Molecular cloning of IGF-1 and IGF-1 receptor and their expression pattern in the Chilean flounder (*Paralichthys adspersus*)

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Abstract

Insulin-like growth factor-1 and insulin-like growth factor-1 receptor (IGF-1 and IGF-1R) play main roles in vertebrate growth and development. In fish, beside contributing to somatic growth, both molecules exhibit pleiotropic functions. We isolated complete cDNAs sequences encoding for both IGF-1 and IGF-1R in the Chilean flounder by using RT-PCR and rapid amplification of cDNAs ends (RACE) techniques. In addition, we analyzed gene expression in pre-metamorphic larvae and different organs of adult fish through whole mount *in situ* hybridization and RT-PCR, respectively. The IGF-1 cDNA sequence displays an open reading frame of 558 nucleotides, encoding a 185 amino acid preproIGF-1. Moreover, IGF-1R contains an open reading frame spanning 4,239 nucleotides, rendering a 702 amino acid subunit alpha and a 676 amino acid subunit beta. The deduced mature IGF-1 and IGF-1R exhibited high sequence identities with their corresponding orthologs in fishes, specially those domains involved in biological activity. RT-PCR showed expression of IGF-1 and IGF-1R transcripts in all studied tissues, consistent with their pleiotropic functions. Furthermore, we observed a strong IGF-1 expression in notochord in larvae of 9 days post fertilization. Similarly, IGF-1R transcripts were observed in larvae of 9 days post fertilization, in territories such as notochord, somites and head. Interestingly, both mRNAs were detected in territories such as notochord, an embryonic midline structure essential for the pattern of surrounding tissues as nervous system and mesoderm. Our results suggest that IGF-1 and its receptor could have an important role in the development of the nervous system, muscle and bone-related structures during larval stages. The present data contributes to the knowledge of the insulin-like growth factor signaling components in an emergent and new commercial important marine fish.

Keywords: IGF-1, IGF-1R, notochord, somites, Chilean flounder.

Introduction

The insulin-like growth factor signaling system plays an important role in promoting the embryonic growth and development in vertebrates (Moriyama et al., 2000). This pathway involves the coordinated function of two ligands, two cell surface receptors and at least six high affinity binding proteins (Moriyama et al., 2000; Wood et al., 2005). The biological effects of the IGF system are mediated mainly by the interaction of IGF-1 ligand with IGF-1 receptor (IGF-1R) modulated through IGF binding proteins (IGFBPs) (Riedemann and Macaulay, 2006). IGF-1 is synthesized as a pre-pro-hormone, which undergoes at least two processing events: cleavage of the signal peptide and the C-terminal peptide (Etherton, 2004; Le Roith et al., 2001). The mature IGF-1 is a single chain polypeptide composed of 70 amino acids, which contains domains A and B separated by a C domain, and a D carboxy domain (Humbel, 1990). The IGF-1 receptor is synthesized as a single chain pre-pro-receptor, with a 30 residues signal peptides that is co-translationally cleaved and a 1,337 amino acids pro-receptor that is processed at a tetrabasic cleavage site to generate alpha and beta subunits (LeRoith et al., 1995). The mature IGF-1R is comprised by two alpha subunits and two beta subunits linked by disulphide bonds, forming $\alpha_2\beta_2$ heterotetramers. The alpha subunits contain an extracellular ligand-binding domain and the beta subunits are composed of a single transmembrane domain and a highly conserved intracellular TK domain (LeRoith et al., 1995).

At a cellular level the IGF-1 acts in an autocrine/paracrine manner to control physiological processes such as protein synthesis, cell proliferation, differentiation, and apoptosis (Jones and Clemmons, 1995). Almost all biological actions of IGF-1 are mediated by the type 1 IGF-1 receptor (IGF-1R) (Whitehead et al., 2000). Once activated, IGF-1R undergoes a conformational change leading to autophosphorylation

of tyrosine residues that serve as recruiting sites for cytoplasmic proteins, including insulin receptor substrate proteins (IRS). IRS molecules are associated with IGF-1R at the cell surface, creating a scaffold for downstream molecules, such as phosphoinositide 3-kinase (PI3K) (Glass, 2005). Once IGF-1 signal transduction activates intracellular PI3K activity, it results in an increased phosphorylation and activation of the Akt/mTOR regulated pathways which increase protein synthesis and suppress protein degradation (Rommel et al., 2001). Other described signaling transduction pathways activated by IGF-1 are the MAP kinases MEK-ERK involved in cellular proliferation and differentiation (Li and Johnson, 2006). In vertebrates, the insulin-like growth factor system has been shown to be unique among growth factors, playing an important role in the early patterning and muscle development: the mRNA microinjection of a non functional IGF-1 receptor in zebrafish induced small sized embryos with the absence of notochord and abnormal somites, on the other hand the over expression of IGF-1 resulted in a greatly expanded development of anterior structures (Eivers et al., 2004).

Over the past decade, IGF-1R and IGF-1 cDNAs partial and complete sequences have been isolated from several vertebrates, including teleost such as coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Psetta maxima*), among others (Elies et al., 1999; Chan et al., 1997; Greene and Chen, 1999; Wood et al., 2005). Additionally, two distinct IGF-1R genes named *igf-1ra* and *igf-1rb* were described in zebrafish (*Danio rerio*) indicating a probable gene duplication during teleost evolution (Maures et al., 2002).

Chilean flounder (*Paralichthys adspersus*) is a marine fish widely distributed throughout the Chilean coast, which is raising a high economic value. However, slow growth rate has been recognized as a major problem in marine fish, increasing the final production cost. Thus, the viability to farm these species requires new knowledge in

order to develop new strategies to improve fish growth (Delgado et al., 2008). In this regard, considering the relevant function of the IGF-1 signaling pathways in promoting growth and skeletal muscle development in fish, we describe here as a first approximation, the isolation and characterization of the full length IGF-1R and IGF-1 cDNAs, which include the codifying regions and the 5' and 3' untranslated regions of both transcripts. Additionally, we studied their mRNA expression in pre-metamorphic larvae and in different tissues of adult fish.

Materials and Methods:

Fish: Chilean flounder fish (*Paralichthys adspersus*) were collected from the Centro de Investigaciones Marinas de Quintay (CIMARQ) (V Region, Valparaíso, Chile). The fish were maintained under natural temperature and photoperiod conditions corresponding to geographic localization of CIMARQ (33°13'S 71°38'W) and were feed twice daily with turbot pellet (Biomar, Chile). Adult fish (36 month old) were sacrificed through an overdose of anesthetic (3-aminobenzoic acid ethyl ester) (300 mg/L). The kidney, gills, intestine, gonads, spleen, liver, stomach, brain, white muscle, esophagus and red muscle tissues were collected, directly frozen in liquid nitrogen and stored at -80°C.

Larvae were obtained after *in vitro* fertilization of eggs by male broodstock sperm. Embryos were maintained under intensive-culture conditions in conic larval culture tanks at 19°C $\pm$ 2°C. Larvae at pre-metamorphic stages were collected, fixed in 4% paraformaldehyde in PBS for 2h at 4°C, dehydrated in methanol and stored at -20°C.

