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Molecular identification of grouper *Igfbp1* and its mRNA expression in primary hepatocytes under Gh and insulin

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ABSTRACT

The insulin-like growth factor (IGF) system plays a pivotal role in the regulation of growth, and IGF binding proteins (IGFBPs) are important regulatory factors in the IGF system. Generally, IGFBPs inhibit IGF actions by preventing its binding to receptors. Under some conditions, the IGFBPs can also enhance IGF actions. IGFBP1 is generally inhibitory to IGFI. In this study, the grouper (*Epinephelus coioides*) *igfbp1* (MK621003) gene was cloned from the liver. The sequence of *igfbp1* cDNA was 1055 bp and contained a 5′UTR of 127 bp and a 3′UTR of 247 bp, and the ORF of grouper *igfbp1* was 741 bp, encoding 246 amino acids. The tissue distribution results showed that *igfbp1* has a higher expression in the liver. In the nutritional status experiment, *igfbp1* expression was significantly increased in the liver after 7 days of fasting and was markedly decreased after refeeding. In in vitro experiments, *igfbp1* expression in grouper primary hepatocytes was significantly inhibited by recombinant grouper Gh (growth hormone) in a dose-dependent manner. Additionally, *igfbp1* expression decreased in grouper primary hepatocytes upon incubation with insulin. This is the first report describing grouper *igfbp1*, and these findings contribute to understanding the roles of IGFBP1 in metabolism and growth in grouper.

1. Introduction

It is well known that the growth hormone/insulin-like growth factors (GH/IGFs) axis plays a vital role in the neuroendocrine regulation of growth (Wood et al., 2005). The GH/IGF system is comprised of growth hormone (GH), GH receptors, IGFs (IGF1 and IGF2), IGF receptors (IGF1R and IGF2R) and IGF-binding proteins (IGFBPs) (Safian et al., 2012). IGF1 synthesis is increased by stimulation of the GH receptor in the liver, and IGF1 plays a crucial role in growth regulation and cell metabolism (Frystyk, 2004; LeRoith and Yakar, 2007). The biological action of IGFs is regulated by the binding of these growth factors with IGFBPs. IGFBPs are a family of proteins composed of six members (IGFBP1-6), and they show high affinity for IGFs (Clemmons, 2011). Each of the six types generally possess one gene in mammals (Allard and Duan, 2018a). One or more of the six types is lacking in some vertebrate species, and others vertebrate species have more than one isoforms of the six types (Allard and Duan, 2018a). Due to a third round of whole genome duplication, many teleost fish have two copies of each Igfbp (Allard and Duan, 2018a; Serrana and Macqueen, 2018). Salmonid fish experienced a fourth round of whole genome duplication and possess four copies of each of the six types of Igfbps (Allard and Duan, 2018a; Macqueen et al., 2013). Thus, there are 22 unique *igfbp* genes in some salmonid species, which belong to 11 paralog pairs (Serrana and Macqueen, 2018). In the circulatory system, the IGFBPs play a vital role in prolonging the half-lives of IGFs and regulating the availability of IGFs for receptor binding (Allard and Duan, 2018a; Bauchat et al., 2001; Shimizu and Dickhoff, 2017). IGFBPs have a higher affinity for IGFs than IGF receptors; thus, the IGFBPs are involved in regulating the bioactivity and bioavailability of IGFs. In humans, 75–80% of circulating IGFs form a ternary complex with IGFBPs and an acid-labile subunit (ALS) (Baxter and Martin, 1989), and another 20% form a binary complex with IGFBPs (Guler et al., 1989). < 1% of IGFs are in the free form in circulation (Shimizu and Dickhoff, 2017). The ternary complex is too large to leave the vascular

Abbreviations: IGF, insulin-like growth factor; IGFBPs, IGF binding proteins; UTR, untranslated regions; ORF, open reading frame; Gh/GH, growth hormone; IGFR, IGF receptor; ALS, acid-labile subunit; RGD, Arg-Gly-Asp; cAMP, cyclic adenosine monophosphate; PCR, Polymerase Chain Reaction; RACE, rapid-amplification of cDNA ends; RT-PCR, reverse transcription PCR; FBS, fetal bovine serum; IRE, insulin response element

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system, leaving IGFs unavailable. Although the binary complexes can cross the capillary barrier, the bioavailability of IGF for receptor binding is still limited by IGFBPs (Shimizu and Dickhoff, 2017). The IGFBPs usually have two actions: the IGF-dependent and IGF-independent actions. To the IGF-dependent actions, the IGFBPs bind to IGF, which increases the half-life of IGF and blocks its potential binding to insulin receptor (Allard and Duan, 2018b). Because of IGFBPs binding IGFs with 10-fold higher affinity than the IGF1 receptor, IGFBPs inhibited IGF actions under most circumstances (Sitar et al., 2006a). The inhibition actions of IGFBPs to IGF including survival, migration, proliferation and differentiation in many cell types (Allard and Duan, 2018b). To IGF-independent actions, IGFBPs can also inhibit cell migration, proliferation and survival but may enhance these processes in a context-specific manner. The study showed that IGFBPs increased intracellular calcium levels with involvement of G-proteins (Seurin et al., 2013). And IGFBPs can modulate gene transcription by interacting with nuclear receptors via importin-dependent mechanisms (Allard and Duan, 2018b).

