, CA) following the manufacturer’s instruction. The RACE products were first screened by nested PCR using two internal primers (forward primer, 5'-GAGCACCCCAACAACAGC-3'; reverse primer, 5'-TGGCAGGATTTGATGCCAGT-3'). The RACE products were then cloned into the pUCm-T vector (Sangon, Shanghai, China) and sequenced at the Biosune Sequencing (Shanghai, China).

Amino acid sequence alignment of IGFBP-4s was performed using the GeneDoc software (Free Software Foundation). Phylogenetic analysis was done using full-length amino acid sequences by the maximum likelihood method with the PhyML V2.4.4 program (13). Bootstrap analyses were run on 1,000 replicate with amino acid substitution of JTT model, with the settings of the y-distribution parameter (a) at 0.74 and the proportion of invariable sites (p-invar) at 0.00. We also constructed a phylogenetic tree using the Neighbor-joining method and the Maximum parsimony method with the MEGA 3.1 software (The Biodisgn Institute, Tempe, AZ).

The genomic structure of the fugu IGFBP-4 gene was obtained by the Blat program (http://genome.ucsc.edu/cgi-bin/hgBlat) using the cloned fugu IGFBP-4 full-length cDNA sequence as probe to search Fugu Assembly Oct.2004. Synteny data among fugu (Takifugu rubripes), tetraodon (Takifugu nigroviridis), and human (Homo sapiens) orthologs were obtained by Muliticontigview (http://www.ensembl.org/Fugu_rubripes/multicontigview).

RNA extraction and quantitative real-time RT-PCR. Total RNA was isolated from tissue samples using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer’s instruction. First-strand cDNA was synthesized using Moloney murine leukemia virus (Promega, Madison, WI) following the manufacturer’s instruction. Oligo(dT)18 were used as first-strand primers. Quantitative real-time RT-PCR (qRT-PCR) was carried out in an iCycler iQ Multicolor real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad Laboratories). Primers used are as follows: β-actin: 5'-GAAGCCCAGCAAACAGG-3' and 5'-GCGATACAGGGCAGCAGCAG-3'; and IGFBP-4: 5'-AGGCCCAAGGCAAAAGC-3' and 5'-TGAATGCCGTCGGTCTAAGC-3'. cDNA (0.5 μl) was used as PCR template. Plasmid DNA was used as controls. Serial dilutions of the plasmids ranging from 10^10 to 10^4 molecules/μl for β-actin and 10^1 to 10^2 molecules/μl for fugu IGFBP-4 were used for generating the standard curve. After a 3-min incubation at 95°C, the amplification was performed as follows: 95°C, 10 s; 60°C, 30 s, for 40 cycles. Each assay for an unknown sample was performed in duplicate along with standard DNA samples and negative controls. The number of molecules of a particular gene transcript was calculated based on the standard curve and normalized by the β-actin mRNA levels.

Construction of IGFBP-4 expression constructs. cDNAs encoding the open reading frame of fugu and human IGFBP-4 were amplified by RT-PCR using KOD plus DNA polymerase (Toyobo, Shanghai, China). The primers used were as follows: fugu IGFBP-4: 5'-CCGATTGGCCACCAACCAGCTGCCTGCT-3' and 5'-CCA-TGATTAAGTCTCCCTGTCGGGAC-3'; and human IGFBP-4: 5'-CCATCGATGCCAACCAGCTGCCTGCCCTTGT-3' and 5'-GAATTTGGCTCTGAAAGGTGTCACGAG-3'. PCR products were used as follows: one cycle of 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 68°C followed by a final extension of 10 min at 68°C. The amplified PCR products were digested with appropriate restriction enzymes (fugu IGFBP-4 with BamH I and Cla I; IGFBP-4 with Cla I and EcoR I) and subcloned into the pcDNA-eGFP vector (21), resulting in the pcDNA-fgIGFBP4-eGFP and pcDNA-huIGFBP4-eGFP construct. Both constructs were verified by DNA sequencing.