RT-PCR and cDNA cloning: Total RNA was isolated from liver using Trizol reagent following the manufacturer protocols (Invitrogen, Carlsbad, CA, USA). A total of 5 μ g of RNA previously treated with DNase I (1U/ μ L) was used for RNA first-strand cDNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR primers for cloning IGF-1 receptor and IGF-1 cDNA (table 1) were designed from a consensus analysis of conserved coding regions of known IGF-1 receptor and IGF-1 ligand sequences from Japanese flounder, turbot and zebrafish. The PCR reaction, containing the cDNA template, 10 μ L of 10X PCR buffer, 200 μ M of each dNTP, 1 μ g of the forward and reverse primers, and 2.5U of Taq DNA polymerase (Promega, Madison, WI, USA), was carried out in a final volume of 50 μ L. The IGF-1R and IGF-1 PCR

products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and the clones were completely sequenced and assembled in only one sequence. The IGF-1R and IGF-1 full-length 5'-terminal region, including the transcription start site, was completed using the First Choice RLM-RACE kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Briefly, a RT-PCR with an adapter primer and the IGF-1R gene specific primers (5IGF-1ROP 5'-GACAGACAGCATCAGACCCCAAAACA-3', 5IGF-1RIP 5'-TGCCAGTCACAGGATACTTG-3') or the IGF-1 gene specific primers (5IGF-1OP 5'-AAAAGCCTCTCTCTCCACACAC-3', 5IGF-1IP 5'-TCTCTCCACACACACAACTGCAG-3') using a CIP/TAP mRNA as a template in a nested reaction. The IGF-1R 3'-region was obtained using the gene-specific primers (3IGF-1ROP 5' - ACCCAGGTCCTACCCCCACTCAA -3', 3IGF-1RIP 5'-TTCTCCCTTCGGGGAAAT GAGTTT -3'). The IGF-1 3'-region was obtained using the gene-specific primers (3IGF-1OP 5' -ACCTGGAGATGTACTGTGCAC-3', 3IGF-1IP 5'-CAAGACTAGCAAGGCAGCTC-3').

Sequence analysis: Amino acid sequences translated from the cDNA sequence were compared with sequences in the GeneBank public database, by using the NCBI-BLAST application (<http://www.ncbi.nlm.nih.gov/blast>). Multiple aminoacid sequences alignment for the IGF-1 receptor and IGF-1 were performed using clustalW (Thompson et al., 1994).

Tissue expression and distribution of IGF-1 receptor and IGF-1 by RT-PCR analysis: Total RNA was extracted from different tissues (kidney, gills, intestine, gonads, spleen, liver, stomach, brain, esophagus, white muscle and red muscle).

Reverse transcription reaction was performed using 1 µg of total RNA previously treated with DNase I. For IGF-1R and IGF-1, gene-specific primers were designed to amplify a 320 (forward: 5'-GCGGGAATTCGATTGCCTTT -3', reverse: 5'-ATCACGAGGGCGTAGTTGTA-3') and 454 pb (forward: 5'-GTCTAGCGCTCTTTCCTTTCAGTG-3', reverse: 5'-TTTTGTCTTGTCTGGTCGCTGTGC-3') fragments respectively. For normalization purposes, gene-specific primers (forward: 5'-AGGGAAATCGTGCGTGACAT-3', reverse: 5'-TCAGGCAGCTCATAGCTCTT-3') were used to amplify a β-actin 116 bp fragment as constitutive gene expression control.

Whole-mount in situ hybridization: A 1,604 bp fragment corresponding to the IGF-1R coding sequence was amplified by PCR using gene-specific primers (5'-GCCTTTCCAGAACATCACAGAG-3', 5'-TTGAACTCCTTCATGACGGAGG-3') and the same cDNA described before as a template. This fragments were cloned into pGEM-T Easy Vector System (Promega, Madison, WI, USA) originating the pL3F4R1604, which was linearized with *NdeI* or *SpHI* restriction enzymes to synthesized sense (control) and antisense riboprobes DIG-UTP-labeled (Roche Diagnostics, Mannheim, Germany) using SP6 and T7 RNA polymerases (Promega, Madison, WI, USA) respectively. A 454 bp fragment corresponding to the IGF-1 coding sequence was amplified by PCR using gene-specific primers (5'-CCTCTCCACTACTGCTGTGTGTC-3', 5'-ATGTCTGTGTGGCGTTGTGCAC-3') and the same cDNA described before as a template. This fragments were cloned into pGEM-T Easy Vector System (Promega, Madison, WI, USA) originating the pLeigfI454, which was linearized with *NdeI* or *NcoI* restriction enzymes to synthesized sense (control) and antisense riboprobes DIG-UTP-labeled (Roche Diagnostics,

Mannheim, Germany) using SP6 and T7 RNA polymerases (Promega, Madison, WI, USA) respectively. The riboprobes were purified using mini Quick Spin Columns (Roche Diagnostics, Mannheim, Germany) to eliminate unincorporated labeled nucleotides. Whole mount in situ hybridization were performed according to Fuentes et al., (2008). Briefly, after bleaching treatment, embryos and larvae were pre-hybridized overnight at 60°C in hybridization buffer and then incubated overnight at 65°C in hybridization buffer including 50 ng of sense or antisense IGF-1R and IGF-1 riboprobes. After hybridization, embryos and larvae were washed in a solution with decreasing formamide concentration in 2X SSC, followed by two wash-steps with SSC 0.2X for 30 min at 65 °C. Larvae were incubated for 4 h in a blocking buffer at room temperature. For immunodetection, samples were incubated overnight at 4°C with anti-digoxigenin-AP antibody (Roche Diagnostics, Mannheim, Germany). After washes with PBT to eliminate non-bounded antibodies and three additional washes with AP-buffer, stains were performed with NBT/BCIP (75 mg/mL and 50 mg/mL, respectively) (Promega, Madison, WI, USA) for 6 h at 37 °C. The experiment was performed four times using n=15 individuals from each developmental stages. After in situ hybridization, observed in a Leica MZ12.5 stereomicroscope and photographed with a Leica DF300 camera.

Results

Cloning and characterization of the IGF-1R and IGF-1 cDNA: The complete Chilean flounder IGF-1R and IGF-1 cDNA sequences (cfIGF-1R and cfIGF-1) were obtained using RT-PCR coupled to RACE method.

The length of the complete IGF-1R cDNA sequence was 5,033 bp, which includes a 622 bp 5'-untranslated region (UTR), an open reading frame (ORF) of 4,239 bp and a 172 bp 3'-UTR. The ORF encodes a putative protein of 1,412 amino acid residues (Fig. 1A). The sequence analysis reveals that the Chilean flounder cDNA IGF-1 receptor is organized into several major domains including a signal peptide sequence of 30 amino acids, an extracellular alpha subunit of 702 amino acids and an intracellular beta subunit of 676 amino acids (Fig. 1A).

The length of the complete IGF-1 cDNA sequence is 980 bp, which includes a 152 bp 5'-untranslated region (UTR), an open reading frame (ORF) of 558 bp and a 270 bp 3'-UTR, encoding a putative protein of 185 amino acid residues (Fig. 1B). The comparative IGF-1 sequence analysis with other species reveals that the ligand deduced protein is subdivided into six structural domains, including a signal peptide sequence of 44 amino acids, the B domain of 29 amino acids, the C domain of 10 amino acids, the A domain of 21 amino acids, the D domain of 8 amino acids and the E domain of 73 amino acids (Fig. 1B).

