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Hypoxia induces the PDZ domain-containing syntenin in the marine teleost *Paralichthys olivaceus*

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ABSTRACT

Syntenin is a scaffolding PDZ domain-containing protein with diverse biological activities, including organization of protein complexes in the plasma membrane, regulation of B-cell development, intracellular trafficking, synaptic transmission, and cancer metastasis. In the present study, we isolated and characterized the cDNA of the olive flounder *Paralichthys olivaceus* syntenin, designated PoSyntenin. The full-length CDS of PoSyntenin with 5′- and 3′-UTR sequences is 2618 bp long and consists of a 909 bp open reading frame preceded by a 161 bp 5′-UTR and followed by a 1551 bp 3′-UTR. The PoSyntenin cDNA encodes a polypeptide of 302 amino acids containing two PDZ domains, which shares 61–80% homology with those of other species, including humans. Expression of the PoSyntenin mRNA was detectable from 1 day post-hatching and constitutively in the brain, spleen, intestine, stomach, eye, liver, kidney, and gill of normal conditioned fish. Expression of the PoSyntenin mRNA was upregulated in the eye, liver, kidney, spleen, brain, gill, and intestine of flounder under hypoxia and was increased by treatment with the hypoxia-mimic $CoCl_2$ (a HIF-1 inducer) in HINAE cells. Taken together, these results suggest that PoSyntenin is a hypoxia target gene that has a potential role in the hypoxia response mechanism of fish.

1. Introduction

Syntenin, also called *melanoma differentiation associated gene-9* (*mda-9*), is a multifunctional adaptor protein. Syntenin has two copies of the PDZ (*p*ostsynaptic density protein, *d*isc large and *z*onula occludens) domain (Hung and Sheng, 2002), which is a prototypical protein interaction module that generally mediates the assembly of dynamic multi-protein complexes at the membrane by binding to the C-termini of target proteins and membrane lipids (Meerschaert et al., 2007; Zimmermann et al., 2002). Through the interaction with various binding partners including syndecan, eIF5A, proTGFα, lymphocyte receptor CD63, TRAF6, IL5Rα, neurexin, neurofaschin, Frizzled 3,7,8, and schwannomin (Fernandez-Larrea et al., 1999; Grootjans et al., 2000; Geijsen et al., 2001; Jannatipour et al., 2001; Koroll et al., 2001; Li et al., 2004; Grembecka et al., 2006; Latysheva et al., 2006; Chen et al., 2008), syntenin participates in the regulation of diverse biological functions, including transmembrane-receptor trafficking, synaptic transmission, activation of the transcription factor SOX4, regulation of Wnt signaling, syndecan recycling through endosomal compartments, cytoskeleton-membrane organization, melanoma metastasis and invasion, apoptosis, and inflammation in the hypoxic developing brain (Koo et al., 2002; Li et al., 2004; Beekman and Coffer, 2008; Kaur et al., 2009).

Hypoxia occurs in marine coastal areas, and is becoming more common with the increasing occurrence of eutrophication and pollution (Karim et al., 2002) Hypoxia often leads to population decline and changes in community structure by eliminating oxygen-sensitive species (Rudolf and Wu., 2002). When hypoxia occurs, the uptake of oxygen from water becomes more difficult for fish, affecting their growth and survival, with various effects on physiological functions such as metabolism and cardiovascular regulation (Duthie, 1982; Van Den Thillart et al., 1994; Lefrançois and Claireaux, 2003; Fritsche and Nilsson, 1989; Chabot and Dutil, 1999; Thetmeyer et al., 1999; Eby et al., 2005). Under hypoxia, fish change their physiological responses to take up more oxygen and adapt. These responses include erythropoiesis, hemoglobin synthesis, angiogenesis, changes in gill surface area, glycolysis, glucose transport, and growth suppression (Nikinmaa and Rees, 2005). Investigation of responses to hypoxia in mammals has revealed a hypoxia-inducible factor (HIF) system. HIF functions as a transcription factor, and binds to hypoxia response element

