Molecular Cloning of Insulin-like Growth Factor-I (IGF-I) and IGF-II Genes of Marine Medaka (*Oryzias dancena*) and Their Expression in Response to Abrupt Transfer from Freshwater to Seawater

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Growth hormone (GH) is known as one of the main osmoregulators in euryhaline teleosts during seawater (SW) adaptation. Many of the physiological actions of GH are mediated through insulin-like growth factor-I (IGF-I), and the GH/IGF-I axis is associated with osmoregulation of fish during SW acclimation. However, little information is available on the response of fish IGF-II to hyperosmotic stress. Here we present the first cloned IGF-I and IGF-II cDNAs of marine medaka, *Oryzias dancena*, and an analysis of the molecular characteristics of the genes. The marine medaka IGF-I cDNA is 1,340 bp long with a 257 bp 5' untranslated region (UTR), a 528 bp 3' UTR, and a 555-bp open reading frame (ORF) encoding a propeptide of 184 amino acid (aa) residues. The full-length marine medaka IGF-II cDNA consists of a 639 bp ORF encoding 212 aa, a 109 bp 5' UTR, and a 416 bp 3' UTR. Homology comparison of the deduced aa sequences with other IGF-I and IGF-II showed that these genes in marine medaka shared high structural homology with orthologs from other teleost as well as mammalian species, suggesting high conservation of IGFs throughout vertebrates. The IGF-I mRNA level increased following transfer of marine medaka from freshwater (FW) to SW, and the expression level was higher than that of the control group, which was maintained in FW. This significantly elevated IGF-I level was maintained throughout the experiment (14 days), suggesting that in marine medaka, IGF-I is deeply involved in the adaptation to abrupt salinity change. In contrast to IGF-I, the increased level of marine medaka IGF-II mRNA was only maintained for a short period, and quickly returned a level similar to that of the control group, suggesting that marine medaka IGF-II might be a gene that responds to acute stress or one that produces a supplemental protein to assist with the osmoregulatory function of IGF-I during an early phase of salinity change.

Key words: cDNA cloning, Hyperosmotic stress, Insulin-like growth factor-I (IGF-I), IGF-II, Marine medaka (*Oryzias dancena*)

Introduction

For aquatic organisms, salinity is a crucial factor for maintaining normal physiological processes. Euryhaline teleosts, which can tolerate wide variations in salinity without compromising life processes, are equipped with effective osmoregulatory mechanisms against dramatic environmental salinity changes. Some species of the genus *Oryzias* including Japanese medaka, *O. latipes*, a well-known experimental model fish with a genomic database

(Wittbroadt et al., 2002), are good model species for analyzing osmoregulatory mechanisms in teleosts. Among them, the marine medaka, *O. dancena*, a fish species inhabiting estuarine waters (Roberts, 1998), has been reported to have better tolerance against hyperosmotic stress than *O. latipes* (Inoue and Takei, 2002, 2003; Kang et al., 2008).

Growth hormone (GH) is known as one of the main osmoregulators in euryhaline teleosts during seawater (SW) adaptation (McCormick, 1995). Many of the physiological actions of GH are mediated through insulin-like growth factor-I (IGF-I) (Froesch et al., 1985; Butler and Le Roith, 2001), and the GH/IGF-I

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axis is associated with osmoregulation of fish during SW acclimation (Madsen and Bern, 1993; Sakamoto and Hirano, 1993; McCormick, 1995, 1996; Kalujnaia et al., 2007; Meier et al., 2009). As in mammals, teleosts possess IGF-I and IGF-II (Reinecke et al., 2005). The teleostean IGF-I is involved in osmoregulation as well as many developmental, physiological, and immunological responses (Reinecke, 2006; Yada, 2007). However, although the expression of the IGF-II gene was demonstrated to be GH-dependent in several fish species, such as rainbow trout and redbanded seabream (Shamblott et al., 1995; Ponce et al., 2008), little information is available on the response of fish IGF-II to hyperosmotic stress.

Here we report the first cloned IGF-I and IGF-II cDNAs of marine medaka and present an analysis of the molecular characteristics of the genes. In addition, to determine whether the IGFs respond to acute hyperosmotic stress, time-course changes of IGF-I and IGF-II transcripts levels were examined after abrupt transfer of marine medaka from freshwater (FW) to SW.