Capped mRNA synthesis and microinjection. Capped mRNA synthesis was carried out using a commercial kit and linearized plasmid DNA as template (Megascript kit; Ambion, Austin, TX). mRNA (800 pg per embryo) was microinjected into zebrafish embryos at the 1–2 somite stage using glass micropipettes attached to a micromanipulator. Injection was driven by compressed N2, under the control of a PV830 Pneumatic PicoPump (World Precision Instruments, Sarasota, FL). Green fluorescent protein (GFP) mRNA injected and wild-type embryos were used as controls. After injection, embryos were placed in embryo rearing medium (37) and kept at 28.5°C.

Western immunoblot. Twenty-five embryos from each treatment group were dechorionated, deyolked, and homogenized in 100 μl of RIPA buffer (50 mM Tris·HCl, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100 pH 7.5) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 100 mM PMSF. The homogenates were briefly centrifuged to pellet cellular debris, and the supernatant was retained. Each sample was subjected to SDS-PAGE (12.5%) and transferred to Immobilon-P membrane (Millipore, Billerica, MA). Western immunoblot analysis was performed as described previously (9) using a GFP antibody at a 1:5,000 dilution (Torrone Pines Biolabs, Houston, TX).

Body size and somite measurement. Body length, defined as the curvilinear distance from the forebrain and midbrain boundary to the tail, was quantified with the MB-Ruler 5.1 software (Markus Bader, Germany). Each line was then measured using a scale taken at the same magnification. Somite number was quantified by counting total somite number per embryo.

Statistics. Data are means ± standard error (SE). Differences among groups were analyzed by one-way ANOVA followed by Fisher post-hoc Test or t-test (SPSS, Chicago, IL). Significance was accepted at P < 0.05.

RESULTS

Identification and cloning of fugu IGFBP-4. Using the human IGFBP-4 amino acid sequence as a query, we searched the fugu genome database and identified an IGFBP-4 like sequence (http://www.ensembl.org; Ensembl ID: SINFRUT00000169066). Next, RT-PCR was performed to amplify a 640-bp DNA fragment of this gene product using RNA isolated from the adult fugu liver. Subsequently, its full-length cDNA was obtained by 5'- and 3'-RACE. The nucleotide and deduced amino acid sequence has been deposited in GenBank (accession no. EU255232). The 1,710-bp cDNA contains an initiation codon ATG that is flanked by sequences resembling the Kozak consensus sequence. The complete open reading frame of 786 bp encodes a protein of 261 amino acids with a putative signal peptide of 30 residues. The 231 amino acid mature protein has a predicted molecular size of 25,789 Da with a putative...
N-glycosylation site at amino acid position 103. The 5’-UTR and 3’-UTR are 550 and 374 bp, respectively. A poly(A) tail-like sequence was found at position 1,684–1,710 bp, but no conserved poly(A) addition signal was found within 30 bp. The overall sequence identity of fugu IGFBP-4 with the six known human IGFBPs is 29% (IGFBP-1), 32% (IGFBP-2), 29% (IGFBP-3), 54% (IGFBP-4), 31% (IGFBP-5), and 27% (IGFBP-6; Table 1). Its overall sequence identity to those of mouse, rat, bovine, and chicken IGFBP-4 is 52–57%. The sequence identity to a partial rainbow trout IGFBP-4 is 87% (Table 1).

The alignment of the fugu IGFBP-4 amino acid sequence with that of known full-length vertebrate IGFBP-4 is shown in Fig. 1. There are two highly conserved regions in the mature protein: the cysteine-rich N-terminal domain (residues 31–110) and the C-terminal domain (residues 176–261). The greatest sequence identity is observed in the N domain, being 75 and 78% identical with that of human and chicken IGFBP-4, and the identity in the C domain is 67–71%. As in most mammalian IGFBPs, two conserved motifs (GCCGCGXXC and CWCV) are found in the N domain (position 61–69) and C domain (position 228–231), respectively. Fugu IGFBP-4 contains 20 cysteine residues: 12 in the N domain and 6 in the C domain. The central L domain is variable among different vertebrate IGFBP-4s (23–32%). It is believed that this domain acts structurally as a hinge between the N and C domains (14).

Like other vertebrate IGFBP-4, fugu IGFBP-4 has two additional cysteine residues in the variable L domain. The positions of these cysteine residues are identical to those of mammalian IGFBP-4s.

