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Molecular divergence of the fish somatomedins: the single family of insulin-like growth factor (IGF)-I and -II from the teleost, flounder

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The teleosts represent ancient real-bony vertebrates in phylogeny and resemble major genetic patterns to higher vertebrates. In the present study, we have defined the single family of insulin-like growth factors (IGFs) from flounder (*Paralichthys olivaceus*), compared to the prototype of IGFs observed in the Agnathan hagfish. In flounder, IGFs are clearly diverged into two major types including type I and II, and they are structurally similar by displaying a multidomain structure consisting of five functional regions as previously found in other vertebrates. However, flIGF-I appears to be more basic (pI 8.03) than the flIGF-II (pI 5.34) in the fully processed form for the B to D domain region. The flIGF-I seems to contain an evolutionary conserved Asn-linked glycosylation in E domain, which is not found in flIGF-II. The most interesting feature is that flIGF-II appeared to be structurally close to hagfish IGF in secondary structures, particularly in B and D domains. This could tell us an idea on the molecular divergence of IGFs from the Agnatha to teleosts during the vertebrate phylogeny. It also support, in part, a notion regarding on how IGF-II is appeared as more embryonic during development. Nonetheless, the biologically active B to D domain region of flIGF-II shows significant sequence homology of 65.6% to flIGF-Is and contains the evolutionary conserved insulin-family signature, as well as a reserved recognition site (Lys) in D domain, necessary to generate proteolytic cleavage for E-peptide. A significant structural difference was found in E domain in which flIGF-I possesses two potential alternative splicing donor site at $Val^{17, 24}$ of E domain. Therefore, it seems so far that IGF-I sorely produces spliced variants due to the spliced E-peptide moiety while IGF-II appears to be maintained in a single type during evolution. IGF-II, however, may be also possible to transcribe unidentified variants, depending on the physiological conditions of tissues in vertebrates *in vivo*.

Key words: Teleosts, Flounder IGFs, Molecular evolution, E-peptide, Asn-linked glycosylation, Spliced variant, single IGF family

Introduction

The proliferating cells during development are highly influenced by intercellular tropic signals such as growth hormone (GH) and growth factors. The insulin-like growth factor (IGF) is one of key molecules mediating the growth of vertebrates. Many studies have been conducted over recent years on the vertebral IGFs. Fish is a good model system to investigate development of the cellular response and tissue physiology involving these chemotrophic signals *in vivo*. The teleost provides enough resources to investigate many biologically important genes in terms of the molecular evolution. It is generally regarded that the phylogenic occurrence of IGFs is one of distinct features in molecular evolution, in which IGFs are found in all vertebrates ranging from teleostean fish species to mammals. IGF includes type-I and -II, and a single common prototype known to stimulate protein synthesis in mammalian myoblast is found in the primitive vertebrate hagfish (Upton et al., 19 97). In fact, IGF is clearly observed in the two diverged types from elasmobranchs (Stephen et al., 1995). The growth promoting effects of IGFs are mainly derived from free IGFs, and the serum GH is highly involved in this event (Hashimoto et al., 1997). It is thought that GH is a potent amplifier on IGFs by stimulating steroidogenesis (Xu et al., 1997). However, there is a fundamental question regarding on why the primitive IGF molecule diverged into two types during evolution. At present, it is presumed that developing vertebrates might

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need certain specific roles from this growth factor to adapt their surrounding environment during the chordate evolution. Therefore, IGF-I and -II appeared from the cartilage fishes might be able to serve their particular roles in the ontogenic development of many vertebrates. Recently, we have characterized the flounder IGFs and we define, herein, the molecular divergence of IGFs from the single teleostean IGF family, based on the amino acid composition and their secondary structure analysis.