Materials and Methods

Fish

Marine medakas (*O. dancena*) were obtained from brood stock maintained at the Institute of Marine Living Modified Organisms (IMLMO), Pukyong National University. Yolk-sac absorbed fry were held in two 50 L FW aquaria and acclimated for 2 weeks. Throughout the study, the water temperature was maintained at $27 \pm 1^\circ\text{C}$. After acclimation, fish were transferred directly from FW to SW or from FW to FW (control group), and sampled at 0, 3, 6, 12, 24, 72, 168 (7 days) and 336 (14 days) h after transfer. Five fish were randomly collected at each time point to analyze the mRNA levels of IGF-I and IGF-II.

Gene cloning

Total RNA was extracted from the whole body of fish using the RNAiso reagent (Takara Bio, Tokyo, Japan) according to the manufacturer's instruction. A 1- μg aliquot of purified total RNA was incubated with 1 μL of random primer (0.5 $\mu\text{g}/\text{mL}$) (Promega, Madison, WI, USA) at 80°C for 10 min, and further incubated at 42°C for 60 min in a reaction mixture containing 4 μL of $5\times$ reaction buffer, 4 μL of 10 mM dNTP mix (Takara Bio), 1 μL of M-MLV reverse transcriptase (Promega), and 0.5 μL of RNase inhibitor (Promega) in a final reaction volume of 20 μL .

Two degenerate oligonucleotides, IGF-I F: 5'-GTSTGYGGRGASMGRGGCTTTTATTTC-3' and IGF-I R: 5'-GGGGGMAGRAAYTAYCGVATKTA G-3', were used to obtain the marine medaka IGF-I cDNA fragment by PCR. Using another two degenerate oligonucleotides (IGF-II F: 5'-GGAGARCT GGTRGAYGCGCTRCAGTTYGTSTG-3' and IGF-II R: 5'-CRGMGRCTMCGRAGRGGYRTCCC-3'), a partial fragment of marine medaka IGF-II cDNA was obtained by PCR. The amplified PCR product was run on an agarose gel (1.5%) and visualized by ethidium bromide (Et-Br) staining. The fragment was purified using a gel purification kit (Cosmo Genentech, Seoul, Korea) and subcloned into a pGEM-T easy vector (Promega). Several clones were sequenced using two vector primers (T7 and SP6). The sequence was submitted to GenBank (<http://www.ncbi.nlm.nih.gov/blast>) to examine the homology with previously known IGF sequences.

To obtain full ORF sequences of IGF-I and IGF-II, 5'-RACE (primer 1: 5'-CAACGGGCTACGGTC CCAATGC-3' in IGF-I; primer 2: 5'-CTGTGCCAA ACCCACCAAGTCCGAAAG-3' in IGF-II; primer 3: 5'-AACCAAAACCCGCGTCTGCCATG-3' in IGF-II) and 3'-RACE (primer 4: 5'-GAGGCAACAA CGGGGGCAGAAA-3' in IGF-I; primer 5: 5'-CCCTTTGCACAGACAAGAGTTTGAGGG-3' in IGF-II) PCR reactions were performed separately using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. All RACE PCR products were purified using a spin column (Labopass; Cosmo Genentech), cloned into a pGEM-T easy vector (Promega), and sequenced. Multiple sequence alignments were generated using the CLUSTALW 1.8 program.

Semiquantitative RT-PCR

The expression of IGF-I and IGF-II in response to hypersalinity stress was analyzed using semiquantitative RT-PCR. Fish were anesthetized with tricaine methanesulfonate (MS222; Sigma, St. Louis, MO, USA), and total RNA was extracted from the whole body using RNAiso reagent, then reverse-transcribed to cDNA as described above to be used for the gene expression assay. Primers used for amplification of each gene were as follows: IGF-I-F (5'-CAACGGGCTACGGTCCCAATGC-3') and IGF-I-R (5'-GAGGCAACAACGGGGGCAGAAA-3') for IGF-I, IGF-II-F (5'-ATGGAGAGCCCGCA AAGACTCGG-3') and IGF-II-R (5'-CTCAACCT GCTAGAGCAGTACTGTGCCAA-3') for IGF-II, Actin-F (5'-ATGGAGAAGATCTGGCATCACACC

TTCTAC-3') and Actin-R (5'-GGCTGGCAGAGA TTTGACAGATTACCTGAT-3') for the β -actin gene (internal control). PCR was performed using Takara rTaq premix and 1 μ L of tenfold diluted cDNA in a 20 μ L reaction. For normalization, the β -actin gene was used as an internal standard. The optimal PCR cycles of the selected genes were determined after PCR products taken every 2 or 3 cycles from 15 to 33 cycles, and were confirmed by agarose gel electrophoresis. PCR samples to be compared were visualized on 1% agarose gels with Et-Br staining. Images of the Et-Br stained bands were imported into the Quantity-One image analysis software implemented in Gel Doc XR imaging device (Bio-Rad, Hercules, CA, USA). The band intensity of IGF-I and IGF-II genes was normalized against that of β -actin to estimate their relative mRNA levels.

