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REGULAR ARTICLE

Expression, mitogenic activity and regulation by growth hormone of growth hormone/insulin-like growth factor in *Branchiostoma belcheri*

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Abstract The growth hormone (GH)/insulin-like growth factor (IGF) axis is unique to all the vertebrate species but its evolutionary origin is ill-defined. We therefore cloned a cDNA encoding *Branchiostoma belcheri* IGF (*BbIGF*). *BbIGF* was expressed in a tissue-specific manner, with the most abundant expression in the hepatic caecum, the putative liver precursor. The recombinant *BbIGF* expressed in vitro showed mitogenic activity capable of stimulating cell proliferation in the flounder gill, a characteristic of vertebrate IGF. Quantitative real-time polymerase chain reaction demonstrated that the recombinant rat GH was able to induce a significant up-regulation of *BbIGF* expression in the hepatic caecum. Moreover, Western blotting revealed the presence of a molecule similar to rat GH receptor in the hepatic caecum. These results suggest that *BbIGF* expression is inducible by exogenous mammalian GH, suggesting the presence of a GH/IGF axis in *B. belcheri*. The relationship between *BbIFG* expression and the origin of the vertebrate liver is discussed.

Keywords Growth hormone · Insulin-like growth factor · Hepatic caecum · Amphioxus · Lancelet · *Branchiostoma belcheri*

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Introduction

Growth hormone (GH), formerly known as somatotropin, is a polypeptide that is secreted by the pituitary gland, that promotes the growth of the body and that influences the metabolism of proteins, carbohydrates and lipids. A major target of GH is the liver. In this tissue, GH binds to GH receptor (GHR) and stimulates the release of somatomedins or insulin-like growth factors (IGFs), polypeptides that have high structural similarity to prepro-insulin and that play a role in regulating cell proliferation, differentiation and apoptosis (for a review, see Pavelić et al. 2007). IGFs are part of a complex system that cells use to communicate with their physiological environment. This complex system, often referred to as the pituitary-liver axis or GH/IGF axis, consists of the two ligands IGF-1 and IGF-2, their receptors IGF1R and IGF2R, a family of six high-affinity IGF-binding proteins (IGFBP 1-6) and associated IGFBP-degrading enzymes (Kelley et al. 2002; Reinecke and Collet 1998). The pituitary-liver axis has been shown to be present in all vertebrate species including lamprey and hagfish (Kawauchi and Sower 2006; Leibush et al. 1998). However, the evolutionary origin of this axis remains ill-defined.

The cephalochordate amphioxus, which occupies a nodal position from invertebrate to vertebrate, possesses the so-called Hatschek's pit, which is an invagination of the dorsal epithelium of the preoral cavity and possible morphological homologue of the vertebrate pituitary (Tjoa and Welsch 1974). This homology is further strengthened by immunohistochemical studies showing that Hatschek's pit contains a vertebrate-like gonadotropic hormone (Chang et al. 1982; Nozaki and Gorbman 1992). The cephalochordate amphioxus also has a hepatic caecum, the pouch that protrudes forwards as an outpocketing of the digestive tube and extends along the right side of the posterior part of the

pharynx, which has long been considered to be the precursor of vertebrate liver (Hammar 1898; Welsch 1975). Our recent studies have shown that the hepatic caecum of *Branchiostoma belcheri* is capable of synthesizing liver-specific proteins including vitellogenin (Han et al. 2006), antithrombin (Liang et al. 2006), plasminogen (Liang and Zhang 2006) and alanine aminotransferase (Lun et al. 2006), providing additional evidence for the homology of the hepatic caecum to vertebrate liver. In agreement, a gene encoding an IGF polypeptide has been identified from *B. californiensis* and its deduced amino acid sequence shows that the peptide is as similar to IGFs as it is to insulin (Chan et al. 1990). Moreover, a gene coding for the IGF peptide receptor has also been identified from the same species (Pashmforoush et al. 1997). These data suggest the presence of both pituitary-like and liver-like tissues in the cephalochordate amphioxus. However, whether the GH/IGF axis is present remains to be demonstrated. In addition, although the molecular structures of the cephalochordate amphioxus insulin/IGF gene and its receptor are becoming clearer, many questions are still unsolved. For example, where is the insulin/IGF gene expressed in the cephalochordate amphioxus? Does the insulin/IGF polypeptide share functional properties similar to those of vertebrate insulin and/or IGFs? Is the insulin/IGF gene expression inducible by GH, as observed in vertebrate species? We have therefore sought to answer these questions and to examine whether exogenous GH can stimulate the expression of the insulin/IGF gene in this primitive chordate.

Materials and methods

Cloning and sequencing of IGF-like cDNA

Total RNAs were extracted with Trizol (Invitrogen) from adult *B. becheri* collected from the amphioxus-containing area in the vicinity of Qingdao, China. To obtain the 3′-end of *B. belcheri* IGF-like cDNA, the rapid amplification of cDNA ends (RACE) was used. A gene-specific primer for 3′-RACE, 5′-GAAGCCCGTCTTTCCGTTCATCAG-3′, was designed by using Primer Premier 5.0 software as based on the nucleic acid sequences of *B. californiensis* insulin/IGF peptide (GenBank accession number: M55302) and insulin/IGF peptide precursor from *B. floridae* genome data (JGI Protein ID: 291016). The 3′-RACE-Ready cDNA was prepared according to the manufacturer's instructions for the SMART RACE cDNA amplification kit (Clontech). The 3′-RACE reaction mixture (final volume 20 μl) contained 10× Advantage 2 PCR buffer, 0.2 mM (each) dNTPs, 10× Universal Primer A Mix (UPM, Clontech), 0.4 μM gene-specific primer, 50× Advantage 2 polymerase mix, and 2 μl 3′-RACE-Ready cDNA (template). The 3′-RACE reaction was carried out under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min. The 3′-RACE products of predicted sizes were gel-purified by using the AXYGEMTM DNA gel extraction kit (AXYGEN), ligated to the T/A cloning vector pGEM-T easy (Promega) at 4°C overnight and transformed into Top10 competent cells (TIANGEN). The positive clones were selected and sequenced with an ABI PRISM 3730 DNA sequencer. The sequences were searched with BLASTx in GenBank for comparative analysis.

Reverse transcription with polymerase chain reaction (RT-PCR) amplification was employed to obtain the full-length cDNA sequence. The sense primer, 5′-CACTCT GACTATAGCAACAGGCAT-3′, was designed based on the 5′-untranslated region (UTR) sequences of the insulin/IGF peptides of *B. californiensis* and *B. floridae*. The antisense primer, 5′-CGAGGTCGGTTGGATACTGATGG-3′, was designed based on the nucleic acid sequence of the 3′-RACE amplification product. First-strand cDNA was synthesized with a reverse transcription system (Promega) by using an oligo d(T) primer. The RT-PCR amplification was performed as follows: initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 60 s and a final cycle of 72°C for 7 min. The PCR product was gel-purified, sequenced and assembled.