Amino acid sequence alignment of cfIGF-1R (FJ438475.1) with different vertebrates orthologs, including mammalian (human, X04434.1 and rat, AF056187.1), birds (chicken, AJ223164.1), amphibians (*Xenopus*, AF055980.1) and fish (zebrafish, AF400275.1 and Japanese flounder, AB065098.1) was performed. The cfIGF-1R sequence was found to have 61%, 61%, 63%, 62% of identity with IGF-1R of human, rat, chicken and *Xenopus*, respectively. Higher degrees of identity were found with

other fish IGF-1R sequences, including a 74% with zebrafish and as much as 97% with another fish belonging to the *Paralichthys* genus, the Japanese flounder (Fig. 2A, Fig. 3A).

A potential proteolytic cleavage sequence R-X-R-R was conserved in all the species compared. The cysteine-rich domain, into the alpha subunit of the cfIGF-1R, contains 24 cysteine residues, which were also observed in all vertebrates IGF-1Rs. In the beta subunit, an IRS-1 and IRS-2 binding site (NPEY and GVLY), a potential ATP binding site (G-X-G-X-X-G-21-X-K), an autophosphorylation motif (YETDYY) and seven tyrosine residues in the tyrosine kinase domain, were highly conserved in all the studied species. A lesser conserved region was found in the carboxyl-terminal, where large insertions were observed in the teleosts IGF-1R compared with those from higher vertebrates (Fig. 2A, Fig. 3A).

Amino acid sequence alignment of the cfIGF-1 (EU017533.1) with those from several other species, including human (M27544.1), rat (NM_001082479.1), chicken (M32791.1), *Xenopus* (M29857.1), zebrafish (BC114262.1) and Japanese flounder (AJ010602.1) was performed. The cfIGF-1 sequence was found to be 60%, 65%, 69%, 66%, 73%, 97% homologous to the IGF-1 of human, rat, chicken, *Xenopus*, zebrafish and Japanese flounder, respectively (Fig. 2B, Fig 3B).

The comparison between the known IGF-1 sequences, reveal higher conserved regions at both B and A domains while the C, D and E domains differ significantly (Fig. 3B). The predicted IGF-1 protein is highly rich in charged amino acid residues and conserves the cysteine residues responsible for maintenance of tertiary structure. Moreover, Chilean flounder IGF-1 contains conserved residues involved in the interaction between IGF-1 with IGF-1 receptor and IGF-1 binding protein (Fig. 2B).

Tissue distribution of IGF-1R and IGF-1 mRNA: IGF-1R and IGF-1 mRNA

RT-PCR experiments were performed to study the expression of mRNA in different tissues of adult fish using β -actin as a constitutive expression control (Fig. 4). The IGF-1R and IGF-1 transcripts were detected in all investigated tissues. High levels of IGF-1R mRNA expression were observed in the testis, intestine, liver, stomach, brain, muscle and oesophagus. The IGF-1R mRNA expression detected in the kidney, gills and spleen was at low levels. Moreover, high IGF-1 mRNA expression was observed in the gills, liver, testis, intestine, spleen, white muscle and brain. Lower IGF-1 mRNA expression levels were detected in the kidney, stomach, oesophagus and red muscle.

In addition, we studied the expression pattern of IGF-1R and IGF-1 mRNA in embryos using whole mount *in situ* hybridization in Chilean flounder larvae from 9.0 days post-fertilization (dpf) (Fig. 5). The cfIGF-1R mRNA was detected at 9 dpf, larvae exhibited IGF-1R mRNA expression in the somites, notochord and cartilaginous tissues in the head. Additionally, cfIGF-1 mRNA was detected only in the notochord. Sense probe was included as a negative control in all *in situ* hybridization experiments. No signal was detected, showing that RNA hybridization was specific.

Discussion

In this work we reported the complete cDNA sequence of the IGF-1 and IGF-1 receptor from the flat fish the Chilean flounder (*Paralichthys adspersus*), an emergent species for aquaculture. These results complement reported data of myostatin and the growth hormone receptor (GHR) (Fuentes et al., 2008; Delgado et al., 2008), all important genes for growth and development in vertebrates (Dayton and White, 2008). The Chilean flounder pre-pro-IGF-1 consists of 185 amino acid residues, which contains a signal peptide and B, C, A, D, and E domains. The cfIGF-1R cDNA consists of 1412 amino acid residues, and like other vertebrates contains a signal peptide and cysteine, trans membrane, juxtamembrane, tyrosine kinase and carboxy domains (Jones and Clemmons, 1995; LeRoith et al., 1995). A multiple alignment of the deduced amino acid sequence of cfIGF-1 and cfIGF-1R with other vertebrate sequences was performed indicating a high degree of conservation of these proteins during vertebrate evolution (Elies et al., 1999; Nakao et al., 2002).

The deduced protein sequence of cfIGF-1 shares an overall identity of 60 to 65% with mammalian IGF-1 sequences, and 73 to 97% with other teleost IGF-1 sequences. Among mature IGF-1, the B and A domains of the peptides are well conserved, while the C, D and E domains differ significantly (Moriyama, 2000). The importance of this high sequence identity of B and A domains in different species can be attributed to the functional roles of these regions, which are involved in the binding of IGF-1 with its receptor such as Arg21 and the Phe23-Tyr24-Phe25 motif in the B domain and Tyr58 (cf_Tyr56) in the A domain (Bayne et al., 1990; Zhang et al., 1994). The amino acidic residues involved in IGF binding with IGFBP: Glu3, Thr4, Glu9, Gln15, and Phe16 in the B domain and Phe47 (cf_Phe45) and Ser49 (cf_Ser47) in the A domain are highly conserved (Clemmons et al., 1992; Magee et al., 1999). The C domain also contains

some conserved residues important in IGF-1R and/or IGFBP binding, such as Tyr31, Arg36 and Arg37. Additionally, the cfIGF-1 sequence showed six conserved cysteine residues CysB6, CysB18, CysA6, CysA7, CysA11, and CysA20 which are also located at the same positions as mammals IGF-1 which is responsible for maintenance of tertiary structure (Hober et al., 1992).

The deduced protein sequence of cfIGF-1R shares an overall identity of 61% with mammalian IGF-1R sequences, and 68 to 97% with other teleost IGF-1 sequences. Among mature IGF-1R, the cysteine, juxtamembrane and tyrosine kinase domains are well conserved, while the C terminal domain differs significantly. Most of the conserved regions are known to be critical for IGF-1R biological activity, such as the ligand-binding motif, tyrosine kinase domain, ATP-binding site, and IRS binding site. The ligand binding motif is located between the amino acidic residues Cys148 and Cys302, showing a variable conservation, however, 24 cysteine residues have a high conservation degree in number and position (Jones and Clemmons, 1995). The tyrosine kinase domain located between the amino acids residues Arg1003 (Cf_Lys1002) and Phe1259 (Cf_Phe1256) contain the tyrosine cluster (Tyr1131, Tyr1135 and Tyr1136) required for the receptor autophosphorylation and the ATP binding sequences (G-X-G-X-X-G-21-X-K) (Gronborg et al., 1993). This domain is absolutely necessary for the biological receptor activity in vertebrates. The juxtamembrane domain contains the Insulin Receptor Substrate (IRS) binding site (NPEY and GVLY) with a high degree of conservation (Moriyama et al., 2000). In contrast, the carboxy-terminus of the receptors is the most divergent region; teleosts allocate insertions in the carboxyl-terminus and this suggests that the function played by this region may differ between mammals and fishes (Kuang et al., 2005). Moreover, isoforms of IGF-1R in teleost fish have been reported: two subtypes of IGF-1R cDNAs were found to be coded by distinct genes in

zebrafish and the Japanese flounder (Maures et al., 2002; Nakao et al., 2002). Additionally two partial cDNAs have been identified in coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*), and it has been suggested that the IGF-1Rs are encoded by two genes in these species (Chan et al., 1997; Greene and Chen 1999). Sequence comparison analysis revealed that the IGF-1R cDNA obtained in this study belongs to the IGF-1Ra. Until now we have been unable to isolate the cDNA sequence of the IGF-1Rb in Chilean flounder.