We next performed phylogenetic analysis using the sequences of known IGFBPs. The results showed that fugu IGFBP-4 is grouped into the IGFBP-4 clade with very high bootstrap support value (Fig. 2), suggesting that this gene is indeed the ortholog of the human IGFBP-4 gene. Recent studies (6, 35) have suggested that a genome duplication event occurred in the teleost lineage ~350 million years ago, before the beginning of the teleost radiation. Fugu and other teleost genome often have two paralogs for single human genes. We therefore searched the Fugu genomes using the fugu and human IGFBP-4 sequences as queries. No additional IGFBP-4 genes were found, suggesting that there is likely one copy of IGFBP-4 gene in the fugu genome.

Fugu IGFBP-4 gene has a similar genomic structure and high synteny to the human IGFBP-4 gene. The genomic structure of fugu igfbp-4 is shown in Fig. 3. The fugu igfbp-4 spans over 8 kb and contains four exons and three introns (Fig. 3A). The first exon contains 550 bp 5’-UTR, the entire signal peptide (30 amino acid), and almost the entire N domain. Exons 2 and 3 are relatively small (164 and 126 bp), and they encode the L domain and part of the C domain. Exon 4 contains 46 aa of the C domain and 374 bp of the 3’-UTR.

To explore the possible synteny relationship between the fugu and human IGFBP-4 genes, we analyzed genes surrounding the IGFBP-4 locus in fugu (T. rubripes), tetraodon (T. nigroviridis), and human genomes. The IGFBP-4 gene is located on the scaffold 244, on chromosome 18, and on the long arm of chromosome 17 in fugu, tetraodon, and human, respectively (Fig. 3B). The genes located upstream of the

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IGFBP, insulin-like growth factor-binding protein. *Comparison was made using corresponding partial sequences. †Comparison was made using ensembl predicted sequences.

Table 1. Amino acid sequence identities between fugu IGFBP-4 and other IGFBPs
IGFBP-4 locus include MLLT6 (myeloid/lymphoid or mixed-lineage leukemia; translocated to, 6), PCGF2 (polycomb group ring finger 2), PIP4K2B (phosphatidylinositol-5-phosphate 4-kinase, type II, β), PSMB3 (proteasome subunit, β-type, 3), and CCDC49 (coiled-coil domain containing 49; Fig. 3B). In the proximity downstream of the IGFBP-4 genes, there are SMARCE1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily e, member 1), CCR7 (C-C motif receptor 7), and KRT222P (keratin 222 pseudo-gene; Fig. 3B). There are some rearrangements in the order of these genes among these genomes. This highly conserved synteny relationship among the fugu, tetraodon, and humans provides further evidence that the fugu gene we cloned is indeed the fugu igfbp-4.

IGFBP-4 mRNA is expressed in a variety of tissues at different ages and is regulated by age and nutritional state. The tissue IGFBP-4 mRNA levels were measured by qRT-PCR. IGFBP-4 mRNA was easily detectable in the muscle, liver, heart, brain, gill, eyes, intestine and stomach, and gallbladder in all three age groups (Fig. 4). Among the tissues examined, muscle had the highest levels. This was followed by the liver, gallbladder, brain, gill, intestine and stomach, heart, eyes, kidney, and testis (Fig. 4). The expression levels in the spleen of three different ages were at the detection limit of the assay.

Age appeared to have an effect on IGFBP-4 mRNA levels in certain tissues. As shown in Fig. 4, the IGFBP-4 mRNA levels in the muscle and eyes of 2.5-yr-old fish (5.39E-02) were significantly higher ($P < 0.05$) than those of 1.5-yr-old fish (2.76E-02) and 0.5-yr-old juvenile fish (2.99E-02). Similar results were also observed in the eyes and gallbladder, although the differences were not statistically significant in the case of the gallbladder. No age-dependent difference was found in the kidney, brain, intestine, and stomach. In the liver, the IGFBP-4 mRNA expression appeared to decrease with age, but the differences were not statistically different.