Sequence and phylogenetic analyses

The cDNA sequence assembled, named *BbIGF*, was analysed for coding probability with the DNATools program. Comparison against the GenBank protein database was performed by using the BLAST network server at the National Center for Biotechnology Information. The SMART program (http://smart.embl-heidelberg.de/) was used to predict the functional sites and domains in the deduced amino acid sequence. The signal peptide was predicted with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The potential glycosylation sites were predicted with NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc). The conserved regions and multiple protein sequences were aligned by using the MegAlign program by the CLUSTAL method in the DNASTAR software package. The phylogenetic tree was constructed by the neighbour-joining method within the PHYLIP 3.6 c software package by using 1000 bootstrap replicates. A three-dimensional (3D) model of BbIGF and of zebrafish IGF1, IGF2 and insulin was predicted by using the template models of the human (PDB code: 1imxA) IGF domain by fully-automated protein structure homology modelling (Schwede et al. 2003; http://www.expasy.org/swissmod/SWISSMODEL.html).

Southern blotting

The genomic DNAs for Southern blotting analysis were isolated from *B. belcheri* and digested with the restriction enzymes (1 U/μg DNA) *Eco*RV, *Sca*I and *Xba*I at 37°C for 24 h or *Bam*HI at 30°C for 24 h. The digested DNAs were separated on a 1% agarose gel by using 1× TBE (89 mM TRIS-borate and 2 mM EDTA) and transferred onto nylon membranes (Osmonics, Trevose, USA). Digoxigenin (DIG)-labelled *BbIGF* riboprobes of about 750 bp were synthesized in vitro from linearized plasmid DNA following the instructions of the DIG-UTP supplier (Roche). Membranes were hybridized at high stringency with the DIG-labelled *BbIGF* riboprobes. The hybridized bands were visualized according to the instructions of the detection kit.

Construction of expression vector

The open reading frame (ORF) of mature *BbIGF* (excluding the signal peptide) was amplified by PCR with the upstream primer 5′-CCGGAATTCGAGTACCTGTGCGGTTC CACCCT-3′ (*Eco*RI site is underlined) and the downstream primer 5′-AAGGAAAAAAGCGGCCGCTTATCAGTT GAGTGATAATTG-3′ (*Not*I site is underlined). The reaction was carried out under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 60°C and extension at 72°C for 1 min. The PCR product was digested with *Eco*RI and *Not*I and sub-cloned into the plasmid expression vector pET32a (Novagen) previously cut with the same restriction enzymes. The identity of the insert was verified by sequencing. The plasmid was designated as pET32a/BbIGF.

Expression and purification of recombinant protein

The cells of *Escherichia coli* BL21 were transformed with the plasmid pET32a/BbIGF and cultured overnight in LB broth containing 100 μg/ml ampicillin. The culture was diluted 1:1000 with LB broth and subjected to further incubation at 37°C for 2 h. The expression of BbIGF was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to the culture at a final concentration of 0.1 mM and the recominant protein was purified as previuously described (Fan et al. 2007). The purity of the eluted samples was analysed by 12% SDS-polyacrylamide gel electrophoresis (PAGE); proteins were stained with Coomassie brilliant blue R-250. Protein concentrations were determined by the method of Bradford with bovine serum albumin as a standard.

Western blotting

Humoral fluids were prepared from *B. belcheri* by the method of Wang et al. (2002). A total of about 1000 *B. belcheri* were cut into pieces on ice and centrifuged at 15,000*g* for 20 min at 4°C. The supernatant was pooled and stored at −70°C until use.

The humoral fluids from *B. belcheri*, the total cellular extracts from IPTG-induced *E. coli* BL21 containing pET32a/BbIGF and the purified recombinant BbIGF were mixed with SDS sample buffer, boiled for 5 min and run on a 12% SDS-PAGE gel. After electrophoresis, the gel was washed with 20 mM phosphate-buffered saline (PBS) containing 0.1% Tween−20 for 15 min and the proteins on the gel were blotted onto nitrocellulose membrane (Hybond, Amersham Pharmacia). The blotted membranes were immunostained by using anti-rat IGF-I or IGF-II antibody (BIOSYNTHESIS, Beijing, China) diluted 1:200 as the primary antibody, followed by staining with horseradish-peroxidase-labelled goat anti-rabbit IgG (Zhongshan, China).

To test the presence of a molecule similar to GHR, both the liver dissected out of rat and the hepatic caecum removed from *B. belcheri* were homogenized in RIPA buffer containing 1% Triton 100, 50 mM TRIS-base, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 0.25% Na-deoxycholate and 1 mM phenylmethane sulphonylfluoride (Fukui et al. 1983) or in 50 mM TRIS-HCl buffer (pH 7.2) with 50 mM NaCl, and centrifuged at 15,000*g* at 4°C for 20 min. The supernatants were pooled and stored at −70°C until used. Both the rat liver and *B. belcheri* hepatic caecum extracts were run on 12% SDS-PAGE gels. The proteins were blotted onto nitrocellulose membrane and immunostained with rabbit anti-rat GHR antibody (ADL, America) diluted 1:100 as the primary antibody.

Northern blotting

Total RNAs were prepared with Trizol (Invitrogen) from adult *B. belcheri* ground in liquid nitrogen. An aliquot of 5 μg RNAs was electrophoresed, blotted onto Nylon membrane (Roche) and hybridized as described by Fan et al. (2007).

In situ hybridization histochemistry

B. belcheri was cut into three to four pieces and fixed in freshly prepared 4% paraformaldehyde in 100 mM PBS (pH 7.4) at 4°C for 8 h. The samples were dehydrated, embedded in paraffin and sectioned at 7 μm. The sections were mounted onto slides coated with poly-L-lysine, dried at 42°C for 36 h and de-paraffinized in xylene for 20 min (two changes of 10 min each) followed by immersion in absolute ethanol for 10 min (two changes of 5 min each). They were then re-hydrated and brought to double-distilled water treated with 0.1% diethylpyrocarbonate. In situ hybridization histochemistry was carried out as described by Fan et al. (2007).

Immunohistochemistry

B. belcheri was cut into three to four pieces and fixed in freshly prepared 4% paraformaldehyde (w/v) in 100 mM PBS (pH 7.4) at 4°C for 24 h. After dehydration, the samples were embedded in paraffin and sectioned at 7 μm. The sections were mounted on slides and dried at 42°C for 36 h. Immunohistochemical staining was performed as described by Liang et al. (2006).

Assays for recombinant BbIGF activities

To test the mitogenic activity of the recombinant BbIGF, FG-9307 cells derived from the gills of *Paralichthys olivaceus* (Tong et al. 1997) were grown in 25 cm^2 cell culture flasks (Corning, America) in minimal essential medium (MEM; pH 7.4) with 2.2 mg/ml $NaHCO_3$, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% bovine calf serum (BCS; Hyclon) at 20°C, harvested and diluted to a concentration of 10^5 cells/ml in MEM with 10% BCS. The cell suspension was agitated and an aliquot of 200 μl was sampled and added to each well of 96-well culture plates (Falcon). The plates were incubated at 20°C for 12 h and the medium was removed. The cells were re-fed with serum-free medium (SFM) and cultured at 20°C for 18 to 24 h. The SFM was then replaced with SFM plus various concentrations (0, 0.1, 1 and 10 μg/ml) of the recombinant BbIGF. The growth of the cells was observed under an inverted microscope and photographed. After 48 h, cell growth was assayed by the tetrozolium (MTT) method (Borenfreund et al. 1988).