Insulin-like growth factor-1 is a mitogenic peptide that circulates in plasma and acts in many tissues through the endocrine, paracrine and autocrine mechanisms (Humbel, 1990). Although IGF-1 is secreted predominantly in the liver in response to growth hormone, it is also produced in essentially all tissues. The biological responses of IGF-1 are mainly mediated by the binding and activation the IGF-1 receptor. The IGF-1R and IGF-1 mRNA expression were found in a wide variety of tissues; the tissue mRNA expression patterns reported in this study are consistent with previous reported data from other teleosts and the known pleiotropic role of the receptor and ligand (Duan et al., 1993; Reinecke et al., 1997; Maures et al., 2002; Tse et al., 2002; Queenie et al., 2003; Radaelli et al., 2003a; Radaelli et al., 2003b; Duval et al., 2002; Clay et al., 2005; Patruno et al., 2006). RT-PCR revealed expression of IGF-1R and IGF-1 mRNA in the kidney, gills, intestine, testis, spleen, liver, stomach, brain, oesophagus and skeletal muscle. The expression of IGF-1 and IGF-1R in the brain suggests the IGF-1 involvement in central nervous system development (Kuang et al., 2005; Greene et al., 1999; Ayaso et al., 2002). The presence of high levels of IGF-1R and IGF-1 mRNA in the gonad supports their important roles such as regulators in hormone synthesis and secretion, germ cell proliferation and differentiation (Weber et al., 2000; Hammond et al., 1991). The presence of IGF-1R and IGF-1 in the gills and intestine is according to

their described roles in osmoregulation in fishes, and seawater adaptability (Sakamoto and Hirano., 1991; Datuin et al., 2001; Ng et al., 2001). Moreover, the high expression levels of IGF-1R and IGF-1 detected in skeletal muscle agree with their participation in skeletal muscle satellite cell proliferation and differentiation mediated by the signaling pathways Ras-MEK-ERK (Li and Johnson, 2006; Castillo et al., 2006) and their hypertrophic role mediated by the signaling pathways PI3K-Akt-mTOR during fish growth (Rommel et al., 2001; Castillo et al., 2006).

The spatial expression of IGF-1R and/or IGF-1 in the notochord and somites has been described in other fish such as zebrafish, tilapia (*Oreochromis niloticus*), gilthead seabream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) (Devoto et al., 1996; Rescan et al., 2001; Radaelli et al., 2003b; Funkenstein et al., 1997; Maures et al., 2002; Eivers et al., 2004; Berishvili et al., 2006). In zebrafish it has been shown that IGF-1R and its ligand IGF-1 mRNA are expressed during early development, from blastula (2 hpf) to the larval stage (96 hpf) showing that IGF-1R is maternally expressed (Ayaso et al., 2002; Maures et al., 2002). We examined the mRNA expression pattern of Chilean flounder IGF-1R and IGF-1 in larvae at pre-metamorphic stage through whole mount *in situ* hybridization. We observed that Chilean flounder of 9 dpf, where larvae begins to feed themselves, showed IGF-1R expression in the somites, head and notochord, whereas IGF-1 expression was found in the notochord. We previously described the expression pattern of the growth hormone receptor (GHR) and myostatin genes during larvae development of Chilean flounder (Fuentes et al., 2008; Delgado et al., 2008). Interestingly, we showed that GHR was expressed in similar territories as IGF-1R, such as the somites, which give rise to muscle and the axial skeleton, and the notochord, an essential structure for the proper formation of the nervous system and mesoderm, the last of which gives rise to the somites later in embryo development

(Richardson et al., 1998; Stemple et al., 1996). Furthermore, ligands just as myostatin and IGF-1 mRNAs, were mostly found in the notochord. Taken together, these observations suggest there could be a synchronization between positive and negative growth signals originated in the notochord that plays a crucial role in the control of somite development.

In summary, the complete cDNA sequence of the IGF-1R and IGF-1 were cloned from the Chilean flounder fish. The protein sequence includes all the structural domains and motifs responsible for the interaction between ligand- receptor and IGF-1 mediated signal transduction. Indeed, our results contribute to the knowledge of the IGF-1 system in the larvae and juvenile stages, both of which are crucial periods for developing successful farming of the Chilean flounder.

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Figure legends

Table 1. Primer sequences used in IGF-1R and IGF-1 cDNA cloning. W = A/T, R = G/A, Y= T/C, S= G/C, K = G/T.

Figure 1.A) Chilean flounder IGF-1R cDNA complete sequence (GenBank number FJ438475). Deduced amino acid sequence is indicated below the nucleotide sequence. Start and stop codon are underlined. 5' and 3' UTR are indicated in small letters. The signal peptide sequence is indicated by cursive letters. Alfa subunit and beta subunit are underlined and boxed respectively. Transmembrane domain is indicated in bold character. B) Chilean flounder IGF-1 cDNA complete sequence (GenBank number EU017533). Deduced aminoacidic sequence is indicated below the nucleotide sequence. Start and stop codon are underlined. 5' and 3' UTR are indicated in small letters. The signal sequence is indicated by cursive letters. B and C domains are underlined and bolded respectively. A, D and E domains are shown by the boxed, gray boxed and black boxed areas respectively.

Figure 2. Amino acid multiple sequence alignments of IGF-1R and IGF-1 in different vertebrates species: A) GenBank numbers: human (*Homo sapiens*) GenBank: X04434.1; rat (*Rattus norvegicus*) GenBank: AF056187.1; chicken (*Gallus gallus*) GenBank: AJ223164.1; African clawed frog (*Xenopus laevis*) GenBank: AF055980.1; zebrafish (*Danio rerio*) GenBank: AF400275.1; Japanese flounder (*Paralichthys olivaceus*) GenBank: AB065098.1, Chilean flounder (*Paralichthys adspersus*) GenBank: FJ438475.1. Conserved cysteine and tyrosine residues are indicated in black boxed areas. The proteolytic cleavage sequence and potential ATP binding sites are shaded. Potential IRS-2 and IRS-1 binding sites are in bold characters. Cystein,

transmembrane, juxtamembrane, tyrosine kinase and carboxy domains area indicated in open boxed areas .B) GenBank accession numbers: human (*Homo sapiens*) GenBank: M27544.1; rat (*Rattus norvegicus*) GenBank: NM_001082479.1; chicken (*Gallus gallus*) GenBank: M32791.1; African clawed frog (*Xenopus laevis*) GenBank: M29857.1; zebrafish (*Danio rerio*) GenBank: BC114262.1; Japanese flounder (*Paralichthys olivaceus*) GenBank: AJ010602.1; Chilean flounder (*Paralichthys adspersus*) GenBank: EU017533.1. Conserved cysteine residues are indicated by black boxed areas. Conserved residues implicated in IGF-1R binding are indicated in a grey boxed area. IGFBP binding is indicated in bold characters. B, C, A, D and E domains are indicated in open boxed areas.