IGFBP1 binds to and mediates the bioavailability of IGF1; it was the first identified member of the IGFBP family, which inhibits IGF action by preventing it from interacting with its receptor (Kajimura and Duan, 2007; Lee, 1993; Lee and Giudice, 1997; Wheatcroft and Kearney, 2009). *Igfbp1* is expressed in many tissues, and the high expression is in the liver (Hoeflich and Russo, 2015; Kamei et al., 2008; Rajaram et al., 1997). Based on cysteine clusters, the protein structure of IGFBP1 can be divided into three regions, including N-terminal, linker and C-terminal domains (Bach, 2018a). The N-terminal domain contains 12 cysteines, and 6 conserved cysteines are located in the C-terminal domain (Bach, 2018b). The N-terminal domain contains a high-affinity IGF-binding domain, and the C-terminal domain contains an integrin binding Arg-Gly-Asp (RGD) motif which contributes to its action (Bach, 2018b). The linker domain contains several sites of posttranslational modifications, which regulate IGFBP1 actions by modulating its circulating half-lives, cell association and stability (Bach, 2018a).

The major source of IGFBP1 is the liver, and insulin is the central regulator of IGFBP1 synthesis (Brismar et al., 1994). The promoting factors, including pro-inflammatory cytokines, glucocorticoids, cAMP, oxidative stress and hypoxia, increase the synthesis of IGFBP1 (Forsberg et al., 2015). Due to insulin deficiency, IGFBP1 levels are increased in subjects with type 1 diabetes (Bach, 2018a). A recent study indicated that IGFBP1 increased insulin sensitivity via its RGD (Arg-Gly-Asp) sequence and focal adhesion kinase activation (Haywood et al., 2017). Moreover, fasting increased and feeding decreased plasma IGFBP1 levels (Hoeflich and Russo, 2015; Lewitt et al., 2014). IGFBP1 also plays a role in growth. In isolated rat hepatocytes, *Igfbp1* mRNA expression was inhibited by growth hormone (GH) (Thissen et al., 1994). In addition, IGFBP1 plays an important role in placental function and inhibits embryonic growth and development by binding to and inhibiting the activities of IGFs (Bach, 2018a; Kajimura et al., 2005).

Until now, Igfbp1 has been identified in a number of fish species (Chen et al., 2018). However, the study of Igfbp1 has never been reported in orange-spotted grouper (*Epinephelus coioides*), one of the most important commercial marine fishes in China. To further confirm the physiological function of Igfbp1 in grouper, the *igfbp1* gene was first isolated and characterized in orange-spotted grouper. The tissue distribution of *igfbp1* was analyzed, and the expression characteristics of *igfbp1* in grouper liver was determined with fasting treatment. Furthermore, the effect of insulin and Gh on *igfbp1* expression was assessed in the primary grouper hepatocytes. To the best of our knowledge, this is the first report of IGFBP1 in *Epinephelus*.

2. Materials and methods

2.1. Animals

For the cloning, tissue distribution experiments and hepatocytes isolation experiments, female orange-spotted grouper (*Epinephelus coioides*) with a body weight of 550–650 g were purchased from the Huangsha aquatic products wholesale market in Guangzhou, China. The fish were acclimated to indoor tanks with recirculating seawater at room temperature under a cyclic light-dark photoperiod (12 h: 12 h) for at least one week. During the acclimation period, the fish were fed commercial pellets twice daily until satiety. All of the animal experiments were approved by the animal care committee of Sun Yat-sen University.

2.2. Molecular cloning of grouper igfbp1

The molecular cloning of *igfbp1* was conducted in grouper by nested PCR coupled to 3′/5′ RACE according to the procedures described previously (Yang et al., 2017). In brief, total RNA was extracted from the liver using TRIzol Reagent (Invitrogen, USA) and the quality of RNA was determined on a 1% agarose electrophoresis gel with ethidium bromide staining. First strand cDNA was synthesized using the M-MLV kit (Invitrogen, USA). Nested PCR was performed, and a partial fragment of grouper *igfbp1* cDNA was isolated. The nested PCR primers were designed based on the conserved ORF regions of Nile tilapia, medaka, rainbow trout and fugu rubripes *igfbp1* (Table 1). Based on the nucleotide sequence obtained, new primers were synthesized and used in 3′/5′ RACE (Table 1). To confirm the composite ORF sequence, RT-PCR was performed using a pair of gene-specific primers designed upstream and downstream of the protein coding sequence. The procedure

Table 1
Primers used in this study.

Name	Sequences (5′—3′)	Purpose	Amplicon size (bp)	PCR efficiency
Partial CDS-F1	GARCCBATYCGCTGYGCYCC	Partial CDS clone		
Partial CDS-R1	GCCRTGTTTGTCRCAGTTKGG			
Partial CDS-R2	AKCTTCTTCCCATTCCARGA			
3′RACE-F1	CACTACCTGTTGGATCTCAA	3′RACE		
3′RACE-F2	ACAGGGTCCATGTCACATTG			
5′RACE-R1	TGCAGTCTGCTGGGATGGCA	5′RACE		
5′RACE-R2	AGTTTCCTCTTGGCCCACAT			
ORF-F	ATGCCTGGATTATATGAGGA	ORF clone	741	
ORF-R	TCAGTGAGTGGCTTCTTGAT			
igfbp1-qRT-F	AGGCAGGTGCTGAGGGA	*igfbp1* Real-time PCR	260	1.982
igfbp1-qRT-R	TGGTGGTCCAAAGGAAGGT			
18S-F	CCTGAGAAACGGCTACCACATCC	Reference gene	221	1.958
18S-R	AGCAATTTAGTATACGCTATTGGAG			
AP	GGCCACGCGTCGACTAGTAC$(T)_{16}$	Universal primers		
AAP	GGCCACGCGTCGACTAGTAC$(G)_{10}$			
AUAP	GGCCACGCGTCGACTAGTAC			

Degenerate bases: Y = C + T; R = A + G; H = A + T + C; V = A + G + C; S = C + G; B = T + G + C; K = G + T.