Three duplicate cultures were tested for each concentration. All the experiments were performed three times. Statistical analysis was performed by using SPSS 13.0 for Windows. The statistical significance of difference between mean values was determined by Student's two-tailed *t*-tests; differences at $P<0.05$ were considered significant. All data were expressed as means±SD.

The blood glucose reduction assay of the recombinant BbIGF was carried out according to Chinese Pharmacopoeia (Zheng et al. 2005). A total of 90 Kunming mice (male: female = 1:1) with body weight of 20–23 g each were divided into six groups and used. Diabetic (high blood glucose) mice were induced by a single injection of alloxan (Sigma, St Louis, USA) of 110 mg/kg body weight via the tail veins. The differences between groups were analysed by Student's *t*-tests as above and the data were expressed as means±SD.

Assay for effect of exogenous GH on *BbIGF* expression

The hepatic caecums were dissected out of *B. belcheri* and cut into pieces (1 mm^3). After being washed three times with MEM containing 2.2 mg/ml $NaHCO_3$, 100 IU/ml penicillin and 100 μg/ml streptomycin, they were cultured in this medium plus 100 ng/ml and 0 ng/ml (control) of rat recombinant GH (Protein Laboratories Rehovot, Israel) at 18°C and sampled at 0, 3, 12, 24 and 36 h, respectively.

Total RNAs were prepared with Trizol from the samples. After digestion with RQ1 RNase-free DNase (Promega) to eliminate genomic contamination, cDNAs were synthesized with a reverse transcription system (Promega) with oligo d (T) primer and used as the template. After qualification of the cDNA template, real-time PCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems, USA) to investigate the expression of *BbIGF*. Two *BbIGF*-specific primers, viz. 5′-CTCATCCGCCCATCAGTA-3′ and 5′-GGTTCTTTCTTGTCCGTTT-3′, were used to amplify a PCR product of 132 bp. The β-actin gene was chosen as the reference for internal standardization. Two *β-actin* primers, viz. 5′-TTCCAGCCTTCATTCCTCG-3′ and 5′-CGGTGTTGGCGTACAGGTC-3′, were used to amplify a β-actin gene fragment of 109 bp. The real-time PCR amplifications were carried out in triplicate in a total volume of 20 μl reaction mixture containing 10 μl 2× SYBR *Premix Ex Taq* (Takara), 0.4 μl ROX Reference Dye II (50×), 1 μl of the 1:5 diluted cDNA, 0.2 μl each of *BbIGF*-specific primers (20 μM) or *β-actin* primers (to amplify *β-actin*) and 8.2 μl PCR-grade water. The real-time PCR program was as follows: denaturation at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 60°C for 15 s and 72°C for 35 s. Dissociation analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected.

After the PCR program, data were analysed with 7500 System SDS Software v 1.4.0 (Applied Biosystems). All analyses were based on the C_T values of the PCR products. The C_T was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. To maintain consistency, the baseline was set automatically by the software. The comparative C_T method ($2^{-\Delta\Delta CT}$ method) based on C_T values for both *BbIGF* and *β-actin* was used to analyse the expression level of *BbIGF*. All data are given in terms of relative mRNA expressed as means±SD. The data obtained from real-time PCR analysis were subjected to a one-way analysis of variance followed by Dunnett two-sided test to determine differences in the mean values among the treatments. Statistical analysis was performed by using SPSS 13.0 for Windows; significance was concluded at $P<0.05$.

Results

Sequence, phylogeny and copy number of *BbIGF*

The cDNA obtained (GenBank number: EU420069) was 1630 bp long with an ORF of 924 bp, a 5′-UTR of 23 bp

and a 3′-UTR of 683 bp. The ORF encoded a polypeptide of 307 amino acids with a molecular mass of approximately 35 kDa. The initiation codon (ATG) was assigned on the basis that there was no ATG in the 5′-UTR nucleotides and that the DNA surrounding the initiation codon ATG had a purine at positions of both −3 and +4, in accordance with the Kozak consensus sequence (Kozak 1987). The 3′-UTR had a polyadenylation signal AATAAA and a polyadenylation tail. The deduced polypeptide contained two potential N-linked glycosylation sites at positions 2–4 and 263–265, respectively. The first one-third (residues 1–101) of the polypeptide was organized much like a typical prepro-insulin with a signal peptide (24 residues), B chain, C-peptide and A chain, whereas its remaining sequence (residues 102–307) could be divided into putative D and E domains reminiscent of proIGF. Sequence comparison