Materials and methods

The deduced amino acid (aa) sequences for flounder IGFs were prepared using the original cDNA sequences of flIGFI-1 (AF016922), flIGFI-2 (E08878), and our recent flIGF-II clone (AF091454) from the nucleotide data bank (GenBank in NCBI). To define the molecular characteristics between these molecules, protein sequence alignments were carried out using the separate three coding regions including leader peptide, B~D domains, and E-peptide. The hagfish IGF sequence (M57735) was provided as the prototype molecule for both IGF-I and IGF-II in the multiple protein sequence comparison. The signal peptide sequence and E-peptide cleavage sites were predicted based on either similarity to human IGF-I (P01343, P05019) and -II (P01344) obtained from the protein data bank (SwissProt) or the method according to von-Heijne (1990). To provide patterns of the amino acid compositions between the flounder IGF-I and IGF-II, the deduced amino acids were categorized into anionic, cationic, neutral, hydrophobic, and hydrophilic based on their side chain properties. The amino acid sequence homology was obtained by Fasta3/Promsed2. The molecular weight (Mw) and isoelectric point (pI) were estimated in the Internet-based PC programs such as Translate and pI in ExPASy against the completely processed (B~D) and incompletely processed (B~E) forms of flounder IGFs. Finally, the secondary structure prediction was conducted by Antheprot/Wisconsin-GCG using the coding region for biologically active B~D domains providing the mature IGF forms.

Results and discussion

The teleost flounder expresses IGFs throughout its development stages from embryonic to adult periods. Both IGFs including IGF-I and -II may exhibit differential expressions depending on the physiological conditions of cells or tissues. In the present study, we have defined the structural characteristics of the flounder IGF family. Depending on our idea regarding that the agnathan IGF molecule may represent one of molecular characteristics close to either IGF-I or IGF-II of flounder, the hagfish IGF sequence was provided in this analysis. The amino acid numbering in this study was based on the order in the aligned sequences to reserve the pattern comparison. The domain-specific primary structural features are shown in Fig. 1. In the leader peptide region, we found no significant relationships between these sequences, except for the extra six amino acid insertion compared to flIGF-I (Fig. 1-a). The hydrophobic $Leu^{36, 37, 40, 42}$ were appeared to be conserved, and the acidic Gln^{9} was in consensus as well. The most interesting features were found in the patterns of B~D domains (Fig. 1-b), in which IGF-I contained 66 aa in contrast to 65 aa observed in flIGF-II. The 17~18th amino acids were both acidic in hagfish and flIGF-II, but were substituted to neutral Gly^{17} in flIGF-I. This pattern was also found in Glu^{71} (acidic E and neutral A). The 32th amino acid was basic Arg in hagfish and IGF-II, but was hydrophilic Tyr in IGF-I. Moreover, hagfish and flIGF-II contain neutral Ser^{38} and Pro^{38}, and the Lys^{51} and Arg^{51} were all basic, respectively. However, flIGF-I contains acidic Gln^{51} in this site, suggesting that the hagfish IGF known as a prototype for both IGF-I and -II may be more IGF-II like molecule. The hagfish IGF sequence showed a short residue of four amino acids between neutral Val^{24} and Pro^{29}, and the 27^{th} amino acid site was basic in all sequences. It appears that flIGF-I contains two extra codons of neutral amino acids (Pro^{58}, Ala^{59}) in D domain (aa59~71), compared to the six amino acid-containing flIGF-II. Therefore, variations in codon number seem to be occurred exclusively in C and D domains, and the remaining B and A domains are likely to be evolutionary stabilized from the Agnatha. Of particular, the 15 aa-long insulin family signature was found in all sequences, and the Lys^{69} providing a motif for proteolytic production of E-peptide from Arg^{1} in E domain was well conserved from hagfish. Recently, the E-peptide region has been largely interested due to the highly mitogenic activity of incompletely processed form (Hodzic et al., 1997: Li et al., 1998).