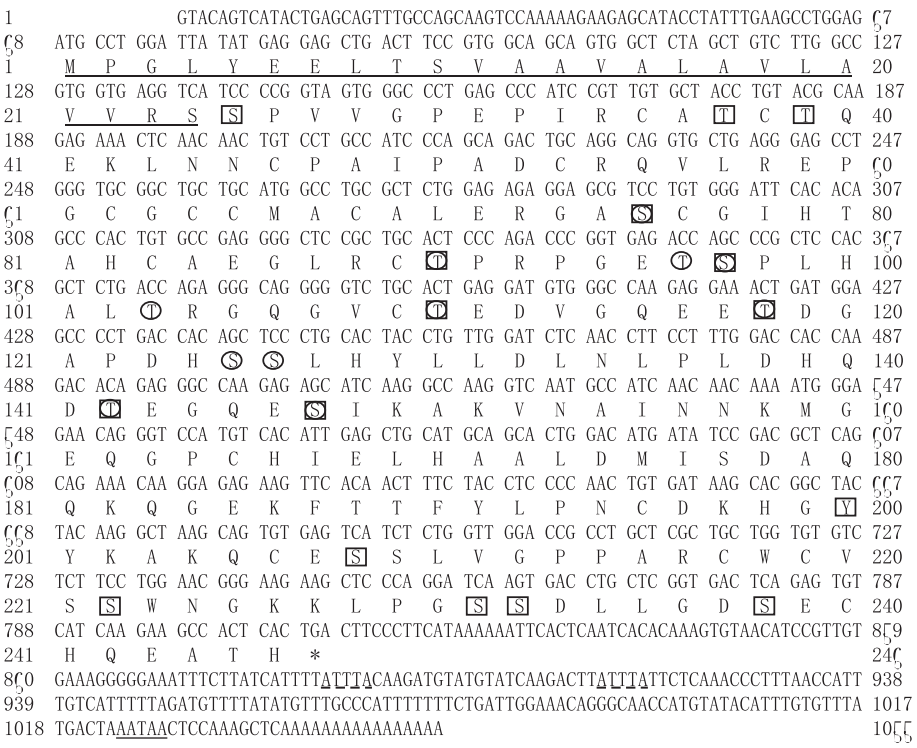

Fig. 1. The cDNA and deduced amino acids of grouper Igfbp1. Single underlined represent signal peptide; box represent predicted phosphorylation sites; round represent predicted glycosylation site. The asterisk represent the stop codon. The dotted line show predicted rapid degradation signal. The double underlined show predicted the polyadenylation signal sequence.

of all PCR was: 94 °C, 3 min; 94 °C, 30 s, 56 °C, 30 s, 72 °C, 1 min, 35 cycles; 72 °C, 10 min. All the PCR products were purified by the E.Z.N.A Gel Extraction Kit (OMEGA, USA) and subcloned into the PCR2.1 vector (Invitrogen, USA) for DNA sequencing. The ORF Finder in NCBI (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used to obtain the ORF, and the signal peptide of Igfbp1 was predicted using the SignalP4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Sequence alignment was performed at protein level using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phosphorylation and glycosylation sites of the Igfbp1 precursor protein were analyzed by NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. The phylogenetic tree was constructed with MEGA6.0 by the neighbor-joining method (bootstrap phylogeny test, 2000 replicates) (Dai et al., 2017).

2.3. Tissue distribution and effects of nutritional status on igfbp1 mRNA expression in the liver

In the tissue distribution analysis, three two-year-old female grouper with body weights of 550–650 g were anesthetized and sacrificed by decapitation. The samples were rapidly collected and snap-frozen in liquid nitrogen, and then stored in −80 °C until RNA extraction.

Fish (prepubertal female) with body lengths of 13–14 cm and body weights of 55–65 g were used in the fasting and refeeding experiment. After acclimation for 10 days, fish were divided into 3 groups (n = 6 fish/group). The control group ('Fed') was fed for 7 days (fed 6 h before sampling on Day 7), the fasted group ('Fasted') was maintained under food deprivation for 7 days, and the refed group ('Refed') was fasted for 7 days and refed 6 h before sampling on Day 7. At the end of the experiments, all fish were anesthetized by MS222 and decapitated. The liver samples were rapidly collected and snap-frozen in liquid nitrogen and then stored in −80 °C until RNA extraction.

2.4. Primary hepatocyte isolation and Gh or insulin treatments

Grouper primary hepatocytes were prepared by the collagenase IV/DNase II digestion method as described previously (Yang et al., 2017). The cells were seeded into 24-well culture plates at a density of 5×10^5 cells/well in 1 mL of L15 medium with 10% FBS (fetal bovine serum) at 25 °C and saturated humidity with plain air in an incubator. After overnight incubation, the medium was changed to serum-free fresh medium and the cells were incubated for 1 h under serum starvation conditions prior to treatment. 1) The cells were incubated with recombinant grouper Gh (rgGh, prepared in our lab) at doses of 0, 1, 10, or 100 nM for 12 h. 2) The cells were incubated with human insulin (91077C, Sigma, USA) at doses of 0, 10, 100, or 1000 ng/ml for 12 h. At the end of the incubation, the samples were collected for total RNA extraction.