Fig.3. Comparison of the Chilean flounder IGF-1 and IGF-1R deduced domains with Japanese flounder, turbot, carp, zebrafish, xenopus, chicken, rat and human IGF-1R and IGF-1. A) GenBank accession numbers: human (*Homo sapiens*) GenBank: X04434.1; rat (*Rattus norvegicus*) GenBank: AF056187.1; chicken (*Gallus gallus*) GenBank: AJ223164.1; African clawed frog (*Xenopus laevis*) GenBank: AF055980.1; zebrafish (*Danio rerio*) GenBank: AF400275.1 and BC163723.1; Carp (*Cyprinus carpio*) GenBank: AY144591.1; turbot (*Psetta maxima*) GenBank: AJ224993.1; Japanese flounder (*Paralichthys olivaceus*) GenBank: AB065098.1, Chilean flounder (*Paralichthys adspersus*) GenBank: FJ438475.1. The top line drawing represents the cDNA structure of human IGF-1R. The comparison between Chilean flounder and other species were performed using 170, 43, 253 and 155 amino acid corresponding to the cystein domain, juxtamembrane domain, tyrosine kinase domain and c-terminal domain, respectively. B) GenBank accession numbers: human (*Homo sapiens*) GenBank: M27544.1; rat (*Rattus norvegicus*) GenBank: NM_001082479.1; chicken (*Gallus*

gallus) GenBank: M32791.1; African clawed frog (*Xenopus laevis*) GenBank: M29857.1; zebrafish (*Danio rerio*) GenBank: BC114262.1; Carp (*Cyprinus carpio*) GenBank: BAA11878.1; turbot (*Psetta maxima*) GenBank: ACL14947.1; Japanese flounder (*Paralichthys olivaceus*) GenBank: AJ010602.1; Chilean flounder (*Paralichthys adspersus*) GenBank: EU017533.1. The top line drawing represents the cDNA structure of human IGF-1. The comparison between Chilean flounder and other species were performed using 29, 10, 21, 8 and 73 amino acid corresponding to B, C, A, D and E domains, respectively.

Fig. 4. Qualitative mRNA distribution of the Chilean flounder IGF-1R and IGF-1 in different tissues assessed by RT-PCR. β -actin was used as constitutive expression control. (figure is representative of four independent experiments).

Fig. 5. Expression pattern of IGF-1 and IGF-1R mRNAs in Chilean flounder larvae. Expression of IGF-1 and IGF-1R mRNA were analyzed at 9 dpf in Chilean flounder larvae, through whole-mount *in situ* hybridization using sense and antisense probes. A, larvae at 9 dpf show strong IGF-1 expression in the notochord (nc). B, higher magnification shows the expression in the notochord (nc), but not in somites (s). C, larvae at 9 dpf show IGF-1R expression in the notochord, somites and the head (h). D, higher magnification shows expression in the notochord and somites. We did not detect positive signals using the sense probe (insets in A and B, respectively). Pictures are representative of four independent experiments. Abbreviations: h, head; nc, notochord; s, somites.

<i>primer name</i>	<i>sequence</i>	<i>nucleotide position</i>
1F(sense)	5'-TGAGWTRACCAGCCTGAAGGAC-3'	1000-1021 igf1r
1R(antisense)	5'-CTTTRAGCAGTAGTTGTGYTG-3'	2582-2603 igf1r
2F(sense)	5'-CTTGTTTTGGRTCTGATGCTG-3'	654- 676 igf1r
2R(antisense)	5'-AGCAGAGCTCAGGGTTCTTCTC-3'	1070- 1091 igf1r
3F(sense)	5'-GCCWTTCCAGAACATCACAGAG-3'	2200- 2221 igf1r
3R(antisense)	5'-ATCAGYTCRAACAGCATGTCAG-3'	4326- 4347 igf1r
4F(sense)	5'-AGCCTCCGTCATGAAGGAGTTC-3'	3796- 3790 igf1r
4R(antisense)	5'-TTGAACTCCTTCATGACGGAGG-3'	3770- 3792 igf1r
5F(sense)	5'-GACCTTGCTCTCAAGTGCAC-3'	1476- 1495 igf1r
5R(antisense)	5'-TGTGACTCGTTGTCTCGTTAG-3'	2100- 2120 igf1r
6F(sense)	5'-AGACATGGATGACAACACCAAG-3'	2644-2665 igf1r
6R(antisense)	5'-TGAAGTGAAGCTTGGTCAGAC-3'	3291-3310 igf1r
7F(sense)	5'-GGATGTCACCAGAGTCTCTGA-3'	4146- 4166 igf1r
7R(antisense)	5'-TTGATGCTGTGATGATCTCCA-3'	4392- 4413 igf1r
1F(sense)	5'-CCTCTCCACTRCTGCTGVGTGTC-3'	233-254 igf1
1R(antisense)	5'-ATRTCTGTGTGSCGTTGTGCWC-3'	499-520 igf1