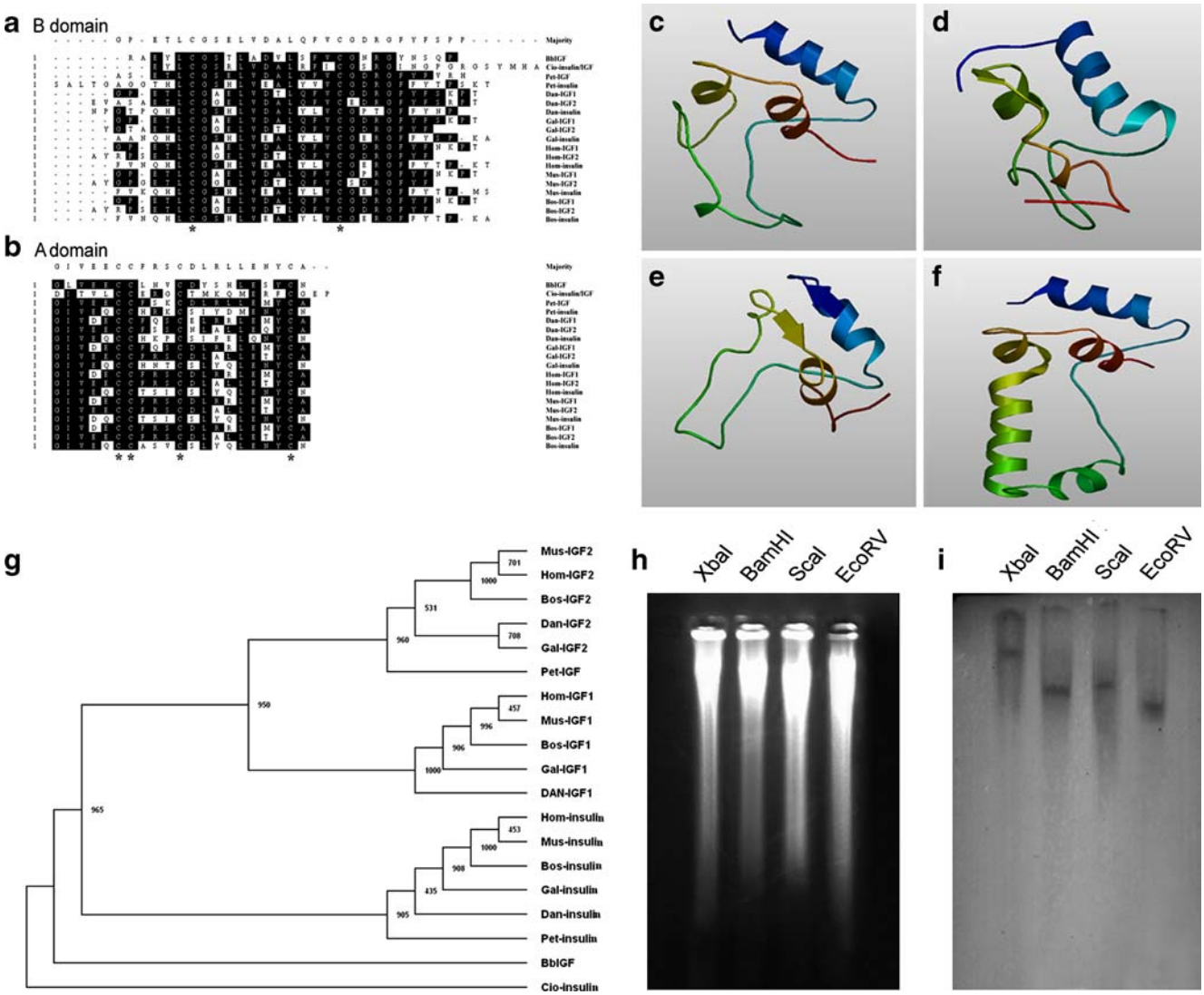

Fig. 1 **a**, **b** Alignment of the B and A domains of BbIGF with their homologous counterparts from the vertebrate insulin/IGF family members and sea squirt insulin/IGF peptide (*residues on black background* amino acids matching the consensus, - gaps introduced into sequences to optimize alignment, *asterisks* conserved cysteine residues). The sequences are (species name and GenBank accession number): BbIGF (*Branchiostoma belcheri*, EU420069), Cio-insulin/IGF (*Ciona intestinalis*, DQ538510), Pet-IGF (*Petromyzon marinus*, BAC15764), Pet-insulin (*Petromyzon marinus*, P68987), Dan-IGF1 (*Danio rerio*, AAK58584), Dan-IGF2 (*Danio rerio*, AAM75746), Dan-insulin (*Danio rerio*, O73727), Gal-IGF1 (*Gallus gallus*, P18254), Gal-IGF2 (*Gallus gallus*, NP_001025513), Gal-insulin (*Gallus gallus*, P67970), Hom-IGF1 (*Homo sapiens*, NM_000618), Hom-IGF2 (*Homo sapiens*, P01344), Hom-insulin (*Homo sapiens*, P01308), Mus-IGF1 (*Mus musculus*, P05017), Mus-IGF2 (*Mus musculus*, P09535), Mus-insulin (*Mus musculus*, P01326), Bos-IGF1 (*Bos taurus*, P07455), Bos-IGF2 (*Bos taurus*, P07456), and Bos-insulin (*Bos taurus*, P01317). **c–f** Comparison of the three-dimensional (3D) structures of BbIGF with zebrafish IGF1, IGF2 and insulin. **c** The 3D structure of BbIGF. **d** The 3D structure of zebrafish IGF1. **e** The 3D structure of zebrafish IGF2. **f** The 3D structure of zebrafish insulin. **g** Phylogenetic tree constructed by the neighbour-joining method in the PHYLIP 3.5c software package (see **a**, **b** for the sequence references). **h**, **i** Southern blotting. **h** Digested DNAs separated on a 1% agarose gel. **i** Blot hybridized with the digoxigenin-labelled *BbIGF* cDNA probe

revealed that the B and A domains of BbIGF shared 47% to 57% and 44% to 51% identities, respectively, with their homologous counterparts of the insulin/IGF family members from the vertebrates including mammalian species, chicken, zebrafish and lamprey, and 23% and 55% identities with their counterparts of sea squirt insulin/IGF peptide. Six conserved cysteine residues required for the formation of inter- and intra-chain disulphide bonds were also identified (Fig. 1a, b). The method further revealed that the protein encoded by the cDNA shared 90% and 87% identity with *B. floridae* and *B. californiensis* insulin/IGF peptides, respectively. These data suggested that the cDNA coded for a hybrid of insulin/IGF peptide of *B. belcheri*, viz. BbIGF, which is structurally more similar to IGFs. This was supported by 3D modelling using BbIGF and zebrafish IGF1, IGF2 and insulin, which showed that BbIGF had a tertiary structure sharing some features characteristic of both IGF and insulin (Fig. 1c-f).

The phylogenetic tree constructed by neighbour-joining method with the sequences of representative IGFs including BbIGF and insulins and sea squirt insulin/IGF peptide revealed that IFGs were grouped together, branching from the insulin clade, whereas BbIGF was located at the base of both the IFG and insulin clades (Fig. 1g). This again supported the idea that BbIGF was a hybrid of insulin/IGF, agreeing with the suggestion of Chan et al. (1990) that cephalochordate amphioxus IGF represents the archetype for IGFs and insulins.

In *B. californiensis*, the insulin/IGF peptide gene has been shown to be a single-copy (Chan et al. 1990). Likewise, Southern blotting of *B. belcheri* genomic DNA has revealed the presence of only one band, indicating that *BbIGF* is also a single-copy gene (Fig. 1h, i).

Characteristics of recombinant BbIGF

An expression vector including the cDNA coding for mature BbIGF and 5′ additional tags of pET32a was constructed and transformed into *E. coli* cells. The recombinant peptide was induced by IPTG and purified by affinity chromatography on a Ni-NTA resin column. The purified recombinant polypeptide (400 μg/ml) with the Trx-tag, His-tag and S-tag yielded a single band of about 50 kDa on SDS-PAGE gels after Coomassie blue staining (Fig. 2a). Western blotting analysis demonstrated that both the supernatant of the cell lysate of IPTG-induced *E. coli* with the expression vector and the purified recombinant peptide reacted with the rabbit anti-rat IGF-1 and IGF-2 antibodies, forming a single band of approximately 50 kDa (Fig. 2a). This established that the purified peptide was BbIGF. Interestingly, *B. belcheri* humoral fluids were also reactive with rabbit anti-rat IGF-1 and IGF-2 antibodies and produced a band of about 35 kDa corresponding to the molecular mass predicted for BbIGF cDNA (Fig. 2a). This