2.5. RNA extraction, cDNA synthesis and Real-time PCR

Total RNA was isolated by TRIzol reagent (Invitrogen, USA), and the total RNA concentration was verified by a UV-spectrophotometer (Nanodrop 2000c, Thermo). One μg total RNA from each sample was digested with DNase I (New England Biolabs, USA), and the first-strand cDNA was then synthesized with M-MLV Reverse Transcriptase (Invitrogen, USA). The synthesized cDNA was used as the template for Real-time PCR using the primers shown in Table 1. Real-time PCR was performed on a LightCycler 480 Sequence Detection System (Roche, Switzerland) with SYBR Green PCR Master Mix (Toyobo, Japan) in a total volume of 10 μl according to the manufacturer's instructions. All Real-time PCR conditions were as follows: 95 °C for 5 min, and 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 30 s. *18S* rRNA was used as the internal reference, and remained stable in various treatments throughout the study. The relative gene expression levels were normalized to the *18S* levels and were calculated by the comparative Ct method.

2.6. Statistical analysis

All data are shown as the means ± S.E.M. Statistical analysis was performed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Fisher's Least Significance Difference (LSD)

test was used to identify the significant difference. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. Cloning and sequence analysis of igfbp1

The full-length cDNA of grouper *igfbp1* was isolated from the liver. The grouper *igfbp1* cDNA was 1055 bp and contained a 5′UTR of 127 bp and a 3′UTR of 247 bp. The ORF of grouper *igfbp1* was 741 bp, encoding 246 amino acids. Based on the amino acids sequence analysis using the SignalP program, the first 24 amino acids were predicted to be the signal peptide (Fig. 1). There were sixteen predicted phosphorylation sites in the cloned grouper Igfbp1 precursor by NetPhos, including nine serine residues, six threonine residues and one tyrosine residue (Fig. 1). Eleven O-glycosylation sites, but no N-glycosylation sites, were predicted in the grouper Igfbp1 by NetNGlyc (Fig. 1). In the 3′UTR of grouper *igfbp1*, there are two rapid degradation signal sequences of mRNA and one polyadenylation signal sequence (Fig. 1). Multiple sequence alignment showed that the amino acids sequence of grouper Igfbp1 displayed a relatively high degree of identity with the counterparts of other fish (Table 2). The structure of grouper Igfbp1 protein is similar to that of other fish, containing a N-domain, a L-domain (linker-domain) and a C-domain (Fig. 2). Twelve cysteine residues are located in the N-domain of grouper Igfbp1 protein, and six cysteine residues are in the C-domain (Fig. 2). In the grouper Igfbp1 protein, the GCGCC motif is located in the N-domain and the CWCV motif in the C-domain (Fig. 2). The phylogenetic tree was constructed using MEGA 6.0 based on Igfbp1 amino acid sequences of grouper and other species, and the phylogenetic analysis showed that grouper Igfbp1 was clustered into Igfbp1B subgroups with other fish with high bootstrap values (Fig. 3).

3.2. Tissue distribution and the effects of nutritional status on igfbp1 expression

Real-time PCR results showed that the expression of *igfbp1* could be detected at high level in the liver and to a lesser extent in the gonad. The expression of *igfbp1* was relatively lower in other tissues (Fig. 4A). To evaluate the effect of nutritional status on *igfbp1* expression in the liver, a fasting and refeeding experiment was performed. As shown in

Table 2
Amino acids identities of grouper Igfbp1 compared to other species.

Species	IGFBP1 isoform	Identity
Epinephelus coioides	IGFBP1 MK621003	100%
Oreochromis niloticus	IGFBP1 ENSONIP00000012143	80.89%
Salmo salar	IGFBP1B1 JX565545.1	71.72%
Salmo salar	IGFBP1B2 JX565546.1	71.54%
Oncorhynchus mykiss	IGFBP1B2 AGH18165.1	71.14%
Cyprinus carpio	IGFBP1Bβ XP_018935478.1	64.02%
Cyprinus carpio	IGFBP1Bα XP_018935476.1	61.51%
Salmo salar	IGFBP1A1 NM_001279140.1	58.54%
Cyprinus carpio	IGFBP1Aα XP_018963913.1	58.02%
Cyprinus carpio	IGFBP1Aβ XP_018981738.1	56.97%
Danio rerio	IGFBP1B NM_001098257.2	56.43%
Salmo salar	IGFBP1A2 JX565544.1	53.25%

