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Cloning and localization of immediate early response 2 (*ier2*) gene in the brain of medaka

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ABSTRACT

Immediate early response (IER) 2 gene, a member of the IER family, is a gene of unknown function which is affected by external stimuli in the brain. In the present study, the full length sequence and localization of medaka (*Oryzias latipes*) *ier2* was investigated in the brain to understand the functions of Ier2 in the future studies. The full length sequence of medaka *ier2* was identified using a 3′-, 5′- rapid amplification of cDNA ends method, and distribution in the brain was identified using *in situ* hybridization. The identified full length *ier2* mRNA consisted of 939 nucleotides spanning along 1 exon. The deduced amino acid sequence consisted of 171 amino acid residues which contains a highly conserved sequence, nuclear localization signal. *ier2* mRNA was distributed in the telencephalon, midbrain and the hypothalamus. This highly conserved primary response gene Ier2 can be used to visualize and map functionally activated neuronal circuitry in the brain of medaka.

1. Introduction

The family of immediate early response (IER) gene is an important regulator of cellular response to stimuli such as stress and hormones. IER5 is upregulated by ionizing radiation, which affects radiosensitivity by disturbing cell cycle checkpoints (Ding et al., 2009). IER3 is stimulated by tumor necrosis factor α (TNFα) to suppress apoptosis and to protect cells by controlling sensitivity to TNFα (Ishimoto et al., 2011; Satsu et al., 2006).

IER2, a member of the IER family, also rapidly responds to stimuli (Lau and Nathans, 1987). *IER2* expression is modulated by signaling pathways, such as the mitogen-activated protein kinase (MAPK) signaling pathway and MAPK-independent Raf signaling pathway (Chung et al., 1998, 2000a). IER2 was initially identified from the human myeloid leukemia cell line (Shimizu et al., 1991) but is involved in various kinds of physiology, such as cancer (Neeb et al., 2012), apoptosis (Chung et al., 2000a; Schneider et al., 2004), pancreatitis (Kowalik et al., 2007), stress response against environmental pollutant (Fry et al., 2007) and angiogenesis (Wu et al., 2015). In the brain, *N*-methyl-D-aspartate (NMDA) administration induces *Ier2* expression in the mouse hippocampus through regulating the MAPK pathway (Chung et al., 2000b). On the other hand, *IER2* gene expression is decreased in the brain by stimuli such as neurotoxin and aging. Neurotoxin ciguatoxin treatment to mice decreases *Ier2* gene expression in the brain (Ryan et al., 2010). During aging, *Ier2* expression in the mouse hypothalamus is also decreased (Fu et al., 2006). Although the presence of IER2 has been shown in the brain, the functions of IER2 in the brain remain unknown. In addition, no functional domain of IER2 has been identified.

In the zebrafish (*Danio rerio*), Ier2 is partially conserved compared to the mammalian sequence, and is functionally equivalent to the human IER2 (Hong and Dawid, 2009). This suggests that the conserved region of Ier2 among different vertebrate species is functionally important. The medaka (*Oryzias latipes*) is an important comparative model because of its

Abbreviation: A, nucleus anterioris of diencephalon; Dm3, area medialis 3 of dorsalis telencephali; Dp, posterioris of dorsalis telencephali; DP, nucleus dorsalis posterioris; ep, Ependyme; Hc, hypothalamus periventricularis caudalis; HD, hapothalamus periventricularis dorsalis; HV, hapothalamus periventricularis ventralis; LZ, zona limitans diencephali; NDTL, nucleus diffuses of torus lateralis; NGp, nucleus glomerulosus posterioris; NVm, nucleus motorius nervus trigeminus; NVs, nucleus sensorius of nervus trigeminus; PMp, nucleus preopticus magnocellularis pars parvocellularis; PPa, nucleus preopticus parvocellularis anterioris; RS, nucleus reticularis superioris; SC, nucleus suprachiasmaticus; TA, nucleus tuberis anterioris; TP, nucleus tuberis posterioris; TS2, torus semicircularis (layer 2); Vd, dorsalis of the ventralis telencephalli; VM, nucleus ventromedialis; Vv, ventralis of the ventralis telencephalli.

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evolutional distance and its available genetical information (Wittbrodt et al., 2002). Medaka *ier2* has been predicted (http://asia.ensembl.org/index.html, accession no. ENSDART00000013843), it could be expressed in the brain and could have similar functions as in other vertebrates. In the present study, we aimed to identify the full length sequence of *ier2* in the medaka and localize it in the brain to understand its function in the brain in future studies.

2. Materials and methods

2.1. Animals

Five month old male medaka (*Oryzias latipes*) were maintained in fresh water at 27 ± 1.0 °C under a controlled natural photo regimen (14 h light/10 h dark cycle). The fish were fed Advanced Fish Diets (ZM, Winchester, UK) twice daily. All experimental procedures were performed in accordance with the guidelines of the Animal Ethics Committee of Monash University (approval no. SOBSB_MY_2010_64).