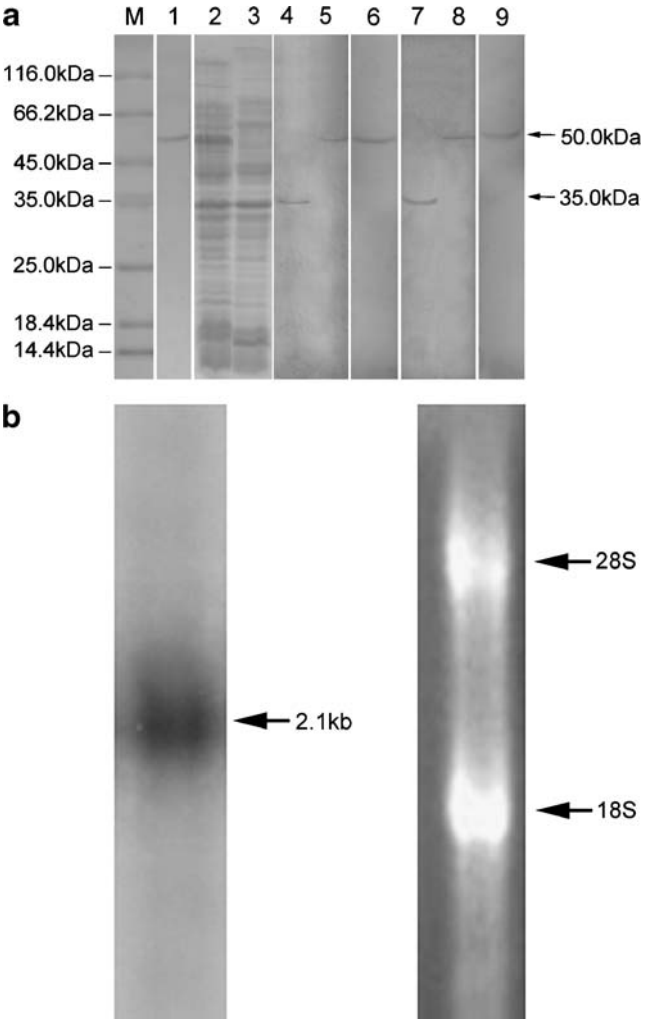

Fig. 2 **a** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (*lane M* standard molecular mass, *lane 1* recombinant BbIGF purified on Ni-NTA resin column, *lane 2* total cellular extracts from IPTG-induced *E. coli* BL21 containing pET32a/BbIGF, *lane 3* total cellular extracts from *E. coli* BL21 containing pET32a/BbIGF before induction, *lane 4* humoral fluids reacted with rabbit anti-rat IGF-I antibody, *lane 5* total cellular extracts from IPTG-induced *E. coli* BL21 containing pET32a/BbIGF reacted with rabbit anti-rat IGF-I antibody, *lane 6* purified recombinant BbIGF reacted with rabbit anti-rat IGF-I antibody, *lane 7* humoral fluids reacted with rabbit anti-rat IGF-II antibody, *lane 8* total cellular extracts from IPTG-induced *E. coli* BL21 containing pET32a/BbIGF reacted with rabbit anti-rat IGF-II antibody, *lane 9* purified recombinant BbIGF reacted with rabbit anti-rat IGF-II antibody). **b** Northern blotting. *Left* Blot probed with DIG-labelled *BbIGF* RNA (*arrow* transcript at approximately 2.1 kb). *Right* Total of 5 μg RNA separated by electrophoresis through a 1.2% agarose formaldehyde-denaturing gel

demonstrated that BbIGF was present in the humoral fluids of *B. belcheri*.

Tissue-specific expression of BbIGF

Northern blotting revealed the presence of a transcript with a single band of about 2.1 kb in *B. belcheri* (Fig. 2b). This

size was ~1.2 kb larger than that of the cDNA clone, suggesting that the transcript had a long 5′ UTR (Sun et al. 2002). In situ hybridization histochemistry showed that the *BbIGF* transcript was specifically expressed in the hepatic caecum and hind-gut (Fig. 3). This was further corroborated by immunohistochemical staining with rabbit anti-rat IGF-1 and IGF-2 antibodies showing that BbIGF was localized in the cytoplasm of hepatic caecum and hind-gut (Fig. 4).

Mitogenic activity of recombinant BbIGF

The mitogenic effect of BbIGF was examined by measuring the growth rate of FG-9307 cells via the MTT method. As shown in Fig. 5, the cell growth was not influenced by lower concentrations (0.1–1 μg/ml) of recombinant BbIGF. However, when a higher concentration (10 μg/ml) of recombinant BbIGF was added to the medium, the cell proliferation was significantly increased, suggesting that BbIGF was capable of stimulating the proliferation of FG-9307 cells. Unexpectedly, BbIGF was not able to reduce blood glucose level, suggesting that it had little insulin activity (see supplemental data S1).

Up-regulation of *BbIGF* by rat GH

Quantitative real-time PCR was employed to quantify *BbIGF* expression in the hepatic caecum following treatment with the recombinant rat GH. Analysis of the dissociation curve of amplification products exhibited a single peak in all cases, indicating that the amplifications were specific.

The results of the time course of the induction of *BbIGF* mRNA are presented in Fig. 6. *BbIGF* expression declined slightly in both the control and GH-treated groups at the initial 3 h time point and remained at a low level up to 12 h, although the expression level in the GH-treated group was continuously higher than that in control. The expression of *BbIGF* started to increase significantly at 24 h after GH treatment and reached a value approximately 3.5-fold higher than the control at 36 h. This showed that exogenous rat GH was able to induce *BbIGF* expression in the hepatic caecum of *B. belcheri*.

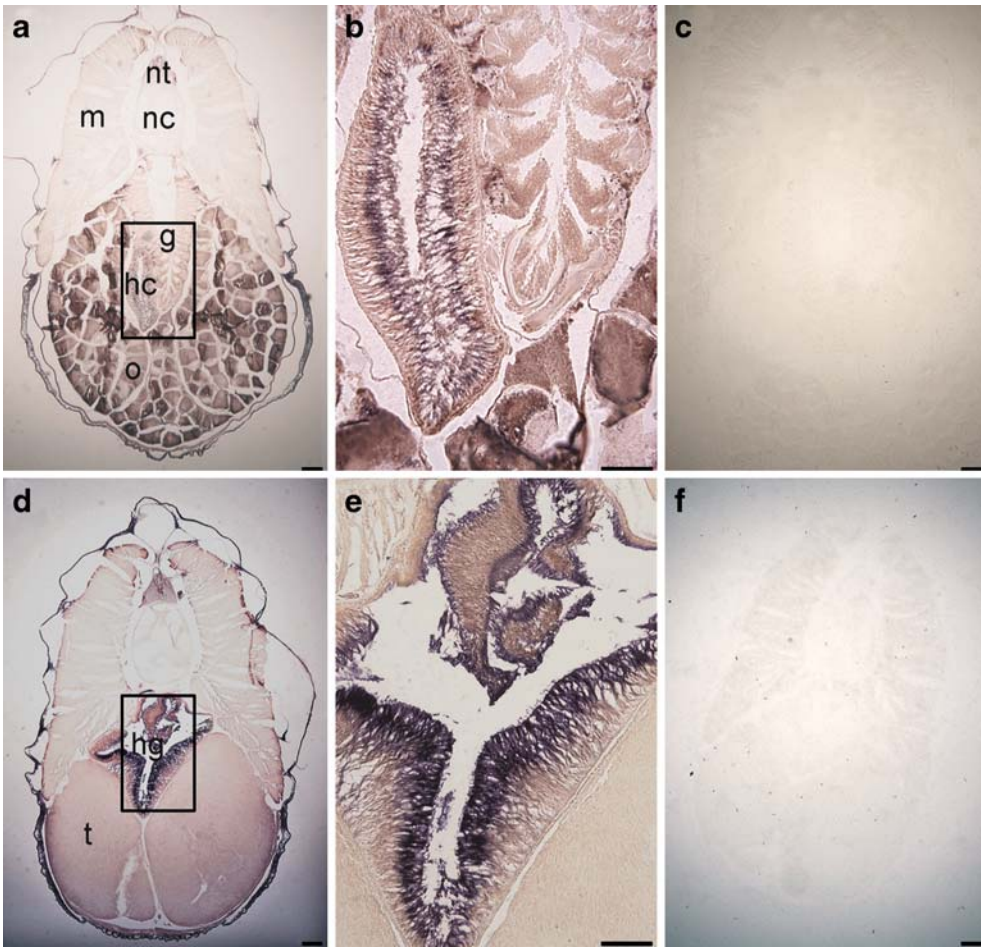

Fig. 3 In situ hybridization histochemistry. **a**, **d** Micrographs showing the presence of *BbIGF* transcripts in the hepatic caeca and hind-gut of male and female *B. belcheri* (*hc* hepatic caecum, *hg* hind-gut, *g* gill, *o* ovary, *t* testis, *m* muscle, *nt* neural tube, *nc* notochord). **b**, **e** Higher magnifications of *boxes* in **a**, **d**. **c**, **f** Micrographs showing no positive signals in control sections.*Bars* 100 μm