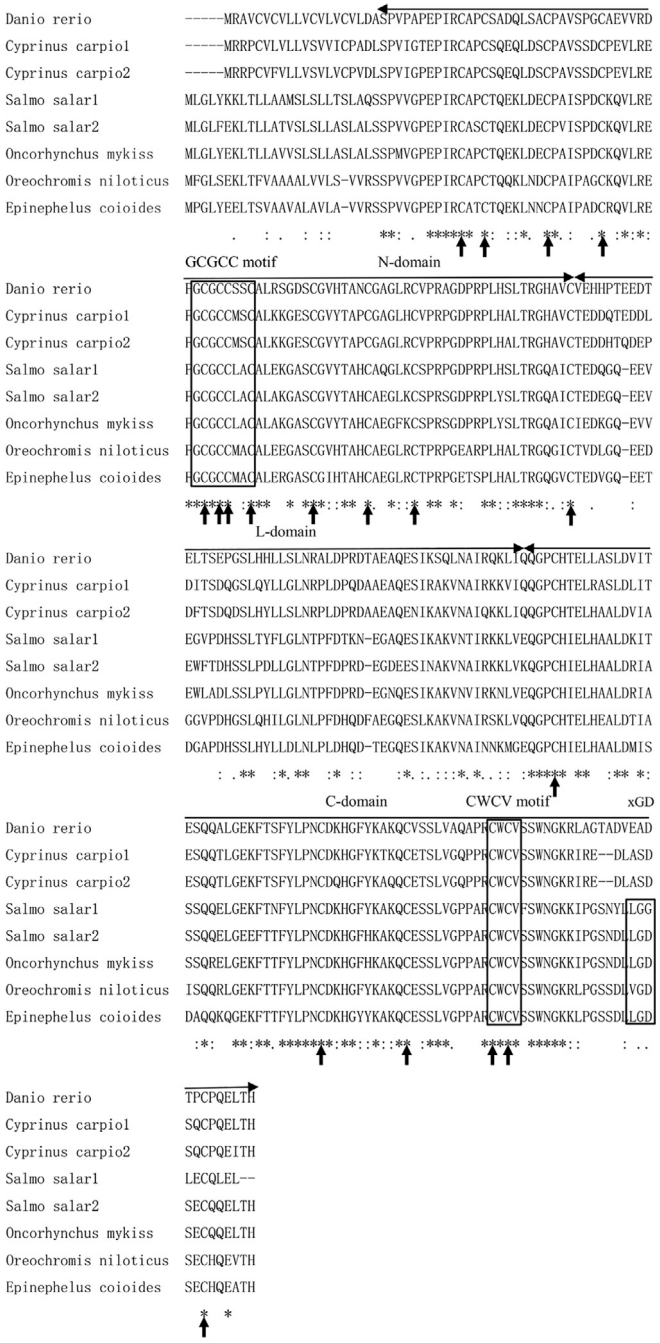

Fig. 2. Amino acid sequence alignment of grouper Igfbp1 with other species. The identical amino acids are noted by (*), and the highly and less conserved amino acids are noted by (:) and (.), respectively. The arrows represent the cysteine residues. *Danio rerio* Igfbp1B (NM_001098257.2), *Cyprinus carpio1* Igfbp1Bα (XP_018935476.1), *Cyprinus carpio2* Igfbp1Bβ (XP_018935478.1), *Salmo salar1* Igfbp1B1 (JX565545.1), *Salmo salar2* Igfbp1B2 (JX565546.1), *Oncorhynchus mykiss* Igfbp1B2 (AGH18165.1), *Oreochromis niloticus* Igfbp1 (ENSONIP00000012143), *Epinephelus coioides* (MK621003).

Fig. 4B, *igfbp1* expression was significantly increased in the liver after 7 days of fasting and was markedly decreased after refeeding.

3.3. The effects of Gh and insulin on the igfbp1 expression in grouper primary hepatocytes

To assess the effect of Gh and insulin on the expression of *igfbp1* in grouper primary hepatocytes, grouper primary hepatocytes were

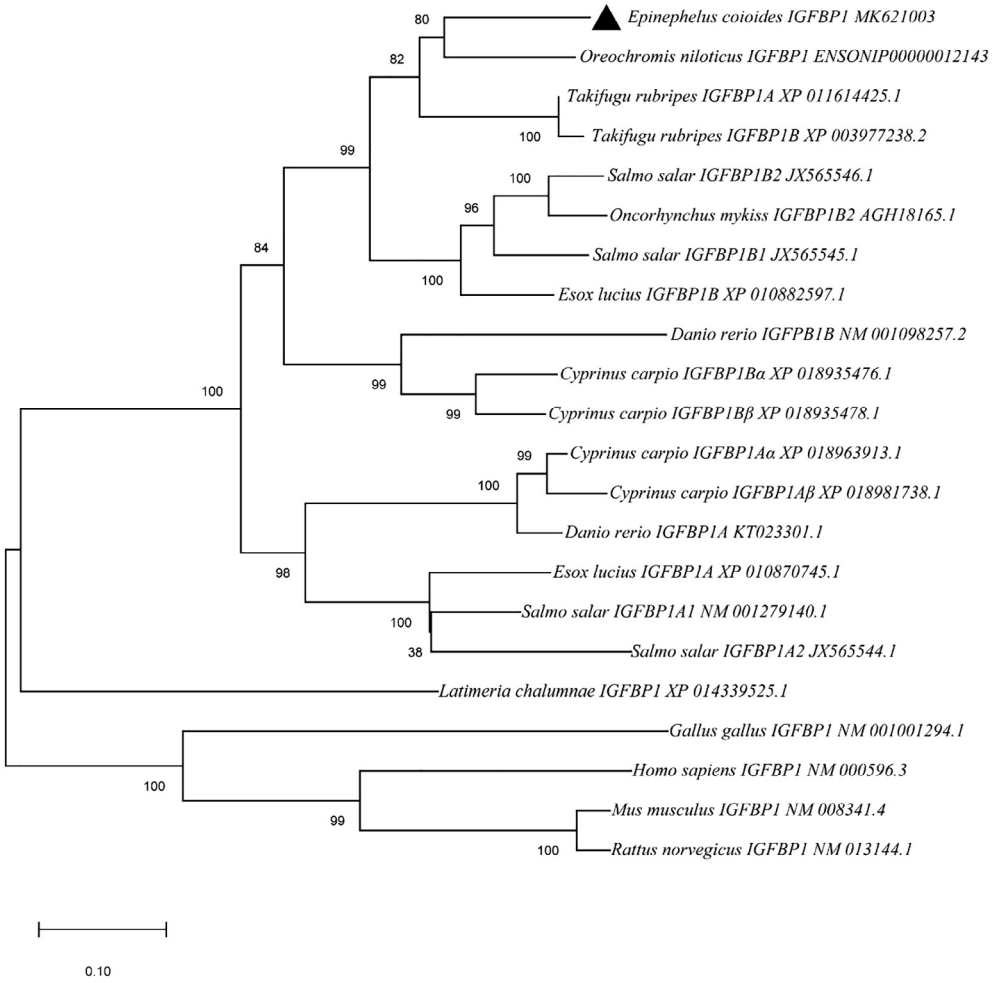

Fig. 3. Phylogenetic tree based on amino acid alignment for IGFBP1 in different species.

isolated and treated with recombinant grouper Gh and human insulin. The *igfbp1* expression was evaluated by Real-time PCR, and the results showed that the *igfbp1* expression was significantly, and dose-dependently, inhibited by recombinant grouper Gh (Fig. 5A). Furthermore, the *igfbp1* expression in grouper primary hepatocytes decreased following incubation with 10 and 100 ng/ml insulin (Fig. 5B).