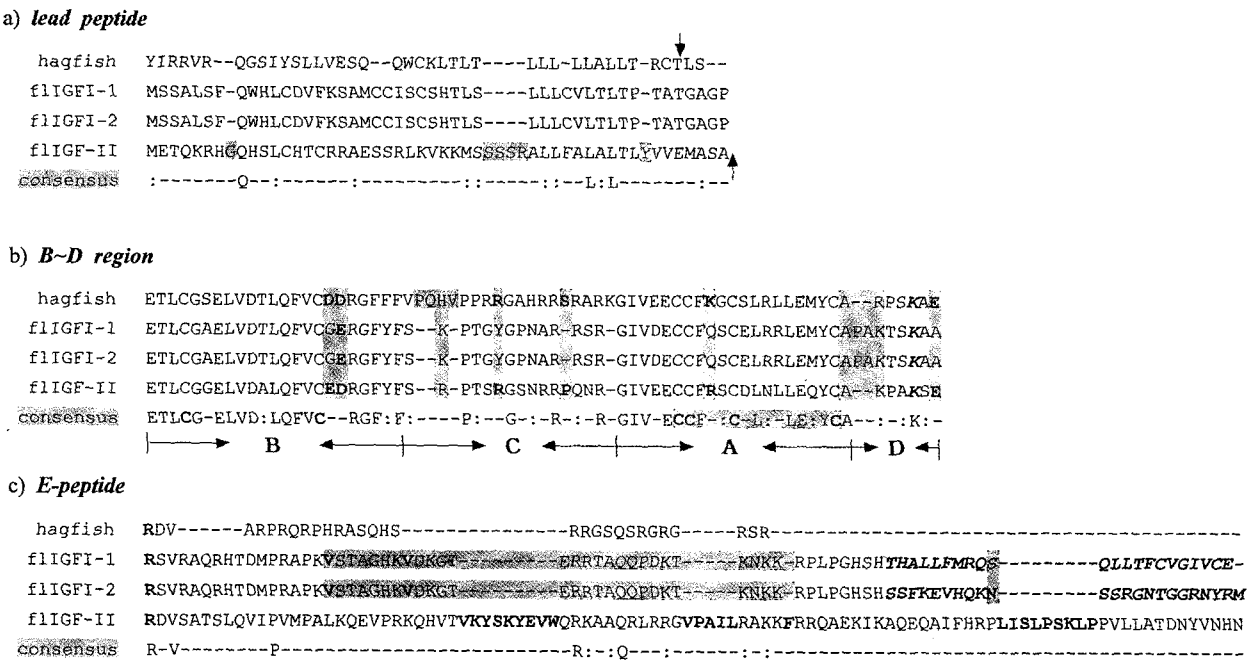

Fig. 1. Multiple sequence alignment for flounder IGFs. The IGF sequences were partitioned into three regions. The arrows indicate signal peptide cleavage, and the structural relationships were highlighted or in bold. ClustalW protein sequence alignment on Pam 250 protein matrix.

On our data, we found that only IGF-I contains alternative splicing donor sites at Val[17, 24], and no potential donor site was found in hagfish or flIGF-II, indicating that IGF-I sorely produces spliced isoforms due to the spliced E-peptide moiety. The E domain of flIGF-II exhibited three insertion sites for short amino acid residues compared to flIGF-I. It, therefore, displays longer E-peptide (98aa) compared to the conventional length of 74aa in flIGFI-2. One distinct feature was the presence of Asn-linked carbohydrate-binding site (N-{P}-[ST]-{P}) found in flIGFI-2, and this site seems to be phylogenically conserved in all vertebrate IGF-I molecules. However, the flIGFI-1 sequence does not show this motif. Even more, its sequence from site[67] to C-terminus exhibits completely different pattern. Therefore, it is likely that this flounder IGF-I clone may represent an E-peptide mutant, even though it has 100% sequence homology to flIGFI-2 in the B~D region. As shown in Table 1., flIGF-II displays 65. 6% of sequence identity in contrast to 24.5~15.1% in the leader and E-peptide regions. However, this high homology for B~D domains between flIGF-I and flIGF-II may not indicate the possible cross reactivity. In the secondary structure analysis (Fig. 2), we used the region corresponding to the complete IGF forms (B~D). Basically, two flIGF-I clones exhibit same pattern in this region. Interestingly, we have found that the hagfish IGF resembles IGF-II-like secondary structure, particularly in B and D domains. Therefore, it is possible to suggest that IGF-II may be appeared earlier than IGF-I in the molecular evolution of IGFs.

