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Identification of an Embryonic Growth Factor IGF-II from the Central Nervous System of the Teleost, Flounder, and Its Expressions in Adult Tissues

KIM, DONG SOO AND YOUNG TAE KIM*

Department of Microbiology, Pukyong National University, Pusan 608-737, Korea

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Abstract The insulin-like growth factor (IGF) is found in all vertebrates and its type-II molecule is regarded as a fundamental embryonic growth factor during development. We have firstly identified, in this study, a cDNA clone corresponding to IGF-II (flIGF-II) from the adult brain of the teleost, *Paralichthys olivaceus*. We also examined the tissue expression of flIGF-II in several adult tissues by RT-PCR. The flIGF-II cDNA contained a complete ORF consisting of 215 amino acids and one stop codon. Its molecular characteristics appear to be similar to the previously identified IGF-II molecules, in which a common primary structure exhibiting B, C, A, D, and E domains is evidently observed. This cDNA clone seems to be cleaved at Ala^{52} for the NH_2-end signal peptide and appears to produce a 98 amino acid-long E-peptide from the Arg^{118}. The functional B~D domain regions, therefore, include 65 amino acids and is able to encode a 7.4-kDa protein. The most prominent structural difference between IGF-I and IGF-II was that the D domain of IGF-II exhibits a two-codon-deleted pattern compared to the 8 amino acid-containing IGF-I. The insulin family signature in the A domain and six cysteins forming three disulfide bridges between the B and A domains were evolutionary-conserved from teleosts to mammalian IGF-II. Interestingly, the E-peptide region appears to provide a distinct hallmark between teleosts in amino acid composition. The flIGF-II shows 85.1% of sequence identity to salmon and trout, 90.6% to tilapia, and 98.4% to perch in amino acid level. In tissue expressions of IGF-II, it is very likely that flIGF-II has a significant expression in the adult brain. However, liver seems to be the main source for IGF-II production, and relatively low signals were observed in the adult muscle and kidney. Taken together, it would be concluded that the functional region for IGF-II mRNA is highly similar in phylogeny and is evolutionary, conserved as a mediator for the growth of vertebrates.

*Corresponding author
Phone: 82-51-620-6366; Fax: 82-51-628-7432;
E-mail: ytkim@dolphin.pknu.ac.kr

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Insulin-like growth factor is one of the anciently-evolved polypeptide growth factors found in vertebrates. In fact, it belongs to the insulin superfamily including all the insulin-related molecules ranging from the molluscan insulin-related peptides (MIP-I, II) to human IGFs [1]. It is known that IGF includes type-I and -II molecules, in which each type is thought to exert fundamental roles on regulation of growth in vertebrates. The somatic growth is highly dependent on the level of plasma growth hormone (GH) which induces anabolic effects on various types of developing tissues. Many reports support this theory by demonstrating the relationships between GH and IGFs using exogenous administration *in vivo* [2, 3]. It has been suggested that the growth-promoting effects of GH may involve expressions of IGFs by enhancing the specific increase of IGF-I [4], serving roles in modulating growth and food utilization as a major lean growth factor [5]. In contrast, IGF-II seems to be related to somatic overgrowth [6]. Interestingly, both IGF-I and IGF-II are expressed in a fetal pattern to provide an enhancement for cell proliferation, indicating that IGFs may generate autocrine/paracrine effects on regulating cells depending on the physiological condition of cells [7]. Recently, an increasing concern has focused on the IGF-II which seems to be more embryonic than IGF-I, being expressed predominantly in all developing tissues as a fetal somatomedin [8, 9]. The IGF-II gene was detected in a terminal location on human chromosome 11 [10]. Molecular studies on IGF-II have been largely conducted in many vertebrates over recent years [11, 12], and fish species have been a good source to investigate the regulatory mechanisms *in vivo* as it develops externally [13, 14]. In the present study, we initially

aimed to acquire this important gene resource from the teleost, flounder (*Paralichthys olivaceus*). Herein, we provide the molecular characteristics and tissue expressions of our newly identified flIGF-II cDNA (GenBank accession number, AF091454) from adult flounder. In addition, the teleost IGF-II may possibly be enough to display biological potency to activate non-teleostean IGF-II systems *in vivo*.