4. Discussion

Since Igfbps were first identified in four teleost species (Kelley et al., 1992), Igfbp1 has also been reported in a variety of fish species (Chen et al., 2018). In our study, the gene sequence of *igfbp1* was isolated from the liver of grouper. Structural analysis showed that the grouper Igfbp1 protein contained four domain: signal peptide, N-terminal domain, linker domain and C-terminal domain. Previous studies indicated that the structure of IGFBP1 in mammalian and other fish also have the N- and C-terminal domains (Chen et al., 2018). In mammalian and fish Igfbp1, the N-terminal domain contains 12 cysteine residue, which forms six disulfide bonds within the domains (Firth and Baxter, 2002; Shimizu and Dickhoff, 2017; Sun et al., 2011; Zhai et al., 2012). The C-terminal domain of IGFBP1 contains 6 cysteine residues that form three disulphide bonds within the domain. The highly conserved cysteine residues located in the N- and C-terminal domains are important for the high-affinity binding to IGFs (Murphy, 1998) and help to shape the globular structure of the N- and C-terminal domains (Duan and Xu, 2005). In our study, 18 cysteine residues were located in the N- and C-terminal domains in grouper Igfbp1. In addition, a GCGCC motif is located in the N-terminal domain of grouper Igfbp1, and a CWCV motif exists in the C-terminal domain. The GCGCC motif is highly conversed in the IGFBP family and it is involved in forming the first four disulphide bonds (Sitar et al., 2006b). The C-terminal CWCV motif is required for high affinity binding to IGFs (Forbes et al., 2012). It is now established that the GCGCC and CWCV motifs are essential for IGFBP1 functions. As an integrin recognition site, the RGD (Arg-Gly-Asp) motif is important for IGFBP1 interaction with cell surface α5β1 integrin (Jones et al., 1993). The independent cellular functions of IGFBP1 mediated by RGD-integrin have been reported in several studies, and recent research indicated that the RGD domain of IGFBP1 can directly modulate insulin signaling and glucose regulation (Haywood et al., 2017). In our study, the RGD sequence in grouper Igfbp1 was replaced by LGD (Leu-Gly-Asp) in grouper Igfbp1. A previous report showed that all mammalian IGFBPs contain the RGD motif, but this motif is absent in fish. In addition, the RGD motif was replaced by PAD in silver carp, zebrafish and common carp Igfbp1 (Chen et al., 2018). The substituted RGD motif in fish may possess different biological functions compared to mammalian IGFBP1. Posttranscriptional modifications are important for biological functions of proteins. In the present study, several phosphorylation and glycosylation sites were located in grouper Igfbp1. The number of phosphates per molecule of IGFBP1 are thought to control its affinity for IGFs (Clemmons, 2011). A study of human IGFBP1 showed that IGFBP1 phosphorylation increased its affinity for IGF1 by 6-fold (Jones et al., 1991). In addition, IGFBP1 phosphorylation could suppress the bioactivity of IGF1 in fetal and placenta growth (Gibson et al., 2001). Posttranslational modifications of IGFBPs, such as glycosylation and phosphorylation, can modify certain properties, such as circulating half-life, cell association and IGF binding affinity (Bach, 2018b). The

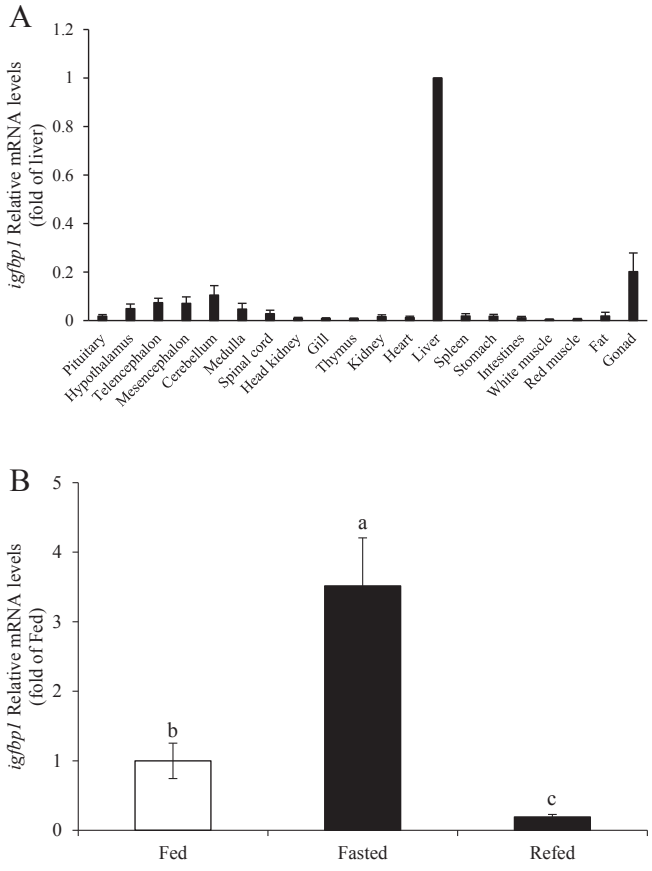

Fig. 4. Analysis the expression pattern of *igfbp1* in grouper. (A) Tissue distribution of *igfbp1* in grouper. The mRNA levels were quantified by Real-time PCR and normalized against *18S* transcripts. All data were represented as the mean ± S.E.M. (n = 3). (B) Effects of fast and refeeding on the mRNA expressions of *igfbp1*. The mRNA expression of *igfbp1* in the liver of groupers were quantified by Real-time PCR. The results were represented as the fold of fed. All data are shown as mean ± S.E.M. (n = 5–6). Significant differences ($P < 0.05$) were indicated by different letters.