A

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1 - cagcctcgagccaccaccctgtggatctgtgagcttattttctgccgccaccctaacctctctctctccacccttctctgccattct
91 - cccctgggcttactcaccatgttctgtggagtctgctgggtgaataaaaccttcaagttatctttgacatataatattttcagttgtttac
181 - caccaggcccggatcgaccgctgttaacgcgcaactcgctgttttcaagagatggagactgaagatggggattaccatgtatccagtcac
271 - aattactggcatgtgtgtccacacagcatccaagtatctctgtgactggcaaaaacggcagactctattaatatatttatattgttcttt
361 - ttttttctctccatgggaactccctgctataacaattcctaacaatttatttagattaaaaaataatattttattctaaatctaactctgg
451 - tagacaagcaaccacgcgttttgcatgctaccatagagaagccacctgggtacacacattattttttctcatttttaaattattgaag
541 - ctctatttttccagaggattttttcacttttggtcccttttgcttattttaatttctgagcggagaatgatgttgaggaccATGAGATC
1 - M R S
631 - TGGCTCACTGATGGGTAGCACGACCTTGTTTTGGGGTCTGATGCTGTCTGTCTCCACTATCTGCATATGGCCCAGTATGGAGAGATTG
4 - G S L M G S T T L F W G L M L S V S T I C I W P T Y G E I C
721 - TGGTCCGGGTATTGACATCCGAAATGACATCAGTGAGTTCAAGCGGCTGGAGAAGTGCACGGTGGTGGAGGGCTACCTGCAGATCCTCTCT
34 - G P G I D I R N D I S E F K R L E N C T V V E G Y L Q I L L
811 - CATCAATGACAAGACCAACAACATCCATCAAGAGGTTTTCCGCTCCCTCAGCTTCCCAAGCTGACCATCAGGAGTACCTGCTGCT
64 - I N D K T N N I H Q E V F R S L S F P K L T I I T D Y L L L
901 - GTTTCGCGTCTCTGGCTTGGACAGCCTCAGCATGCTCTTCCCAACCTGAGCATCATCCGTGGGCGGAGCTCTTCTACAACACGCCCCT
94 - F R V S G L D S L S M L F P T N L S I I R G R Q L F Y N Y A L
991 - CGTGATCTATGAGATGACCAAGCTGAGGACCTTGGCTGTACAACTGAAGAACATCACCAGGAGGACGAGGATGAGAAACCC
124 - V I Y E M T S L K D I G L Y N L K N I T R G A T R I E K N P
1081 - TGAGCTCTGCTACCTTGACTCGGTGGATGGTCTCTCATTATGGATGCGGAGTTCAACAACATTATCAATGGAAATAAGGAAGGTAAGGA
154 - E L C Y L D S V D W S L I M D A E F N N I I N G N K K A K E
1171 - GTGCGACAATGTCTGTCCAGGAATCATGGAGGATAAACCACCTTTGTAAGAGGACGTTGTTCAATGACAACACGACTACCGCTGCTGGAC
184 - C D N V C P G I M E D N P L C K R T L F N D N Y D Y R C W T
1261 - CTCTACCCAGTGCCAGAAAGTTTGCCAGAACCTGCAATATGCTGTACCGACAAGGGGAGTGTGCCACGCCAGCTGCGTGGGCAC
214 - S T Q C Q K V C P E H C K Y A C T D K G E C C H S Q C L G T
1351 - CTGCACTGAACCCCAACACGACATGGCCTGTTCCACCTGCCCTCCACTACTTCCACGAGGACCGCTGCGTGCCAGACTGTCCTCGGGGAC
244 - C T P N I I D S V D A A Q Q S L K D C N T V I E G N L D I N I R
1441 - TTACAAGTTCGAGAGCTGGCGCTGCATCACCATGGGACCTTGCTCTCAAGTGCACCTGCCTGTGACCCCTCAGTTTGTTCATCCACGGGGG
274 - Y K F E S W R C I I T M G P C S Q V H L P V D P Q F V I H G G
1531 - AGAATGTATGATGAATGCCCTCTGGCTTCACAGAACGAGACATCGAATGTTTTCAGCGCCTGCAACGAGTGTGGACACAGT
304 - E C M H E C P S G F T R N E T N R M F C S A C N G L C D K V
1621 - CTGCACACCCCAACATCATGACTCTGTGGATGCTGCTCAGTCTCTGAAGGACTGCACCGTGCATCGAGGGCAATCTGGACATCAACATTCG
334 - C T P N I I D S V D A A Q Q S L K D C N T V I E G N L D I N I R
1711 - TCGCGAAATAACATAGCGTCTGAGCTGGAGAGCTTCATGGGATTGATCCAGACAGTGAAGGGCTATGTGAAGATTTCGACACTCCCACGC
364 - R G N N I A S E L E S F M G L I Q T V K G Y V K I R H S H A
1801 - GCTTGGCTCGTGTGCTTCCCTCAAGAGCCTGCGTTACATCAATGGGAGGAACTCATCGACAACATGTATGCTTCTCCGCCATCAACAA
394 - L G S L S F L K S L R Y I N G Q E L I D N M Y S F S A I N N
1891 - CCAGCACTTGCACTACCTGTGGGACTGGAGTCAGCACAACTGACTATTTCGAGCTGGAGCGCTCTTCTTTCGCCGGAACCCCAAACTCTG
424 - Q H L Q Y L W D W S L H N L T I R A G R L F F R R N P K L C
1981 - CATGTCTGAGATCCACACCATGTGGGAAAAGACGAAGATCACCAGCAAGCCGAGGAGGGTATTTCGCCAACAACGGTGAAGAGCCAG
454 - M S E I H T M W E K T K I T A K P E E G D F R N N G E R A S
2071 - CTGTGAAAGTCACACCTTGACGTTCAAGACTAACGAGACAAGATGCATGATCAAGCTGACGTGGGAGCCATCAGCCACGACACTT
484 - C E S H T L T F K T N E T T S H M I K L T W E R Y Q P P D F
2161 - CGGAGACCTCATGAGCTTCATGCTCTACTTCAAGAGTGCCTTTCCAGAACATCACAGAGTTCGACGGACAGGACGGCTGCGGCTCAAA
514 - G D L I S F I V Y F K E S P F Q N I T E F D G Q D C G S N
2251 - CAGCTGGCACATGTGTGGACGTGGATCTACCTCAGGATAAAACAGTGAACCAAATGTGAGTCTTCGCGACCTGAAGCCCTGGACCCAGTA
544 - S W H M V D V D L P Q D K T S E P N V S L P H L K P W T Q Y
2341 - TGCCATCTTCGTGAAGGCCATCACCTGCAGGTGGAGGACAAACACTACTGGGGCCAAGAGTGACATCATCTACATCCGACGCCACCC
574 - A I F V K A I T L Q V E D K H I T G A K S D I I Y I R T R P
2431 - ATCGTCTCCTTCTGTGCTTAAAGACGCCCGCGCTTTTGCCAACTCCTCAACCAAGCTGGTGGTGAAGTGGTGCSCCCCGTGTFTCCCAA
604 - S V V S P K D A R A F A N S S T K L V V K W S P P V P N
2521 - CGGCAACCTGACCTACTACCTGGTCCGTGGCAGCAGAGCCCGAGGACAGGAGCTATACCAACAACACTACTGCTCCAAGAGAGCTGAA
634 - G N L T Y Y L V R W Q Q Q P E D R E L Y Q H N Y C S K E L K
2611 - GATCCCGATCAGGATTTACGCGACAGGGCTCACAGACATGGATGACAAACCAAGCCCAAGTCAAACTTTGGGGGGGGGAGAGAAGG
664 - I P I R I S A T G L T D M D D N T K P H Q V K L G G G R E C A
2701 - GCCCATGTGCCTTGCAAGAAGACGACGAGGAGAAGGACCGGAGAGGACGACCGCTCTTCTGAAGATTTTCGAGAAGTCTCTCCACAA
694 - P M C L A E D A E E K D R E K D D R V F L K I F E N F L H N
2791 - TGCCATCTTTCTGCGGAGACCTCCAGACCGTCGACGCGAGAGATGTGTTTCGGCGTGGCCAACGACACGCTGTTTCACGACAGCGCCGGGAA
724 - A I F L P R P P D R R R R D V F G V A N D T L F H D S A G K
2881 - GGGGAACACCAACCTCGGCCGGGCAACAGTACAGATGGCGTTCCTCTATTAAGAGTACCCTTCATGGGAGGACAGAGTCAAGCAGA
754 - S N T T T L G P G N S T D G V P P I K E Y P F M E D K S S A E
2971 - ATATTAGACATCCCAACCTGCAGCCCTTCACAGTCTACCCGCTTGACATCCACGCTGCAATGAGGAGGTGGGCGCTGCAGCGCCGG
784 - Y L D I P N L Q P F T V Y R L D I H A C N E E V G R C S A G
3061 - AGCATTGCTCTTCTCCAGACCAACCTGCGGTCAAAGCAGACGACATCCCTGGAAAAGTGATCTATGAGCGCAGTGACAAGGTTGAGGG
814 - A F V F S Q T K P A V K A D D I P G K V I Y E R S P P A D E W E V
3151 - TTCTGTGGTGTGCACTGGCCAGAGCCCATCATGCCAACGGACTCATCTGTATGATGAGATCAAGTTCCGTTTGGGAKTGAAGCTGA
844 - S V V L H W P E P I M P N G L I L M Y E I K F R L G T E P E
3241 - GAAACACGAGTGTGTGTCGCGCAGCACTACCGTGAGCACAGAGGAGCTCGTCTGACCAACCTCAGTTTCAGGAACTACTCTGCCCGTGT
874 - K H E C V S R Q H Y R E H R G A R L T N L S S G N Y S A R V
3331 - GCGCGCCACTTCTCTAGCAGGGAACGGCTCCTGGACAGAGAGCGTGTGTTTCTACGTGCTCCACCAACAGAGACGATGGTGTGCTCTT
904 - R A T S L A G N G S W T E S V F F Y V P P P K R D D G V A F
3421 - TTATTTGGTCATCATAATTCATCATAGCGACGCTCCTCTATGCCAGCCTCACCACCATCTCTCTTTGTGAACAAAAAGGAGAACAG
934 - Y L V I I P I I A T L I A S L T T I L F F V N K K R N S
3511 - CGACAGACTGGGAAATGGAGTCTTTATGCCTCTGTCAATCCAGAATACTTCACGCTGCTGAGATGTACGTCCTCCGGATGAGTGGGAGGT
964 - D R L G N G V L Y A S V N P E Y F S A A E M Y V P A D E W E V
3601 - AGCGAGGAGAAGATCACTATGCACAAGGAGCTGGGCCAGGTTCTCTTCGAGATGGTGTATGAAGTTTAGCCAAAGGTGTGGTCAAGGA
994 - A R E K I T M H K E L G Q G S F G M V Y E G L A K S V G K D
3691 - TGAGCCTGAGACGGCAGTAGCCATCAAGACGGTCAACGAGTTCGGCGAGCATGAGGAGCGCATCGAGTTTTTGAGCAGAGCCTCCGTCAT
1024 - E P E T R V A I K T V N E S A S M R E R I E F L D E A S V M
3781 - GAAGGAGTTCAACTGTCCATGTGGTCCGCTCTGGGTGTGGTCTCTCAGGGTCAGGCCCACTGTGGTCACTGATGACGACG
1054 - K E F N C H H V V R L L G V V S Q G Q P T L V I M E L M T R
3871 - TGGAGATCTCAAGACCCACCTGCGTCTGCGCAAGGAAACTCCACCACCCAGGTCTACCCCACTCAAAAAATGATCCAGATGGC
1084 - S D L K S H L R K E N A S T T Q V L P P L K K M I Q M A
3961 - GGGGGAATCGCTGACGGCATGGCGTACCTTAACGCCAACAGTTTGTCCACAGAGATCTGGCAGCCAGGAACTGCATGGTGGCTGAGGA
1114 - S E I A D G M A Y L N A N K F V H R D L A A R N C M V A E D