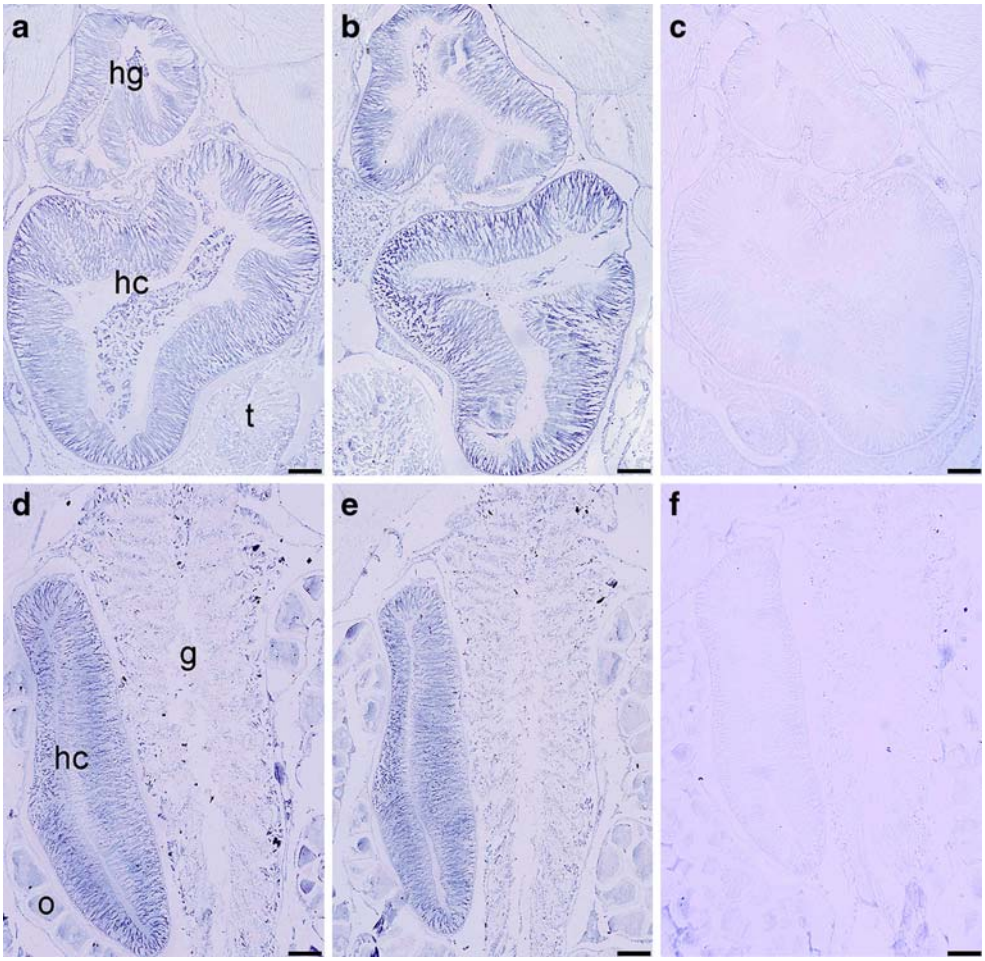

Fig. 4 Immunohistochemical localization of BbIGF. **a**, **d** Sections reacted with rabbit anti-rat IGF-I antibody and showing the presence of BbIGF in hepatic caecum and hind-gut (*hc* hepatic caecum, *hg* hind-gut, *g* gill, *o* ovary, *t* testis). **b**, **e** Sections reacted with rabbit anti-rat IGF-II antibody and showing the presence of BbIGF in the hepatic caecum and hind-gut. **c**, **f** Micrographs showing the absence of BbIGF in control sections. *Bars* 100 μm

Presence of GHR-like immunoreactivity in hepatic caecum

GHR is an integral membrane protein in the vertebrate liver and RIPA is a suitable buffer for the extraction of this molecule (Fukui et al. 1983). Western blotting showed that the homogenates of both the *B. belcheri* hepatic caecum and rat liver prepared with RIPA reacted with the rabbit anti-rat GHR antibody, producing a single immunostained band at approximately 85 kDa (Fig. 7). In contrast, neither of the homogenates extracted with 50 mM TRIS-HCl buffer containing 50 mM NaCl were reactive with the GHR antibody. These data suggested the presence of a

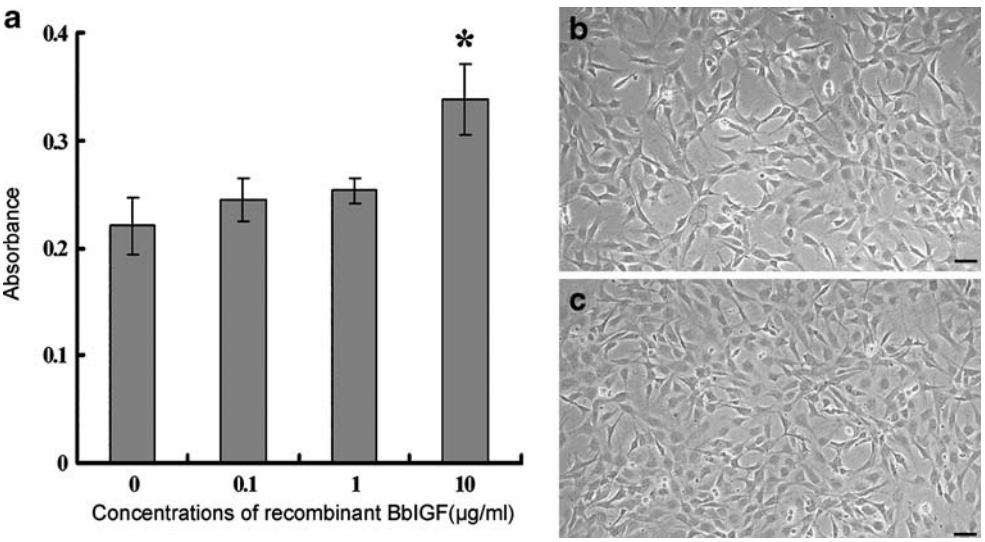

Fig. 5 Mitogenic activity of recombinant BbIGF. **a** Effects of recombinant BbIGF on growth of flounder gill cells incubated with serum-free medium containing various concentrations (0, 0.1, 1, 10 μg/ml) of recombinant BbIGF. After 48 h, cell growth was assayed by the MTT method. Data are expressed as means±SEM (n=3). *Significant differences at P<0.05. **b** Cells grown in the absence of recombinant BbIGF. **c** Cells grown in the presence of 10 μg/ml recombinant BbIGF. *Bars* 100 μm