IGFs mediate cellular responses relating the growth of vertebrates, and the IGF system seems to be highly conserved from bony fishes to mammals (Duan et al., 1998). It may be also related to physiology of many organs including kidney, gonad, and gastrointestinal tract (Fostier et al., 1994; Hirschberg and Adler 1998). Of particular, IGF-I involves osmoregulation via autocrine/paracrine action (Koppang et al., 1998), which might be one of key factors in adapting changing environment during vertebrate evolution. In contrast, IGF-II is more embryonic and is regarded as a fetal growth factor (Lund et al., 1986; Veness-Meehan et al., 19 97). IGFs essentially exert their functions through IGF receptor. It has been known that IGF-I and -II are mediated via the type-I receptor, and this receptor tyrosine kinase is abundantly expressed in fish skeletal muscle and brain (Parrizas et al., 1995; Leibush et al., 1996; Willis et al., 1998). Likely the

Table 1. Molecular identity on the domain-based amino acid sequences of flounder IGFs

Regions	*lead peptide*				*B~D domain*				*E-peptide*			
aa identity	1	2	3	*	1	2	3	*	1	2	3	*
1. flIGF-II	–				–				–			
2. flIGFI-1	24.5%	–			65.6%	–			16.4%	–		
3. flIGFI-2	24.5%	100%	–		65.6%	100%	–		15.1%	70.7%	–	
*hagfish	17.4%	23.3%	23.3%	–	65.7%	63.7%	63.7%	–	14.1%	15.5%	15.4%	–

*the hagfish IGF is to provide the control comparisons for IGF-I and -II.

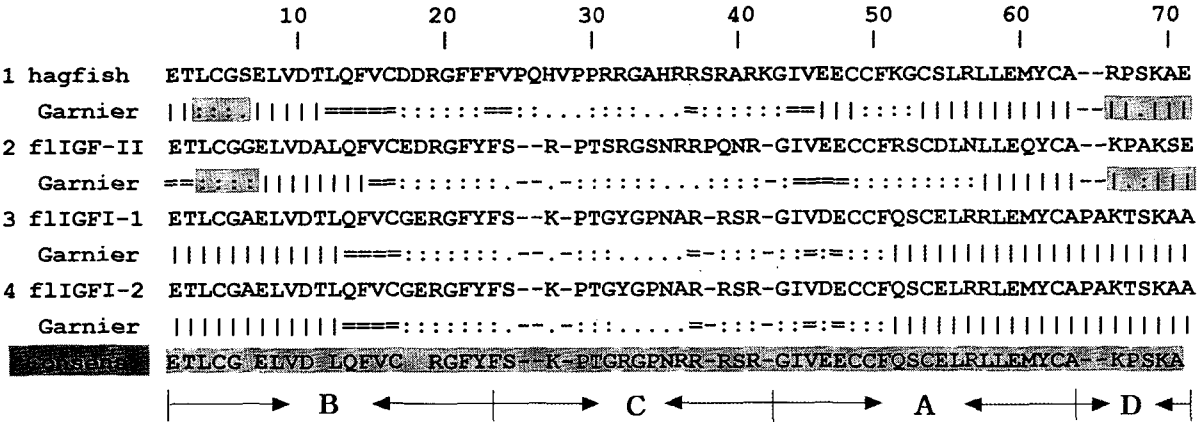

Fig. 2. Secondary structure analysis on the hagfish IGF and flIGFs using the Garnier prediction. The symbols indicate structural patterns as follow: |, helix; =, sheet, :, turn, ., coil. The consensus sequence shows only 100% homologous amino acids, and the distinct patterns observed were highlighted in hagfish and flIGF-II.

ligand IGFs, IGF receptor also appears to be highly conserved in many vertebrates (Elies et al., 1996). Surprisingly, the protochordate amphioxus contains an insulin-like peptide (ILP) gene which displays the basic structural determinants for insulin and IGF-binding (Pashmforoush et al., 1996), indicating the molecular divergence for IGF and insulin during the chordate phylogeny. So, it is very likely that IGF and its receptor molecules seem to form the insulin- and insulin-receptor superfamily ranging from protochordate to vertebrates.

In this study, we found that the flounder IGFs showed structurally similar patterns, but they might have different ways to functions *in vivo*. The flIGF-I could encode a 7.8 kDa basic protein with an isoelectric point (pI) of 8.03 for the complete form, but flIGF-II was acidic (pI 5.34) with a slightly smaller Mw (7.4 kDa). At this moment, we have little understanding on the potential mechanisms relating the acidic and basic properties of flIGFs. Many questions are needed to be answered in the physiological actions of these fish hormones *in vivo*. We are currently aiming to develop *in vitro* model system to investigate and further utilize these important genetic resources in life sciences.

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