Statistics

Semiquantitative RT-PCR data were analyzed using the Student *t*-test, and significant differences were determined at $P < 0.05$.

Results

Complementary DNAs and deduced amino acid sequences of marine medaka IGF-I and IGF-II

The marine medaka IGF-I cDNA was 1,340 bp long with a 257 bp 5' UTR, a 528 bp 3' UTR, and a 555 bp ORF encoding a propeptide of 184 amino acid (aa) residues (GenBank Accession No. HQ176000). Conserved domain analysis revealed that the marine medaka IGF-I had two cleavage regions, an N-terminal signal peptide (1 to 44 aa), and a C terminal E domain (113 to 184 aa), which made the propeptide into a mature peptide of 68 aa residues. The mature peptide consisted of domains B (45 to 73 aa), C (74 to 83 aa), A (84 to 104 aa), and D (105 to 112 aa). At the aa level, marine medaka IGF-I showed high similarity with the IGF-Is of other fish species (64–87%). Mouse (*Mus musculus*) and human (*Homo sapiens*) IGF-Is showed 58% and 59% identity, respectively, with marine medaka IGF-I. Multiple alignments of IGF-I proteins showed that the B and A domains of the mature form IGF-I were highly conserved from fish to mammals. The signal peptide region and the C-terminal E domain were highly variable according to fish species (Fig. 1).

The full-length marine medaka IGF-II cDNA consisted of a 639 bp ORF encoding 212 aa, a 109 bp 5' UTR, and a 416-bp 3' UTR (GenBank Accession No. HQ176001). Conserved domain analysis revealed that marine medaka IGF-II consisted of a signal

peptide region (1 to 44 aa) in the N-terminal region, the domains of mature forms (B, 45 to 76 aa; C, 77 to 87 aa; A, 88 to 108 aa; D, 109 to 114 aa) in the middle region, and the C-terminal E domain (115 to 212 aa). When comparing deduced aa sequences, marine medaka IGF-II showed high similarity (66–79%) with previously reported teleostean IGF-II genes of other fish. The aa identity of marine medaka IGF-II with mouse IGF-II was 51% and that with human IGF-II was 48%. Multiple alignment of IGF-II proteins showed a similar pattern to that of IGF-I (Fig. 2).

Time-course expression of IGF-I and IGF-II transcriptions in response to hyperosmotic stress

The transcription of marine medaka IGF-I in response to abrupt transfer of fish from FW to SW had significantly decreased at 6 h post-transfer and then was restored to the level of the control group at 12 h post-transfer (Fig. 3A). The expression level of IGF-I in the group that underwent hyperosmotic stress was higher than that of the control group from 24 h to the end of the experiment (14 days post-transfer). The transcription of marine medaka IGF-II had also significantly decreased at 6 h post-transfer. The expression of IGF-II had acutely increased at 12 and 24 h post-transfer, and then returned to a level similar to that of the control group toward the end of the experiment (Fig. 3B).

Discussion

In vertebrates, IGF-I and IGF-II are produced as preprohormones, consisting of an N-terminal signal peptide, followed by B, C, A, D, and E domains (Jones and Clemmons, 1995). Then, mature IGFs are produced by proteolytic cleavage of signal peptide and E domain. In the present study, we cloned the complete cDNA of IGF-I and IGF-II of marine medaka. Conserved domain search analysis revealed that marine medaka IGF-I and IGF-II contained N-terminal and C-terminal cleavage regions, resulting in propeptides from which functional, mature peptides consisting of B, C, A, and D domains are produced. Homology comparison of the deduced aa sequences with other IGF-Is showed that marine medaka IGF-I shared high structural homology with orthologs from other teleosts as well as mammalian species. Marine medaka IGF-II also showed high similarity with mammalian IGF-IIs, suggesting conservation of IGFs throughout vertebrates.