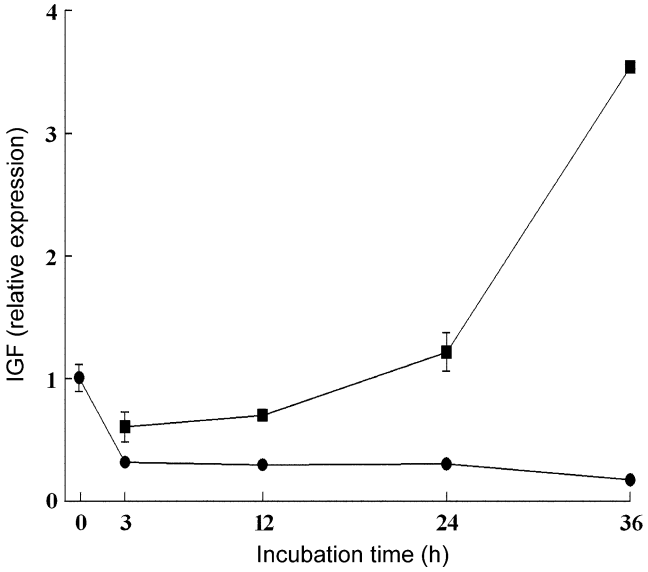

Fig. 6 Effects of exogenous recombinant GH on *BbIGF* expression (*filled circles* absence of exogenous GH, *filled squares* presence of exogenous GH). The time points following exposure are indicated on the *x-axis*; the *y-axis* indicates the expression ratio relative to the *β-actin* gene (*vertical bars* means±SD; *n*=3)

molecule similar to rat GHR in the hepatic caecum of *B. belcheri*.

Discussion

BbIGF is functionally a vertebrate-like IGF

In this study, we have cloned and characterized the cDNA of an IGF polypeptide in *B. belcheri*. The deduced 307-amino-acid polypeptide, BbIGF, has an IGF/insulin-like domain at residue positions 23–101, making it structurally a hybrid of insulin/IGF resembling the insulin/IGF molecules of *B. californiensis* and *B. floridae*. Southern blotting has demonstrated that *BbIGF* is a single-copy gene, a finding in agreement with that of the *B. californiensis* insulin/IGF gene. Our phylogenetic analysis has shown that BbIGF is located at the base of both the IGF and insulin clades. All these data suggest that *BbIGF* is a representative of the archetypical gene from which both IGFs and insulin originate, as initially proposed by Chan et al. (1990).

The cDNAs for IGFs have been documented in a variety of animals including mammalian species (Daughaday and Rotwein 1989; Rinderknecht and Humbel 1976), non-mammalian vertebrates (Clay et al. 2005; Kajimoto and Rotwein 1989, 1990; Kawauchi et al. 2002) and invertebrates (Sherwood and McRory 1997; Sherwood et al. 2006), although the functional properties of non-mammalian IGFs remain poorly understood. We demonstrate here, for the first time, that BbIGF is able to stimulate the proliferation of fish gill cells, although it is not able to reduce high blood glucose levels. This indicates that BbIGF has a conserved mitogenic activity similar to that of vertebrate IGFs (Duan 1997; Pavelić et al. 2007; Pozios et al. 2001; Upton et al. 1997), further suggesting that BbIGF is functionally more closely related to IGF than to insulin. However, a high blood glucose reduction activity of BbIGF cannot be ruled out at present because the recombinant peptide is a protokaryotic expression product that may fold improperly and lack adequate post-translational modification thereby impairing its function.

Homology between hepatic caecum and liver

Vertebrate IGFs including IGF-1 and IGF-2 have been shown to be expressed in various tissues including liver, intestine, skeletal muscle, heart, spleen, brain and ovary (Daughaday and Rotwein 1989; Patruno et al. 2006) but, in all cases, liver is the primary organ producing IGFs (Daughaday and Rotwein 1989; Patruno et al. 2006). Our results show that BbIGF is expressed in the hepatic caecum and hind-gut of *B. belcheri*, in accord with the expression pattern of IGF in *B. floridae* (Reinecke et al. 1993). These data clearly support the hypothesis of homology between the two structures. Moreover, the presence of BbIGF in the humoral fluids, as evidenced by immunoblotting, suggests that the peptide synthesized in the digestive system of *B. belcheri* can be secreted into the blood, circulating via the blood stream throughout the body, a feature that appears to be necessary for vertebrate IGFs acting on diverse target cells.

Induction of *BbIGF* expression by mammalian GH

The GH/IGF axis is unique to all vertebrate species including the extant representative species of Agnatha,

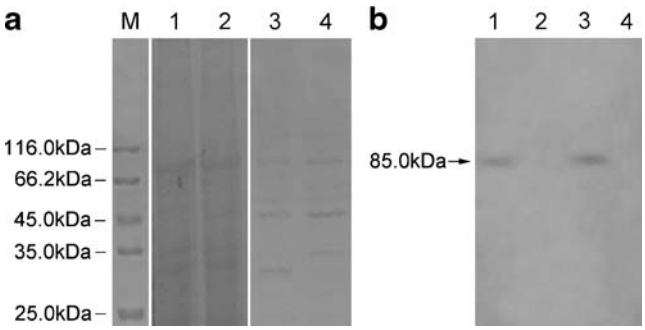

Fig. 7 Presence of GHR-like immunoreactivity in *B. belcheri* as revealed by Western blotting. **a** SDS-PAGE (*lane M* standard molecular mass, *lane 1* homogenate of hepatic caecum extracted with RIPA, *lane 2* homogenate of hepatic caecum extracted with 50 mM TRIS-HCl buffer plus 50 mM NaCl, *lane 3* homogenate of rat liver extracted with RIPA, *lane 4* homogenate of rat liver extracted with 50 mM TRIS-HCl buffer plus 50 mM NaCl). **b** Western blotting showing the presence of GHR-like molecule in the hepatic caecum and rat liver (*lanes 1–4* as in **a**)

lamprey and hagfish (Kawauchi and Sower 2006; Leibush et al. 1998). Previous studies including ours have merely suggested the presence of a pituitary-liver-like axis in cephalochordates (Tjoa and Welsch 1974; Chang et al. 1982; Nozaki and Gorbman 1992; Han et al. 2006; Liang et al. 2006; Liang and Zhang 2006). Here, we show that exogenous recombinant mammalian GH is able to induce the expression of *BbIGF* in the hepatic caecum in a dose-dependent manner. GH generally acts via binding to its own receptor. Accordingly, we have demonstrated the presence of a GHR-like membrane molecule in the hepatic caecum. These data establish, for the first time, a definitive link between GH and IGF in *B. belcheri*. However, analysis of the genome sequence of *B. floridae* has not as yet revealed the presence of GH or GHR. Therefore, our results at present only suggest the possibility of the presence of a GH/IGF axis in *B. belcheri* and await the cloning of the genes for GH and GHR in this animal.