To determine whether there is any gender difference, we analyzed and compared tissue IGFBP-4 mRNA levels in male and female fish. These fish were of the same age (1.5 yr old) and fed normally. As shown in Fig. 5, the levels of IGFBP-4 mRNA in the female fish were similar to those in the male fish. Although the IGFBP-4 mRNA levels in the ovary appeared to be higher than testis (Fig. 5), this difference was not statistically significant.

We next examined the possible effect of food deprivation on tissue IGFBP-4 mRNA expression. As shown in Fig. 6, the IGFBP-4 mRNA levels in the muscle, liver, gallbladder, brain, and eyes of the fastest fish group were significantly higher in the fastest fish compared with those of the fed fish (Fig. 6). There was also a notable increase in IGFBP-4 mRNA abundance in other tissues, but the differences were not statistically significant (Fig. 6).

Overexpression of fugu and human IGFBP-4 results in developmental delay and growth retardation in zebrafish embryo. To determine whether the biological action of IGFBP-4 is conserved in fish, we performed in vivo functional studies. Since it is difficult to perform genetic studies in fugu, we used the zebrafish model. For this, capped mRNAs encoding fugu and human IGFBP-4 were generated and introduced into zebrafish embryos by microinjection. The successful expression of the IGFBP-4::GFP protein was confirmed by fluorescent microscopy (Fig. 7A) and Western immunoblot (Fig. 7E).

As shown in Fig. 7B, embryos injected with fugu IGFBP-4 and human IGFBP-4 were morphologically normal, but they were smaller in size and developmentally delayed compared with GFP mRNA injected or wild-type control embryos. The body lengths of fugu IGFBP-4 and human IGFBP-4 injected embryos were $1.87 \pm 0.08$ and $1.90 \pm 0.09$ mm, which were significantly smaller than the $2.11 \pm 0.07$ and $2.09 \pm 0.07$ mm of the wild-type and GFP-injected embryos ($P < 0.05$). At 24 h postfertilization, wild-type embryo and GFP mRNA injected control embryos had $27.8 \pm 1.2$ and $27.4 \pm 1.7$ somites, respectively. In comparison, embryos injected with fugu IGFBP-4 and human IGFBP-4 had only $22.0 \pm 1.3$ and $22.1 \pm 1.6$ somites ($P < 0.05$). They were developmentally equivalent to wild-type or GFP-injected embryos at 18–19 h postfertilization. These data suggest that the biological activity of IGFBP-4 is conserved between teleosts and humans.
DISCUSSION

In this study, we cloned and characterized the fugu IGFBP-4 gene and determined its spatial and temporal expression pattern and biological activity. To our knowledge, this is the first time the full-length structure and biological activity of IGFBP-4 have been determined in a teleost fish. Furthermore, we have determined the genomic structure of the Fugu IGFBP-4 gene and demonstrated its conserved synteny. Our results suggest that the genomic and primary structure of IGFBP-4 is conserved in fugu.

The fugu IGFBP-4 protein shares a similar domain arrangement with other known IGFBPs. It has a highly cysteine-rich N domain, a cysteine-rich C domain, and a central L domain. Fugu IGFBP-4 also contains two motifs that are common in IGFBPs: a GCCCGXXC motif in its N domain and a CWCV motif in its C domain (22). Like mammalian IGFBP-4, fugu IGFBP-4 contains 20 cysteine residues. Twelve are located in the N-terminal domain, six in the C-terminal domain, and the remaining two in the L domain. The cysteine residues in the N-terminal domain are arranged into two Zn-finger motifs (CX2CX7C). The variable L domain contains an N-glycosylation site (Asn103). It is believed that this region acts structurally as a hinge between the N- and C-terminal domains (14). It has been reported that the N-terminal sequence (Leu72-Ser91) and the C-terminal sequence (Cys205-Val214) are necessary for human IGFBP-4 to bind IGF with high affinity (31). The corresponding regions of the fugu IGFBP-4 are highly conserved. Several residues known to be critical for high affinity IGF binding, including V78, L99, L102, M103, G210, and Q216, are conserved in fugu IGFBP-4. The high degree of conservation is probably not surprising given their involvement in IGF binding. It is also in good agreement with several biochemical studies showing that fish IGFBPs bind human IGF-I and IGF-II with high affinity and specificity (2, 9, 33). Therefore, the IGF-binding determinants of IGFBP-4 may have been preserved during the several hundred million years of vertebrate evolution.