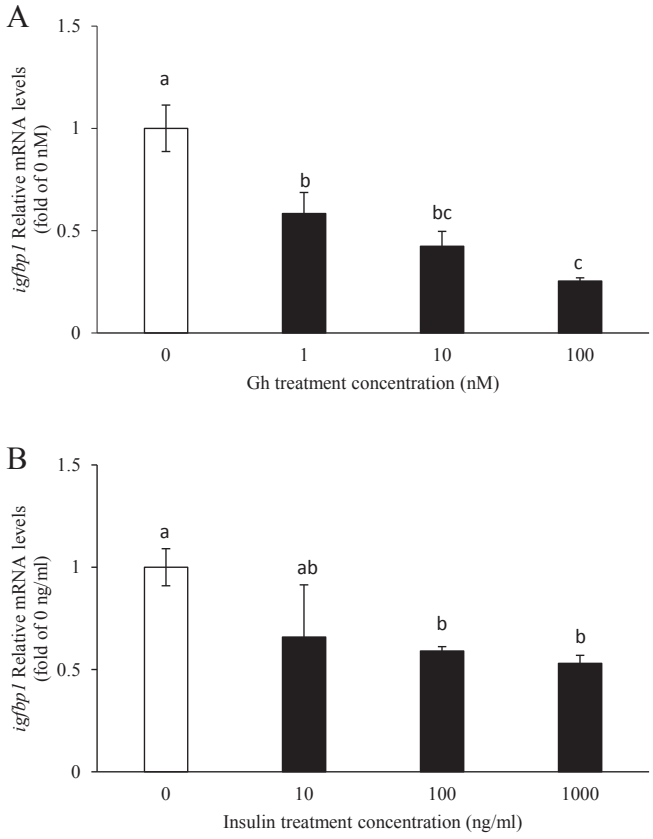

Fig. 5. Effect of insulin/Gh on the mRNA expression of *igfbp1* in primary grouper hepatocytes. (A) Gh, (B) insulin. The cells were seeded in 24-well plates at 5×10^5 per well in 1 mL L15 with 10% FBS. The next day, cells were placed in L15 without FBS for 1 h serum starvation. And then, experiments were performed by the addition of vehicle or recombinant grouper Gh protein (1, 10, 100 nM) or human insulin (10, 100, 1000 ng/ml) for 24 h. At the end of the experiments, the mRNA expression was determined by Real-time PCR. All data are shown as mean ± S.E.M. (n = 5–6). Significant differences ($P < 0.05$) were indicated by different letters.

posttranslational modifications sites may take part in regulating the bioactivity of grouper Igfbp1. Sequence alignment at the protein level showed that grouper Igfbp1 has a high degree of identity with those of other fish. For example, the identities of Igfbp1 between grouper and tilapia, Atlantic salmon and rainbow trout were 80.89, 71.72 and 71.14%, respectively. Based on amino acid sequences, phylogenetic analysis affirmed that grouper Igfbp1 could be grouped into the teleost clade. Cloned grouper Igfbp1 was clustered into Igfbp1B subgroups with other fish. The cloned grouper Igfbp1 may therefore belong to the B isoform of Igfbp1 in teleosts. In summary, based on these analyses, the sequence we obtained is the prospective Igfbp1 of grouper.

In the present study, the *igfbp1* expression of grouper was highest in the liver, and expression was detected in brain and gonad. Previous studies indicated that the *Igfbp1* is mainly expressed in the liver, where it takes part in the acute metabolic regulation of IGF activity (Hoeflich and Russo, 2015). Moreover, *Igfbp1* is also expressed in ovarian granulosa cells and in decidualized stromal cells of the uterine endometrium, where IGFBP1, together with other factors, regulates ovulation, decidualization, menstrual cycles and blastocyst implantation (Lee and Giudice, 1997). In the common carp, *igfbp1a* mRNA was widely expressed in all tissues and mainly expressed in the liver (Chen et al., 2018). In goldfish, *igfbp1* was also predominantly expressed in the liver and widely expressed in all tissues, including the brain region (Chen et al., 2016). *Igfbp1a* expression is distributed in many tissues of salmon, but the liver has a relatively low expression. However, *igfbp1b* was mainly expressed in liver, and other tissues had very little expression (Shimizu et al., 2011). Relatively speaking, zebrafish *igfbp1a* was only expressed in the liver (Maures and Duan, 2002). Therefore, the multiplicity expression of *igfbp1* is generally in teleost. *IGFBP1* expression in the brain may have important neuroprotective functions during hypoxia-induced ischemic brain injury (Chen et al., 2018). For example, *igfbp1* expression of Atlantic croaker was increased by hypoxia, and Igfbp1 protects the brain from hypoxia-induced oxidative stress (Rahman and Thomas, 2011). In the common carp, *igfbp1a* expression was increased by hypoxia induced in the brain (Chen et al., 2018). We suppose that the Igfbp1 is involved in the regulatory function of the grouper brain, and this needs to be investigated in the future.