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References

Borenfreund E, Babich H, Martin-Alguacil N (1988) Comparison of two in vitro cytotoxicity assays: the neutral red (NR) and tetrozolium (MTT) tests. Toxicol In Vitro 2:1–6

Chang C, Chu Y, Chen D (1982) Immunocyctochemical demonstration of luteinizing hormone (LH) in Hatschek's pit of amphioxus (*Branchiostoma belcheri*). Kexue Tongbao 27:1233–1234

Chan SJ, Cao QP, Steiner DF (1990) Evolution of the insulin superfamily: cloning of a hybrid insulin/insulin-like growth factor cDNA from amphioxus. Proc Natl Acad Sci USA 87:9319–9323

Clay LA, Wang SY, Wolters WR, Peterson BC, Waldbieser GC (2005) Molecular characterization of the insulin-like growth factor-I (IGF-I) gene in channel catfish (*Ictalurus punctatus*). Biochim Biophys Acta 1731:139–148

Daughaday WH, Rotwein P (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr Rev 10:68–91

Duan C (1997) The insulin-like growth factor system and its biological actions in fish. Am Zool 37:489–501

Fan C, Zhang S, Liu Z, Li L, Luan J, Saren G (2007) Identification and expression of a novel class of glutathione-S-transferase from amphioxus (*Branchiostoma belcheri*) with implications to the origin of vertebrate liver. Int J Biochem Cell Biol 39:450–461

Fukui Y, Saito I, Shiroki K, Shimojo H, Takebe Y, Kaziro Y (1983) The 19-kDal protein encoded by early region 1b of adenovirus type 12 is synthesized efficiently in *Escherichia coli* only as a fused protern. Gene 23:1–13

Hammar JA (1898) Zur Kenntnis der Leberentwicklung bei Amphioxus. Anat Anz 14:602–606

Han L, Zhang SC, Wang YJ, Sun XT (2006) Imunohistochemical localization of vitellogenin in the hepatic diverticulum of the amphioxus *Branchiostoma belcheri tsingtauense*, with implications of the origin of the liver. Invert Biol 125:171–176

Kajimoto Y, Rotwein P (1989) Structure and expression of a chicken insulin-like growth factor I precursor. Mol Endocrinol 3:1907–1913

Kajimoto Y, Rotwein P (1990) Evolution of insulin-like growth factor I (IGF-I): structure and expression of an IGF-I precursor from *Xenopus laevis*. Mol Endocrinol 4:217–226

Kawauchi H, Sower SA (2006) The dawn and evolution of hormones in the adenohypophysis. Gen Comp Endocrinol 148:3–14

Kawauchi H, Suzuki K, Yamazaki T, Moriyama S, Nozaki M, Yamaguchi K, Takahashi A, Youson J, Sower SA (2002) Identification of growth hormone in the sea lamprey, an extant representative of a group of the most ancient vertebrates. Endocrinology 143:4916–4921

Kelley KM, Schmidt KE, Berg L, Sak K, Galima MM, Gillespie C, Balogh L, Hawayek A, Reyes JA, Jamison M (2002) Comparative endocrinology of the insulin-like growth factor-binding protein. J Endocrinol 175:3–18

Kozak M (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 15:8125–8148

Leibush BN, Lappova YL, Bondareva VM, Chistyacova OV, Gutierrez J, Plisetskaya EM (1998) Insulin-family peptide-receptor interaction at the early stage of vertebrate evolution. Comp Biochem Physiol [B] Biochem Mol Biol 121:57–63

Liang YJ, Zhang SC (2006) Demonstration of plasminogen-like protein in amphioxus with implications of the origin of vertebrate liver. Acta Zool (Stockh) 87:141–145

Liang YJ, Zhang SC, Lun LM, Han L (2006) Presence and localization of antithrombin and its regulation after acute lipopolyssacharide exposure in amphioxus, with implications for the origin of vertebrate liver. Cell Tissue Res 323:537–541

Lun LM, Zhang SC, Liang YJ (2006) Alanine aminotransferase in amphioxus: presence, localization and up-regulation after acute lipopolysaccharide exposure. J Biochem Mol Biol 39:511–515

Nozaki M, Gorbman A (1992) The question of functional homology of Hatschek's pit of amphioxus (*Branchiostoma belcheri*) and the vertebrate adenohypophysis. Zool Sci 9:387–395

Pashmforoush M, Chan SJ, Steiner DF (1997) Structure and expression of the insulin-like peptide receptor from amphioxus. Mol Endocrinol 10:857–866

Patruno M, Maccatrozzo L, Funkenstein B, Radaelli G (2006) Cloning and expression of insulin-like growth factors I and II in the shi drum (*Umbrina cirrosa*). Comp Biochem Physiol [B] Biochem Mol Biol 144:137–151

Pavelić J, Matijević T, Knezević J (2007) Biological and physiological aspects of action of insulin-like growth factor peptide family. Indian J Med Res 125:511–522

Pozios KC, Ding J, Degger B, Upton Z, Duan C (2001) IGFs stimulate zebrafish cell proliferation by activating MAP kinase and PI3-kinase-signaling pathways. Am J Physiol Regul Integr Comp Physiol 280:R1230–R1239

Reinecke M, Collet C (1998) The phylogeny of the insulin-like growth factors. Int Rev Cytol 183:1–94

Reinecke M, Maake C, Falkmer S, Sara VR (1993) The branching of insulin-like growth factor 1 and insulin: an immunohistochemical analysis during phylogeny. Regul Pept 48:65–76

Rinderknecht E, Humbel RE (1976) Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell-growth-promoting activities: evidence for structural homology with insulin B chain. Proc Natl Acad Sci USA 73:4379–4381

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385

Sherwood NM, McRory JE (1997) Ancient divergence of insulin and insulin-like growth factor. DNA Cell Biol 16:939–949

Sherwood NM, Tello JA, Roch GJ (2006) Neuroendocrinology of protochordates: insights from *Ciona* genomics. Comp Biochem Physiol [A] Mol Integr Physiol 144:254–271

Sun M, Richards S, Prasad DV, Mai XM, Rudensky A, Dong C (2002) Characterization of mouse and human B7–H3 genes. J Immunol 168:6294–6297

Tjoa LT, Welsch U (1974) Electron microscopical observations on Kölliker's and Hatschek's pit and on the wheel organ in the head region of Amphioxus (*Branchiostoma lanceolatum*). Cell Tissue Res 153:175–87

Tong SL, Li H, Miao HZ (1997) The establishment and partial characterization of a continuous fish cell line FG-9307 from the gill of flounder *Paralichthys olivaceus*. Aquaculture 156:327–333

Upton Z, Francis GL, Chan SJ, Steiner DF, Wallace JC, Ballard FJ (1997) Evolution of insulin-like growth factor (IGF) function: production and characterization of recombinant hagfish IGF. Gen Comp Endocrinol 105:79–90

Wang CF, Zhang SC, Lu Y, Xu TT (2002) Presence and induction by bacteria of D-galactoside-specific lectins in the humoral fluids of amphioxus *Branchiostoma belcheri tsingtaunese*. Inflammopharmacology 9:241–248

Welsch U (1975) The fine structure of the pharynx, cyrtopodocytes and digestive caecum of amphioxus (*Branchiostoma lanceolatum*). Symp Zool Soc Lond 36:17–41

Zheng XY, Jiang ZJ, Shao ML, Li ZJ, Yuan YL (2005) Chinese pharmacopoeia, 2nd edn. Chemical Industry Press, Beijing