Despite the overall structural similarity, fugu IGFBP-4 has diverged significantly from its mammalian and chicken homologues. The overall sequence identities between the full-length fugu IGFBP-4 protein and those of mammalian and chicken IGFBP-4s are 52–57%. Most of these changes occur in the L domain. To date, the only available sequences of any fish IGFBP-4 is a partial sequence obtained in rainbow trout (Oncorhynchus mykiss; Ref. 18). The partial rainbow trout sequence IGFBP-4 covers partial L domain and the entire C domain. The identity between the two fish IGFBP-4s is much higher (87%).

The architecture of the fugu IGFBP-4 gene is similar to that of humans. Both contain four exons and three introns. The IGFBP-4 to bind IGF with high affinity (31). The corresponding regions of the fugu IGFBP-4 are highly conserved. Several residues known to be critical for high affinity IGF binding, including V78, L99, L102, M103, G210, and Q216, are conserved in fugu IGFBP-4. The high degree of conservation is probably not surprising given their involvement in IGF binding. It is also in good agreement with several biochemical studies showing that fish IGFBPs bind human IGF-I and IGF-II with high affinity and specificity (2, 9, 33). Therefore, the IGF-binding determinants of IGFBP-4 may have been preserved during the several hundred million years of vertebrate evolution.

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70-kb region of the chromosome segment, where the IGFBP-4 gene resides, shows a high degree of similarity in terms of gene content and organization between fish and humans. The conserved synteny map also shows that fugu and tetraodon have a more compact arrangement than that of humans in this region. This is consistent with the fact that tetraodontiform fishes have compact genomes (3), characterized by short intergenic and intronic sequences and a small proportion of repetitive elements (1).

In fugu, the IGFBP-4 gene is expressed in various tissues, including muscle, brain, liver, kidney, heart, gill, eyes, intestine and stomach, gallbladder, testis, and ovary. Our gene expression analysis suggests that there is no difference in the tissue levels of IGFBP-4 mRNA between male and female fish. This is in good agreement with previous reports in other species. In mammals, IGFBP-4 mRNA is expressed in the adrenal gland, testis, spleen, heart, liver, lung, kidney, stomach, hypothalamus, and brain cortex with liver being the site of the highest expression (45). In rainbow trout, the IGFBP-4 transcript was found at comparable levels in all tissues examined, except for the spleen and gills (18). These results indicate that the tissue distribution of fugu IGFBP-4 is conserved among fish and mammals.

An interesting finding made in this study is that fugu tissue IGFBP-4 mRNA abundance appears to be influenced by age and nutritional state but not by gender. In some tissues, such as muscle and eyes, the IGFBP-4 mRNA levels were higher in the older fish (2.5 yr) compared with juvenile (0.5 yr) or young adults (1.5 yr). Although it was reported that circulating IGFBP-4 levels did not change with age in plasma in mammals (34, 39), a recent study (28) indicated that IGFBP-4 mRNA expression increases with age in mice. Our study in fugu indicates that fasting caused a significant increase in IGFBP-4 mRNA abundance in several tissues. In rats, fasting decreased IGFBP-4 mRNA levels in the heart, skeletal muscle, and liver (4). In rainbow trout, liver and muscle IGFBP-4 mRNA abundance was reduced by starvation (12). The difference between our results and other studies may be due to species differences and/or differences in experimental conditions.