MATERIALS AND METHODS

IGF-II cDNA Cloning by RT-PCR

A fully grown flounder (*P. olivaceus*) was purchased from a nearby fish market. The mRNA samples were collected from the whole brain using an mRNA isolation system (Stratagene, La Jolla, U.S.A.). A set of PCR primers was constructed based on the sequences of other teleost IGF-IIs reported previously, as follows: a 22-mer forward primer of 5'-CAT GGA (A/G)AC CCA G(A/C) A AAG ACA C-3' and a 24-mer reverse primer of 5'-TCA (A/T)TT GTG GTT GAC (G/A)TA GTT GTC-3'. The optimal annealing temperature was provided at 61°C in a thermal cycler (MJ research, PTC-100, Watertown, U.S.A.). The main PCR program was 94°C for 1 min, 61°C for 45 sec, and 72°C for 1 min in a 40 cycle reaction following the primary PCR at 48°C for 1 h to induce the first strand cDNA synthesis. The amplified PCR product was then separated in 1.5% agarose gel, followed by cloning into pGEM-T plasmid DNA (Promega, Wisconsin, U.S.A.). Subsequently, transformation and PCR screening were conducted to identify the recombinant plasmid using XL-1 blue competent cells. The resulting transformant containing insert cDNA was selected, and then the plasmid DNA was prepared in a small scale to carry out DNA analysis.

DNA Sequencing and IGF-II Tissue Expressions

DNA sequencing was performed in standard thermal *Taq*-cycling PCR reactions using T7 and SP6 sequencing primers in a DNA analyzer (Perkin Elmer, ABI-310). To examine the tissue expression for IGF-II in adult flounder, mRNAs from the brain, liver, kidney, and muscle were purified and quantified. Approximately, 500 ng of mRNA samples were added to PCR reactions and RT-PCR was carried out as mentioned above.

Comparative Sequence Analysis of Vertebrate IGF-IIs

To define the molecular evolution of IGF-II, several fish IGF-II sequences were imported from the SwissProt data bank/GenBank as follow: *Oncorhynchus mykiss* (rainbow trout, Q02816), *Squalus acanthias* (spiny dogfish, Z50082), *Oncorhynchus keta* (chum salmon, g2570061), *Tilapia* sp. (AF033801), *Lates calcarifer* (barramundi perch, AF007943), and the hagfish IGF sequence (M57735). The human IGF-II (P01344) sequence was provided to compare the protein primary structure with teleosts. Finally, all the DNA sequence data were analyzed using the Internet-based programs such as ClustalW, MAP, and FASTA through the ExPASy tools (Geneva, Switzerland).

RESULTS AND DISCUSSION

In the current study, we have isolated a cDNA clone from the central nervous system (CNS) of adult flounder. This clone appeared to represent the firstly identified flounder IGF-II (flIGF-II) by a database search (FASTA) using the deduced amino acid sequence (Fig. 1). It contained a full-length ORF by displaying 216 amino acids including one stop codon. Hence, our IGF-II specific primers designed in this study seems to be specifically effective to amplify the teleost IGF-II mRNA in RT-PCR cloning. Structurally, flIGF-II exhibits similar primary structure for IGFs, in which it includes signal peptide, B, C, A, D, and E domains from the NH_2-end. The signal peptide is likely to be processed at Ala^{52} based on the eukaryotic prediction [15]. So, the signal peptide-processed flIGF-II can produce a 24.6-kDa core protein with an isoelectric point (pI) of 10.03. However, like insulin, IGF-II has a mature form by a proteolytic removal of the E domain at the post-translational level. As a result, flIGF-II cDNA includes a 65 amino acid-long polypeptide for the biologically active B~D domains, which encodes a completely processed 7.4-kDa protein