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4051 - CTTTCATCGTGAAGATTGGAGATTTTGGCATGACCAGAGACATATATGAGACAGATTACTACCGCAAAGGTGGTAAGGGTCTGCTCCCTGT
 1144 - F I V K I G D F G M T R D I Y E T D Y Y R K G G K G L L P V
 4141 - CCGCTGGATGTCACCAGAGTCTCTGAAGGATGGAGTCTTTACTACTAACTCTGATGTTTGGTCGTTTGGGGTGTACTGTGGGAGATTTC
 1174 - R W M S P E S L K D G V F T T N S D V W S F G V V L W E I S
 4231 - CACCCTGGCTGAGCAGCCGTACCAGGGTCTGTCCAATGAGCAGGTGCTCCGCTTTGTCATGGAGGGAGGACTGCTGGAGAAACCACAAA
 1204 - F L A E Q P Y Q G L S N E Q V L R F V M E G G L L E K P Q N
 4321 - CTGTCCTGACATGCTGTTTGTAGCTGATGCGAATGTGTTGGCAGTACAATCCTAAATGCGTCCATCCTTTGTGGAGATCATCAGCAGCAT
 1234 - C P D M L F E L M R M C W Q Y N P K M R P S F V E I I S S I
 4411 - CAAAGATGAACGTGATTCTCCCTTCAGGGAATGGGTTTCTTCTACAGTGAGAAGAACAAGCCGCTGACACCGAGGAGCTGGACATGGA
 1264 - K D E L D S P F R E M G F F Y S E K N K P P D T E E L D M E
 4501 - GGTAGAAAACATGGAGAACATCCACTGGACCCTGCATCCACGAGCAGCCCTCTGCTGCCGCCGCCCTCGTCGGGGTGCACGGGAGG
 1294 - V E N M E N I P L D P A S T R Q P S A A A A P S S G C T G G
 4591 - AACGCCGCCGCCCTCTGCGCAGCAGTTATCCCCATGCAAGGCCGAGTACTCCTTTACTGGGACCTGTGTCTCCCTCCTCCCCGGGCC
 1324 - F P P P S A Q Q L S P M Q G P S T P L L G P V S P S S P G E
 4681 - GGTGCTCTCAGCCTTGGCGTCTCCGGGCCAAGCTTTGGACAAGCACTCAGGACATGTCTCGGCCAACGGGCTGTGGTGGTGTCTCGGCC
 1354 - V A S A L A S P G Q A L D K H S G H V S A N G P V V V L R E
 4771 - CAACCTTGATGAGATGCAACCTTATGCACACATGAACGGGGCAGAAAGGACGAACGGGCATTACCCTGCCCCAGTCGTCGGCTGCTG
 1384 - N L D E M Q P Y A H M N G G R K D E R A L P L P Q S S A C *
 4861 - Atatcagaccagtcctcattctctcttacacaggttaaaccaaacctctctccatctcttaaggagggtgggtgaggtgaagaatga
 4951 - cttgcttttctactgtgtgccagtagatggtctctccactcctttaagggtcaaactcaaaatgtaaaactgaaaaaataaaaaa