Several studies (26, 36, 44, 46) have shown that targeted overexpression of IGFBP-4 in transgenic mice resulted in decreased tissue growth. For example, overexpression of IGFBP-4 in osteoblasts reduces bone formation and severely impairs skeletal growth and causes global postnatal growth retardation in mice (43). Targeted overexpression of IGFBP-4 in smooth muscle cells led to smooth muscle tissue hypoplasia in transgenic mice and inhibited neointimal expansion in a porcine model of neointimal hyperplasia (26, 36, 44). Additionally, overexpressing IGFBP-4 using a lymphoid tissue promoter (H-2Kb) decreases thymus growth in transgenic mice (46). In contrary to the accepted dogma that IGFBP-4 is a growth-inhibitory gene, IGFBP-4 knockout mice showed reduced growth in fetal and postnatal stages (30). While local administration of IGFBP-4 inhibited IGF-I-induced bone formation, systemic administration of IGFBP-4 increased bone formation parameters in mice (23, 24). The reason(s) for these discrepancies is not immediately clear, but IGFBP-4 proteolysis may play a role. In support of this view, a protease-resistant mutant form of IGFBP-4 inhibits DNA synthesis, cell migration, and muscle growth in response to IGFs (5). Recent in vitro studies (27) also suggest that IGFBP-4 can both promote and inhibit the effects of IGF-II and that these actions may be regulated by proteolysis. Proteolysis of IGFBP-4 is well documented in mammalian cell culture systems and may be a major regulatory mechanism of IGFBP-4 action (7). For instance, pregnancy-associated plasma protein-A (PAPP-A), an IGF-dependent protease, can cleave IGFBP-4 and thereby alter the binding affinity of IGFBP-4 for IGFs. In addition, the IGF-independent actions of IGFBP-4 have also been reported (29, 42).

In this study, we examined the biological action of fugu IGFBP-4 in vivo in zebrafish embryos, due to the difficulty of performing transgenic studies in fugu. Overexpression of either human or fugu IGFBP-4 resulted in a comparable degree of
Fig. 7. Overexpression of fugu IGFBP-4 and human IGFBP-4 inhibits zebrafish embryo growth and development. A: fluorescent micrograph of zebrafish embryos injected with GFP, fugu IGFBP-4:eGFP, or human IGFBP-4:GFP mRNA, respectively. B: morphology of wild-type (WT), GFP mRNA injected, fugu IGFBP-4 mRNA injected, and human IGFBP-4 mRNA injected zebrafish embryos at 19 h postfertilization (hpf; top) and 24 h postfertilization (bottom). Scale bar = 250 μm. C and D: body length and somite number of wild-type, GFP mRNA injected, fugu IGFBP-4 mRNA injected, and human IGFBP-4 mRNA injected zebrafish embryos at 24 hpf. Values are means ± SE. Graphs represent mean values of total number embryos resulting from 3 microinjection experiments. Number of total embryos is indicated in each group. * P < 0.05. E: Western immunoblot analysis of wild-type, GFP, fugu IGFBP-4:GFP, or human IGFBP-4:GFP mRNA injected embryos. Injected embryos were raised to 24 hpf. Lysates were prepared and subjected to SDS-PAGE followed by immunoblot analysis using a GFP antibody.
delayed embryonic development and growth retardation. Our findings suggest that the fugu IGFBP-4 is an inhibitory IGFBP and that the growth inhibitory action of IGFBP-4 is conserved in vertebrates. It should be pointed out that the biological activities of fugu and human IGFBP-4 were determined using a heterologous system (zebrafish embryos). While the zebrafish is uniquely positioned to provide insight into the functional aspects of conserved genes due to its versatility and amenability to genetic and experimental manipulation, future studies will be needed to determine the physiological function(s) of the fugu IGFBP-4 gene using a homologous system.

**Perspectives and Significance**

IGFBP-4 is a secreted protein that binds and regulates IGF actions in controlling growth, development, reproduction, and aging. This study reports the full-length structure of IGFBP-4, its tissue distribution, physiological regulation, and biological action in a teleost species. Sequence comparison, phylogenetic, and synteny analyses indicated that the chromosomal location and the gene and protein structure of the fugu IGFBP-4 are similar to its mammalian orthologs. In fugu, IGFBP-4 mRNA is widely expressed in most tissues. The IGFBP-4 expression is similar to its mammalian orthologs. In fugu, IGFBP-4 mRNA and synteny analyses indicated that the chromosomal location of the gene and its tissue distribution, physiological regulation, and biological actions in controlling growth, development, reproduction, and aging will be needed to determine the physiological function(s) of IGFBP-4 in vertebrates. These findings are not only relevant in understanding the functional evolution of IGFBP-4 but also demonstrate the utility of the zebrafish model system in studying IGFBP physiology.

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