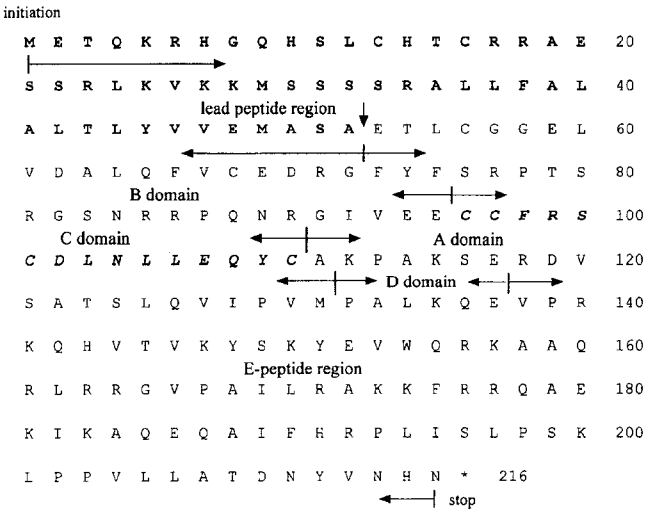

Fig. 1. The complete deduced amino acid sequence for the ORF of flIGF-II cDNA.

Signal peptide predicted was indicated in bold, and the insulin family signature was marked in italics in bold. A characteristic mutidomain structure is found in the ORF, including B, C, A, D, and E domain regions. The flIGF-II contained 52 amino acid-long signal peptide indicated by the vertical arrow and one stop codon.

(pI 5.34). The three disulfide bridges providing molecular foldings for flIGF-II gene product appeared to be formed between the B and A domains (Cys^{56}-Cys^{97}, Cys^{68}-Cys^{110}, and Cys^{96}-cys^{101}) based on the similarity to huIGF-II (P01344). A distinct motif was the presence of the 15 aa-long insulin family signature (C-C-{P}-x(2)-C-[STDNEKPI]-x(3)-[LIVHFS]-x(3)-C) found in the A domain (Cys^{96}~Cys^{110}). In multiple sequence analyses, we compared six fish IGF-II sequences to the huIGF-II to examine the molecular evolution (Fig. 2). We found in this comparison that IGF-II had a phylogenically conserved pattern throughout the domains. In particular, the B, A, and D domains appeared to be highly conserved between molecules ranging from the elasmobranch to humans. Interestingly, the C domain is likely to be a hallmark to distinguish the species in class level during the vertebrate phylogeny, since the number of amino acids was phylogenically exclusive between teleosts (15 aa), aves (13 aa, data not shown), and mammals (12 aa). Unfortunately, the hagfish IGF sequence has no particular relationship to either IGF-I or IGF-II. It seems that IGF-II in cartilage fishes may represent the prototype for vertebrate IGF-IIs as its sequence identity was 37.8% to hagfish IGF, 33.2%~34.4% to teleosts, and 32% to humans. In contrast, flIGF-II shows 33.2% to dogfish, 47.1% to huIGF-II, and over 85.1% to other teleosts (85.1% to salmon and trout, 90.6% to tilapia, and 98.4% to perch). One of the most striking features is that the mature form (B~D) for flIGF-II exhibits 93.8% of sequence homology to salmon and trout, 96.1% to tilapia, and 100% to perch, and even

(A) <Signal peptides>

```
                    10        20        30        40        50
hagfish   YIRRVRQG-----------SIYSLLVESQQWCKLTLTLLLLLALLTRCTL----S
dogfish   MGRHSALGLSGNRQVSPCTGTRPFKVVGSRSPVQPLCILLALTVCIG-TSEARLE
salmon    METQKRHEHHSVCHTCRRTENTRMKVKMMSSSNRVLVIALALTLYIV-EVAS--A
trout     METQKRHEYHSVCHTCRRTENTRMKVKMMSSSNRVLVIALALTLYIV-EVAS--A
tilapia   METQQRYGHHSLCHTCRRTQNSRMKVQRMSSTSRALLFALALTLYVV-EMAS--A
perch     METQQRHGQHSLCHTCRRAESSRMKVKKMSSSSRALLFALALTLYVV-EMAS--A
flIGF-II  METQKRHGQHSLCHTCRRAESSRLKVKKMSSSSRALLFALALTLYVV-EMAS--A
human     --MGIPMG----------------------KSMLVLLTFLAFASCCIAAYRPS
consensus                                 *  .   *.
```