B

1 - tcgcggatccgaacactgcgctttgctggtttgatgaaaatgaaatgaaatagtgtgtttgtatatatgagcatgagctgctgctggtgct
 91 - gctgctgctgctgctgctgctgctgttcccttttcgccgggctttgaacttgcgagaccgctggggATGTCTAGCGCTCTTTCTTTTCAGTGGC
 1 - M S S A L S F Q W H
 181 - ATTTATGTGATGTCTTCAAGAGTGCAGTGTGCTGTATCTCTTGTAGCCACACCCTCTCACTACTGCTGTGTCTCACCTGACTCCGA
 11 - L C D V F K S A M C C I S C S H T L S L L L C V L T L T P T
 271 - CGGCAACAGGGGCGGACCGGAGACCTGTGCGGGCGGAGCTGGTCGACACGCTGCAGTTTGTGTGTGGAGAGAGGCTTTTATTTCA
 41 - A T G A G P E T L C G A E L V D T L Q F V C G E R G F Y F S
 361 - GTAAACCAACAGGTTATGGCCCCAATGCACGGCGGTACGCGGCATTGTGGACGAGTGTCTTCCAAAGCTGTGAGCTGCGGCACCTGG
 71 - K P T G Y G P N A R R S R G I V D E C C F Q S C E L R H L E
 451 - AGATGTACTGTGACCTGCCAAGACTAGCAATGCCGCTCGCTCTGTGCGTGCACAACGCCACACAGACATGCCGAGAGCACCTAAGGTTA
 101 - M Y C A P A K T S N A A R S V R A Q R H T D M P R A P K V S
 541 - GTACCGCAGGGCACAAGTGGACAAGGGCACAGAGCGTAGGACAGCACAGCAGCAGACAAGACAAAAACAAGAAGAGACCTTTACCGG
 131 - T A G H K V D K G T E R R T A Q Q P D K T K N K K R P L P G
 631 - GACATAGTCACTCACAAGCTTTGCTTTTCATGCGCCAAAGCCAGCTGCTTACATTTTGTGTAGGAATTGTATGTGAATGAtgttaacctg
 161 - H S H S Q A L L F M R Q S Q L L T F C V G I V C E *
 721 - ttcagaggattgataccactcactatctgttcatattagatataaaactacaaccagcaaaacatgtatgttatcattcactcggattgat
 811 - gcacatgttttttagtatctcatagtatctatgagttggtcagactaaatctggttgtgtgtgtggataaaagcagatcaataactgagct
 901 - tcaacacattatgtctttaatcaaacaaaaacttaaaaaaaaaaacctatatagttggagtcgtatattaattcggatccgcg

B

	B domain														
human	MGKISSLP	QLFKCCF	CDFLKVKM	HTMSSSHLF	YALCLLT	FTSSAT	-AC	PETL	CSAELV	59					
rat	MGKISSLP	QLFKICL	CDFLKIKI	HIMSSSHLF	YALCLLT	FTSSAT	-AC	PETL	CSAELV	59					
chicken	MEKINSLS	QLVKCCF	CDFLKVKM	HTVSYIH	FFYLGCLLT	LTSSAA	-AC	PETL	CSAELV	59					
xenopus	MEINNLSL	QLFKCYF	CDILKLMH	KMSCIHL	LYLVLC	FLTLTH	SAA	-AC	PETL	CSAELV	59				
zebrafish	MS-----	SGHFFQ	GHWCDF	FKCTMR	CLPSTHT	LSLVLC	VLATPAT	LEA	PETL	CSAELV	55				
J_flounder	MS-----	SALSFQ	WHLCDV	FKSAMCC	ISCSTHLS	LLVCVLT	LTPTAT	GA	PETL	CSAELV	55				
Ch_flounder	MS-----	SALSFQ	WHLCDV	FKSAMCC	ISCSTHLS	LLVCVLT	LTPTAT	GA	PETL	CSAELV	55				
	*	.	.	.	.	.	.	.	.	.	.	*			
	B domain		C domain		A domain		D domain								
human	DALQFV	CDRGFY	FNKPT	GYGSS	RRAPQ	TIVDE	CFPS	CDLR	REMY	CA	FLKPA	KSAR	119		
rat	DALQFV	CDRGFY	FNKPT	GYGSS	RRAPQ	TIVDE	CFPS	CDLR	REMY	CA	FLKPT	KSAR	119		
chicken	DALQFV	CDRGFY	FNKPT	GYGSS	RRRLH	HKIIVDE	CFPS	CDLR	REMY	CA	FLKPT	KSAR	119		
xenopus	DTIQFV	CDRGFY	FSKPT	GYGSS	NNRRSH	HHIIVDE	CFPS	CDLR	REMY	CA	FLKPA	KSAR	119		
zebrafish	DTIQFV	CDRGFY	FSKPT	GYGSS	NNRRSH	HHIIVDE	CFPS	CDLR	REMY	CA	FLKPT	KSAR	115		
J_flounder	DTIQFV	CDRGFY	FSKPT	GYGPN	ARRS--	RGIVDE	CFPS	CDLR	REMY	CA	FLKPT	KSAR	113		
Ch_flounder	DTIQFV	CDRGFY	FSKPT	GYGPN	ARRS--	RGIVDE	CFPS	CDLR	REMY	CA	FLKPT	KSAR	113		
	*	.	.	.	.	.	.	.	.	.	.	.	*		
	E domain														
human	SVRAQR	HTDMP	KTKQ	-----	-----	-----	-----	-----	-----	-----	-----	-----	EVHLKN	140	
rat	SIRAQR	HTDMP	KTKQ	-----	-----	-----	-----	-----	-----	-----	-----	-----	EVHLKN	140	
chicken	SVRAQR	HTDMP	KTKQ	-----	-----	-----	-----	-----	-----	-----	-----	-----	EVHLKN	140	
xenopus	SVRAQR	HTDMP	KTKQ	-----	-----	-----	-----	-----	-----	-----	-----	-----	EVHLKN	140	
zebrafish	SLRAQR	HTDIP	PTP	-----	-----	-----	-----	-----	-----	-----	-----	-----	EVHFKN	148	
J_flounder	SVRAQR	HTDMP	APKVS	TAGHK	VDKG	TERR	TAQQ	PD	TKN	KKR	PLP	GHSH	SFKVE	HQKN	173
Ch_flounder	SVRAQR	HTDMP	APKVS	TAGHK	VDKG	TERR	TAQQ	PD	TKN	KKR	PLP	GHSH	SQALL	FMRQS	173
	*	.	.	.	.	.	.	.	.	.	.	.	.	.	*
	E domain														
human	ASRGS	SAGN	KNYRM	153											
rat	TSRGS	SAGN	KTYRM	153											
chicken	TSRGT	GNR	NYRM	153											
xenopus	TSRGT	GSR	GFRM	153											
zebrafish	SSRGT	GGR	NYRM	161											
J_flounder	SSRGT	GGR	NYRM	186											
Ch_flounder	QLLT	FCV	GIVCE	185											
	.	.	.	.											

Figure 3

	<i>cysteine domain</i>	<i>juxtamembrane domain</i>	<i>Tirosine_kinase domain</i>	<i>C_terminal domain</i>	<i>Whole protein</i>
Ch_flounder/j_flounder	95	97	96	94	97
Ch_flounder/turbot	63	95	91	38	67
Ch_flounder/carp	72	95	92	63	76
Ch_flounder/zebrafish IGFR1a	69	92	92	66	74
Ch_flounder/zebrafish IGFR1b	58	92	92	50	68
Ch_flounder/xenopus	50	93	90	48	62
Ch_flounder/chicken	52	90	91	47	63
Ch_flounder/rat	48	86	91	42	61
Ch_flounder/human	49	88	91	41	61

	<i>B domain</i>	<i>C domain</i>	<i>A domain</i>	<i>D domain</i>	<i>E domain</i>	<i>Whole protein</i>
Ch_flounder/j_flounder	97	100	95	88	73	97
Ch_flounder/turbot	100	100	91	88	49	96
Ch_flounder/carp	97	58	95	25	27	74
Ch_flounder/zebrafish	83	67	95	38	27	73
Ch_flounder/xenopus	97	67	86	50	26	66
Ch_flounder/chicken	93	42	91	38	26	69
Ch_flounder/rat	93	42	86	38	25	65
Ch_flounder/human	86	42	86	38	23	60

Figure 4

Figure 5
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