To assess the regulatory role of Igfbp1 in food intake and energy balance in grouper, a fasting and refeeding experiment was performed. In this experiment, *igfbp1* mRNA expression was detected in grouper and was significantly increased in the liver upon fasting for 7 days. After refeeding, the *igfbp1* expression was markedly decreased in the liver. Thus, IGFBP1 is involved in the regulation of food intake and energy balance. A review of IGFBPs indicated that the IGFBP1 levels were greater in the fasting state and decreased after eating (Bach, 2018b). The results of human studies showed that the levels of IGFBP1 in plasma are increased by food deprivation, and plasma IGFBP1 levels return to nearly normal levels after refeeding (Hoeflich and Russo, 2015). Similarly, IGFBP1 levels were increased 5.5-fold by an overnight fast. Moreover, after fasting for 4 days, the levels of IGFBP1 could be

increased another 3.2-fold, and the IGFBP1 levels return to the normal after refeeding (Clemmons, 2011). The results showed that the mRNA expression of *igfbp1* and *igfbp2* was increased in fasted zebrafish, and *igfbp1* expression was returned to prefast level upon refeeding (Wood et al., 2005). Furthermore, the study of Igfbp1 in salmon indicated that the Igfbp1 levels were higher in fasted fish than in fed fish (Shimizu et al., 2006). Fasting was also associated with increased levels of 30-KDa Igfbp in goby (*Gillichthys mirabilis*) serum, as well as in the levels of 25-KDa Igfbp in striped bass (Kelley et al., 2001). As discussed above, IGFBP1 is involved in the regulation of energy balance and food intake; the mRNA and protein levels of IGFBP1 are increased by fasting, and the levels of IGFBP1 are decreased after refeeding/eating. In our present study, the expression of *igfbp1* was sharply decreased after refeeding in the grouper liver. The rapidly increased glucose and insulin levels after refeeding inhibit the expression of *igfbp1* in grouper liver. In our present study, *igfbp1* expression was inhibited by insulin in the in vitro experiment.

To evaluate the effect of Gh on the expression of *igfbp1*, an in vitro experiment was performed. Primary grouper hepatocytes were treated with recombinant grouper Gh, and the *igfbp1* mRNA expression was inhibited in a dose-dependent manner. Similarly, the results in primary rat hepatocytes showed that GH inhibited the expression of *Igfbp1* by activation of the protein kinase C pathways (Lee and Giudice, 1997). Hypophysectomy led to a two-fold increase in IGFBP1 concentration in the plasma upon low-level GH treatment. In GH-deficient humans, the levels of circulating IGFBP1 have been shown to be increased (Clemmons, 2011). IGFBP1 was decreased in GH-deficient humans after injection of GH (Clemmons, 2011). Thus, there may be a negative correlation between GH levels and IGFBP1 levels in the mammalian circulation. In 2017, Shimizu and Dickhoff reviewed the effect of GH on IGFBPs in fish (Shimizu and Dickhoff, 2017). Even so, there is little available information on the regulation of Igfbp1 by Gh in fish. In coho salmon primary hepatocytes, *igfbp1* mRNA expression was decreased by Gh treatment (Pierce et al., 2006). Furthermore, the *igfbp1a* expression was inhibited by GH in primary cultured carp hepatocytes (Chen et al., 2018). However, in hypophysectomized tilapias, *igfbp1b* mRNA expression was no significantly altered in the liver after GH injection (Breves et al., 2014). The effect of Gh on *igfbp1* expression in grouper is similar to that of in coho salmon and common carp. In common carp, GH inhibits *igfbp1* expression via MAPK and PI3 kinase signaling pathways (Chen et al., 2018). The regulatory mechanism of Gh on *igfbp1* expression in grouper should be clarified in future studies.

To assess the effect of insulin on the mRNA expression of *igfbp1* in primary hepatocytes, human insulin was used to treat the cells. The result showed that *igfbp1* mRNA expression was significantly decreased by insulin treatment. In isletoctomized goby, the plasma levels of 24- and 30-KDa Igfbps were induced, and the Igfbps levels were restored to basal conditions after insulin treatment (Kelley et al., 2001). In coho salmon circulation, there was a negative correlation between insulin and Igfbp1b levels (Shimizu and Dickhoff, 2017). In our present study, the expression of *igfbp1* was also inhibited by insulin in grouper. This suggests that IGFBP1 is negatively regulated by insulin in these species. In contrast, insulin increased *igfbp1* mRNA, but it did not change the medium IGFBP1 immunoreactivity in vitro using primary salmon hepatocytes (Pierce et al., 2006). It is speculated that the negative correlation of insulin on Igfbp1 may be species-specific in fish. Insulin is an inhibitor of IGFBP1 in mammals. IGFBP1 mRNA and protein levels were inhibited by insulin treatment and the IGFBP1 levels were inversely related to insulin in the circulation after meals (Shimizu and Dickhoff, 2017). In mammals, *Igfbp1* transcription was inhibited by insulin via an insulin response element (IRE) located at the 5′ transcription start site (Lee and Giudice, 1997). The protein kinase B/Akt pathway was involved in the insulin inhibition of *Igfbp1* transcription (Murphy, 1998). However, the regulatory mechanism by which insulin inhibitis *igfbp1* transcription in fish has rarely been reported, and it may be a key point in future studies.

5. Conclusion

In conclusion, in our present study, the grouper *igfbp1* gene sequence was cloned, and the mRNA expression of *igfbp1* was mainly found in the liver and brain region of grouper. In the fast and refeeding experiment, *igfbp1* expression in the liver was significantly increased in the fasted group, and *igfbp1* expression in the liver was sharply decreased in the refeeding group. In the in vitro experiment, *igfbp1* expression was markedly suppressed by Gh/insulin in cultured primary grouper hepatocytes. To the best of our knowledge, our data provides the sequence of grouper Igfbp1 for the first time. The data provides evidence for the regulatory effect of insulin and Gh on *igfbp1* expression in grouper. The regulatory mechanism underlying the impact of insulin and Gh on *igfbp1* expression is currently under investigation.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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