(B) <Region for completely processed IGF-II>

```
                    10        20        30        40        50        60        70
hagfish   ETLCGSELVDTLQFVCDDRGFFFVPQHVPPRRGAHRRSRARKGIVEECCFKGCSLRLLEMYCARPSKAE
dogfish   ETLCGSELVDTLQFICAERGFYFVSKVVG------RRSRQNRGIVEECCFRSCDLLILETYCAVPPEAA
salmon    ETLCGGELVDALQFVCEDRGFYFSR---PTSRSNSRRS-QNRGIVEECCFRSCDLNLLEQYCAKPAKSE
trout     ETLCGGELVDALQFVCEDRGFYFSR---PTSRSNSRRS-QNRGIVEECCFRSCDLNLLEQYCAKPAKSE
tilapia   ETLCGGELVDALQFVCEDRGFYFSR---PTSRGNNRRP-QTRGIVEECCFRSCDLNLLEQYCAKPAKSE
perch     ETLCGGELVDALQFVCEDRGFYFSR---PTSRGSNRRP-QNRGIVEECCFRSCDLNLLEQYCAKPAKSE
flIGF-II  ETLCGGELVDALQFVCEDRGFYFSR---PTSRGSNRRP-QNRGIVEECCFRSCDLNLLEQYCAKPAKSE
human     ETLCGGELVDTLQFVCGDRGFYFSR---PASRVSRR----SRGIVEECCFRSCDLALLETYCATPAKSE
consensus *****.****.***.* .*** .*        *          ********..* * .** *** * . .
```

(C)

<E-peptides>

```
                    10        20        30        40        50        60        70        80        90        100
hagfish   RDVARPRQR-----P---HR----------ASQHSRRGSQ-----------------------------------------SRGRGRSR----------
dogfish   RSTPSPAQQ-----RTLMHRIVGKRSLGIEQERLTRPGGHLQQYLNNASPAS------SPKAEILDWPGEGEASPPIGDLTWRARYPPRHWVWLSAALPSPSTS
salmon    RDVSATSLQIIPMVPTIKQDVPRK-HVTVKYSKYEAWQRKAAQRLRRGVPAILRARKFRRQAVKIKAQEQAMFHRPLITLPSKLPP-V-----LPPTDNYVSHN
trout     RDVSATSLQIIPMVPTIKQDVPRK-HVTVKYSKYEAWQRKAAQRLRRGVPAILRARKFRRQAVKIKAQEQAMFHRPLITLPSKLPP-V-----LPPTDNYVSHN
tilapia   RDVSATSLQVIPVMPALKQEVPKKQHVTVKYSKYEVWQRKAAQRLRRGVPAILRARKYKRHAEKIKAKEQAIFHRPLISLPSKLPP-V-----LLTTDNFVSHK
perch     RDVSATSLQVIPVMPALKQEVPRKQHVTVKYSKYEVWQRKAAQRLRRGVPAILRAKKFRRQAEKIKAQEQVIFHRPLISLPSKLPP-V-----LLTTDNYVNHK
flIGF-II  RDVSATSLQVIPVMPALKQEVPRKQHVTVKYSKYEVWQRKAAQRLRRGVPAILRAKKFRRQAEKIKAQEQAIFHRPLISLPSKLPP-V-----LLATDNYVNHN
human     RDVSTPP----TVLP---DNFPR--YPVGKFFQYDTW-KQSTQRLRRGLPALLRARRGHVLAKELEAFREAKRHRPLIALPTQDPAHG-----GAPPEMASNRK
consensus *
```

Fig. 2. The amino acid sequence of flIGF-II was aligned with other IGF-II sequences.

Comparison was carried out according to the domain regions for the signal sequence (**A**), B~D domain (**B**), and E peptide (**C**), respectively. Six cysteins and evolutionary-conserved amino acids are marked in bold. Note the significant homology between IGF-IIs found in Fig. 2B. Dots indicate similar amino acids. Multiple sequence alignment was determined using ClustalW on Pam250 matrix.

displays 83.5% to huIGF-II. Therefore, this suggests that IGF-II is an anciently evolved molecule that appeared after the agnatha (jawless vertebrates) in chordate phylogeny, and the highly conserved patterns were maintained, particularly in the B and A domains, over several hundred million years of phylogeny. Recently, the potential roles of the E-peptide have become interesting, since many types of cancerous cells turned out to express the highly mitogenic prematured IGF-II (high molecular weight form) which seems to include the B to E domains region [16, 17]. Of course, this expression could be dependent on the physiological condition of cells *in vivo*. In flIGF-II, the E-domain seems to be processed at Glu^{117} to generate a 98 aa-long E-peptide (Arg^{118}~COOH end), due to the consensus motif for a proteolytic processing [18]. However, little is known about the growth-modulating roles of E-peptide, so far.