2.2. Cloning of full length ier2 cDNA

Full length *ier2* mRNA sequence was identified by a 3′- and 5′-rapid amplification of cDNA end (RACE). The primers were designed based on the genomic sequence of a predicted medaka *ier2* gene (accession no. ENSDART00000013843). Medaka were anaesthetized by immersing in a 0.01% solution of benzocaine (Sigma, St. Louis, MO, USA), killed by decapitation and the fresh brains were dissected. Total RNA was isolated from the medaka whole brain with TRIzol (Invitrogen). For the 5′-RACE, 1 μg of the total RNA was converted to cDNA in 20 μl volume with SuperScriptIII (Invitrogen), 2 pmol of gene specific primer 5′- GTGTGCTGTGCGCTGACGTCGTG-3′. Polycytosin was added at the 3′ end of the cDNA with terminal transferase (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. The polycytosine tailed cDNA was subjected to PCR reaction in 20 μl volume containing 1 × HotStarTaq Master Mix, 10 pmol each of 5′RACE abridged anchor primer (Invitrogen) and a gene specific primer 5′- AATTACGCGCCTGTGCGTCG-3′. The reaction program consisted of 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 4 min followed by 72 °C for 10 min. Furthermore, 1 μl of the PCR reaction mixture was subjected to nested PCR. Nested PCR was performed in a 20 μl reaction mixture containing 1 × HotStarTaq Master Mix, 10 pmol each of AUAP (Invitrogen) and a gene specific primer 5′- CTCCGCCTGCTGATCACACGC-3′. The reaction program consisted of 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 4 min followed by 72 °C for 10 min. The amplified DNA was subjected to direct sequencing using a BigDye Terminator v3.1Cycle Sequencing kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

For the 3′-RACE, 1 μg of total RNA was converted to cDNA with SuperScript III (Invitrogen) and 50 pmol of oligo d(T)-containing adapter primer (AP) in 20 μl volume according to the manufacturer's instruction. The converted cDNA was subjected to PCR in a 10 μl reaction containing 1 × HotStarTaq Master Mix, 10 pmol each of Abridged universal amplification primer (AUAP, Invitrogen), and a gene specific primer 5′- CCGAGGCCAAGAGGATCA-3′. The reaction program consisted of 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 3 min followed by 72 °C for 10 min. The primer sequence was designed based on the identified 5′-RACE sequence. The complete nucleotide sequence of *ier2* mRNA was confirmed by sequence analysis of each PCR product. Functional domain regions were further analysed by the simple modular architecture research tool (SMART) (Letunic et al., 2012; Schultz et al., 1998) and nuclear localization signals were predicted by cNLS Mapper (Kosugi et al., 2008, 2009a,b).

A phylogenetic analysis by an unweighted pair group method using arithmetic average and multiple sequence alignment were performed with Genetyx 8.0 (Genetyx Corporation, Tokyo, Japan) using amino acid sequences of Ier2 in *Homo sapiens* (NM_004907), *Macaca mulatta* (NM_001266014), *Rattus norvegicus* (NM_001009541), *Mus musculus* (NM_010499) and *Danio rerio* (NM_001142583).

2.3. Localization of ier2 mRNA in the adult medaka brain using in situ hybridization

Medaka were anaesthetized by immersion in 0.01% of benzocaine solution, killed by decapitation and the fish brains dissected. The brains were fixed in buffered 4% paraformaldehyde for 6 h, cryoprotected in 20% sucrose, and embedded in Tissue Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). Coronal sections (12 μm) were cut on a cryostat and thaw-mounted onto 3-aminopropylsilane-coated glass slides. The RNA probes were synthesized by in vitro transcription from the pGEM-T Easy vector (Promega, Madison, WI, USA), containing sequences amplified with the 5′RACE. Sense and antisense digoxigenin (DIG)-labeled riboprobes were synthesized using MAXIscript (Ambion, Austin, TX, USA) and DIG RNA labeling mix (Roche Diagnostics). DIG-*in situ* hybridization was performed as indicated previously (Kitahashi et al., 2009; Parhar et al., 2004) with minor modifications. Briefly, sections were permeabilized with 0.2 M HCl for 10 min followed by proteinase K (1 μg/ml) treatment for 15 min, and hybridized with DIG-labeled riboprobes (0.5 μg/ml) at 55 °C overnight in a humidified chamber. After hybridization, sections were washed twice in 2x SSC (1x SSC: 0.15 M NaCl, 30 mM sodium citrate) at room temperature for 15 min and in 1x SSC and 0.1x SSC at 55 °C for 1 h sequentially. The hybridization signals were detected using anti-DIG antibody conjugated with alkaline phosphatase (Roche Diagnostics; diluted 1:500) and visualized with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Roche Diagnostics). Brain regions were referred to the Brain atlas of the medaka fish (Anken and Bourrat, 1998).

3. Results and discussion

3.1. Full length ier2 mRNA sequence

The identified full length *ier2* mRNA (Genbank accession no. KF431969) consisted of 939 nucleotides spanning 1 exon when compared to the medaka genome. The coding region starts at the 116th base of exon 1 and extends to the 631st base of the exon 1. The deduced amino acid sequence consist of 171 amino acid residues (Fig. 1A). The phylogenetic analysis of the deduced amino acid sequence against the Ier2 amino acid sequences in other vertebrates exhibited that the deduced aa sequence was close to zebrafish, but distant from mammals (Fig. 1B). The sequence for nuclear localization signals was seen at 109th–131st amino acid in the medaka, and conserved in zebrafish, human, rhesus monkey, and rodents (Fig. 1A).

The presence of nuclear localization signal was seen in similar regions of the Ier2 among vertebrates. The zebrafish Ier2 is localized in the nucleus (Hong and Dawid, 2009), which has similar functions to the human Ier2 (Hong and Dawid, 2009). Therefore, Ier2 might be localized in the nucleus and possess conserved function due to its similarity among vertebrates. The deduced medaka Ier2 contained several potential phosphorylation sites (data not shown). Similarly, IER3 is phosphorylated by extracellular