Previous studies have demonstrated that teleost GH

Fig. 2. Multiple alignment of deduced amino acid sequences of marine medaka IGF-II along with orthologues from other teleosts and mammals. Amino acids identical from fish to mammals are indicated by black boxes. Regions of signal peptides and each domain are indicated on the top line of alignment. GenBank accession number and scientific name of each species are as follows; AAB64195 *Lates calcarifer* (barramundi perch), AAS58520 *Epinephelus coioides* (orange-spotted grouper), BAF80635 *Pagrus auriga*, AAO47743 *Siganus guttatus* (orange-spotted spinefoot), AAY46224 *Sparus aurata* (gilthead seabream), BAF80637 *Diplodus sargus* (white seabream), AAW29441 *Dicentrarchus labrax* (European seabass), ABC60230 *Jenynsia maculata*, CAA17123 *Takifugu rubripes* (Fugu rubripes), ABC60254 *Limia melanogaster* (blackbelly limia), ABC60245 *Neoheterandria tridentiger*, ACS92730 *Micropterus salmoides* (largemouth bass), ABY88873 *Oreochromis niloticus* (Nile tilapia), ABC60234 *Poeciliopsis presidionis* (Sinaloa livebearer), ABC60252 *Poecilia reticulata* (guppy), ABC60248 *Phallichthys tico*, ABC60246 *Pseudopoecilia festae*, ABO36528 *Salmo salar* (Atlantic salmon), AAC17496 *Oreochromis mossambicus* (Mozambique tilapia), ABC60247 *Xenophallus umbratilis*, NP001118169 *Oncorhynchus mykiss* (rainbow trout), CAA65862 *Oncorhynchus keta* (chum salmon), ABC60229 *Ilyodon amecae*, ABC60249 *Brachyrhaphis terrabensis*, ABC60251 *Cnesterodon decemmaculatus*, ABC60250 *Brachyrhaphis rhabdophora*, NP001001815 *Danio rerio* (zebrafish), ABK55615 *Ctenopharyngodon idella* (grass carp), P09535 *Mus musculus* (house mouse), P01344 *Homo sapiens* (human).

Fig. 3. Change of mRNA expression of marine medaka IGF-I (A) and IGF-II (B) genes by acute transfer from freshwater to seawater. Semi-quantitative RT-PCR was performed using cDNA synthesized using total RNA from marine medaka after transfer to seawater at various time points ranging from 3 h to 14 d. Scanning densitometry analysis was done to determine the relative mRNA levels of marine medaka IGF-I or IGF-II based on the normalization against β -actin control. Histograms indicate the means of five marine medaka examinations and standard errors were indicated by T bars. *Significantly different from the control group ($P < 0.05$).

and IGF-I are closely associated with SW adaptation (McCormick, 2001). McCormick et al. (1991) reported that exogenous application of IGF-I resulted in improved salinity tolerance of rainbow trout after SW transfer. Sakamoto and Hirano (1993) also observed increased IGF-I mRNA levels in the gill and kidney by transfer of immature trout to 80% SW. Mancera and McCormick (1998) reported that killifish injected with recombinant bovine IGF-I (rbIGF-I) showed improved SW tolerance. In the present study, transferring fish from FW to SW increased the levels of IGF-I mRNA in marine medaka, and the expression level was higher than that of the control group, which was maintained in FW. The significantly higher IGF-I level was maintained throughout the experiment (14 days). This result is in accordance with previously reported responses of IGF-I levels in other fish species that were abruptly introduced to SW, suggesting that marine medaka IGF-I is deeply involved in the adaptation to abrupt salinity changes.

In contrast to IGF-I, the increased level of marine medaka IGF-II mRNA was maintained for only a short period, then quickly returned a level similar to that of the control group. This implied that acute hyperosmotic stress induces a transient increase in the expression of the IGF-II gene. This result suggests that marine medaka IGF-II might be involved in the acute stress response or that it is a supplemental protein that aids in the IGF-I osmoregulatory function during the early phases of salinity change. However, given that little information is available regarding the

function of teleostean IGF-II in relation to osmoregulation, further research is needed to explain this result.

In conclusion, we cloned the cDNAs encoding marine medaka IGF-I and IGF-II and assessed the response of both genes to acute salinity change by measuring mRNA levels. This is the first report of the transient increase of IGF-II in response to acute SW transfer, and understanding the exact role of IGF-II in SW adaptation requires further examination.

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