IGFs are known to have exclusive patterns in cytological localization, in which IGF-II is detected only in insulin-immunoreactive cells [19]. Overexpressing IGF-II causes many phenotypic defects including somatic overgrowth [20] and acts as an angiogenic factor during hepatocarcinogenesis [21]. We have also defined the mRNA signals in the adult tissues including brain, liver, kidney, and muscle by RT-PCR (Fig. 3), and the data indicate that liver could be the main source for IGF-II mRNA production. The adult flounder brain also appears to contain abundant IGF-II mRNA signal, and all the tissues examined exhibited *in vivo* IGF-II expressions.

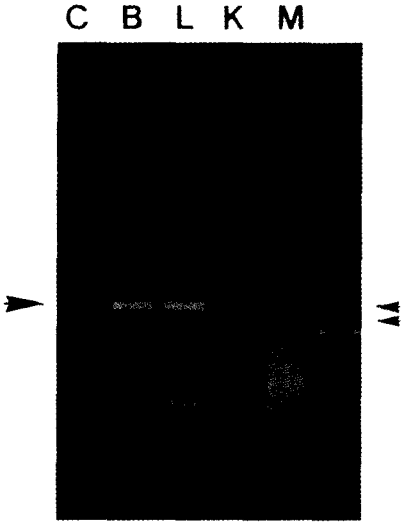

Fig. 3. The tissue expressions for flIGF-II on the adult brain (B), liver (L), kidney (K), and muscle (M) by RT-PCR.

The strongest cDNA signal was found in liver, but brain has also significant IGF-II expression. In brain, another amplified PCR product of approximately 500 bp is observed. Small arrows indicate 600 bp and 700 bp marker DNA (100 bp ladder). Large arrow shows the amplified IGF-II fraction. C is for negative control PCR. 1.5% agarose gel.

The main mechanism in controlling the expression of the serum IGF-II in teleosts may be highly involved with the regulatory control of GH which is able to induce transcriptional factors for the enhancer-binding proteins in IGF-II gene [22]. However, it is stressed that, in any circumstances, either IGF-I or IGF-II acts as a prominent growth factor during the vertebrate development in a highly regulated manner [23]. As we discussed above, the teleost IGFs are significantly similar to higher vertebrates and may even be effective to activate the IGF systems ranging from humans to invertebrates [24]. Among the most interesting biological roles of IGFs may be those on muscles and osteoblasts [25, 26], both of which may result in increases of skeletal growth and body weight. GH seems to enhance these responses in synergism as a potential amplifier for IGF-II in stimulating steroidogenesis [27]. IGF-II may probably exert important biological roles in the development of CNS. Unfortunately, we have little knowledge on the potential roles for IGF-II in neurogenesis, so far. However, IGF-II is regarded as a paracrine survival factor which exerts additional roles in the neuronal replacement for projecting neurons [28]. Hence, our next concern is to define the relationships between IGF-II and neurodegeneration compared to IGF-I on neurons [29, 30].

The class osteichthyes has many advantages to investigate the vertebral phylogeny, as well as the molecular evolution. In particular, the teleoste representing the major modern fish species is a wonderful source to acquire biologically important genetic resources. In the present study, we have characterized a single dominant growth factor, IGF-II, from CNS of flounder and defined its molecular evolution. It is now possible to conclude that the IGF-II gene appeared in the age of fishes, the Devonian period, and was firstly identified in cartilage fishes. It is a little surprising on that why the vertebrates had maintained this single category for peptide growth factors (IGFs) for some 450 million years of evolution. Was there any alternative way to keep the growth of vertebrates in phylogenic evolution? Many questions on the growth mechanisms relating to IGFs still remained to be answered. Therefore, we would like to provide more detailed evidence to enhance our knowledge on the anciently evolved IGFs. Within this aim, some biotechnical applications are currently being designed using our flIGF-II cDNA and its *in vitro* product, which seems to be potent enough to activate the evolutionary-conserved IGF-involved growth systems in all vertebrates.

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