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(HRE) motifs in the promoter region of HIF-mediated genes, including erythropoietin (EPO), vascular endothelial growth factor (VEGF), nitric oxide synthase (NOS2), matrix metaloproteinases (MMPs), glucose transporter (GLU) 1,3, insulin-like growth factor-2 (IGF2), and bcl-2/adenovirus EIB 19 kDa-interacting protein 3 (BNip3) (Zelzer et al., 1998; Wanner et al., 2000; Matrone et al., 2004; Wenger et al., 2005). GLUT1,3 of the Atlantic cod (*Gadus morhua*), IGFBP1 and Leptin of the zebrafish (*Danio rerio*), Leptin receptor (omLepR_L) of the marine medaka (*Oryzias melastigma*), and lactate dehydrogenase-B of the killifish (*Fundulus heteroclitus*) have been reported to be hypoxic-responsive genes in fish (Hall et al., 2005; Kajimura et al., 2006; Wong et al., 2007; Rees et al., 2009; Chu et al., 2010). Although these genes are potentially related to hypoxia response and several genes involved in oxidative stress responses have been reported, less is known about the molecular mechanism of nonmammalian vertebrates to hypoxic exposure.

In the present study, we isolated and characterized the cDNA sequence of the olive flounder *Paralichthys olivaceus* syntenin. Expressional analysis of flounder syntenin mRNA in fish under hypoxia revealed a possible role for syntenin in the hypoxia response mechanism of fish.

2. Materials and methods

2.1. Cloning of flounder P. olivaceus syntenin cDNA

An expressed sequence tag (EST) clone, 1before-2-3a_B07, showed a high degree of sequence identity with syntenins of salmon and zebrafish. Based on the EST sequences, 5′ Rapid Amplification cDNA Ends (RACE) was performed using a SMART™ RACE cDNA amplification kit (Clontech) following the manufacturer's instructions. Briefly, total RNA was isolated from olive flounder *P. olivaceus* gills using TRIzol® reagent (GibcoBRL). First-strand cDNA was synthesized from mRNA isolated using the protocol described above. RACE was performed to obtain a full-length olive flounder syntenin cDNA sequence. Based on the partial sequence, an internal antisense primer was designed (syntenin SP, 5′-ATT CCA TGC CCA AGA ATG A-3′) and used in combination with the universal primer supplied with the kit to amplify the 5′ end of the PoSyntenin transcript. PCR was performed for 5 min at 94 °C, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension for 2 min at 72 °C. A final extension step was performed at 72 °C for 7 min. The amplified products were inserted into the pGEM-T Easy vector (Promega). DNA sequencing was performed with the universal T7 and SP6 primers (Promega) and the internal primers using an autosequencer (ABI3730xl; Applied Biosystems). The full-length PoSyntenin cDNA sequence was obtained by combining the DNA sequences of the partial sequences and the 5′-RACE PCR products.

2.2. Sequence analysis

The nucleotide and deduced amino acid sequences were annotated using the BioEdit and BLAST programs in the non-redundant database of the National Center for Biotechnology Information (NCBI BLAST, http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990).

2.3. Alignment and phylogenetic tree analyses

The relevant sequences were retrieved from GenBank for multiple sequence alignments using CLUSTAL W version 1.8 (Thompson et al., 1994). Sequence homology was calculated using GENETYX version 8.0 (SDC Software Development). A phylogenetic tree was created based on the amino acid distances between the aligned sequences using the neighbor-joining method with 1000 bootstrap replications using the MEGA software (version 4.1).

2.4. Fish maintenance and hypoxia treatment

Olive flounders were maintained in the Genetics and Breeding Research Center of the National Fisheries Research and Development Institute (NFRDI) (Geoje, Republic of Korea). Fish were fed a commercial fish diet (Suhyup Feed; 52% crude protein, 11% crude fat) three times per day. The temperature in the rearing tanks was maintained at 20 ± 1 °C. Hypoxia exposure experiments were conducted using 300 fish of approximately 11–15 cm in body length at 20 ± 1 °C. Oxygen levels were monitored using a dissolved oxygen (DO) meter; the levels were 9.54, 7.15, 6.53, 5.66, 4.50, 3.41, and 1.65 ppm at 0, 15, 30, 60, 90, 120, and 150 min, respectively, after the bubbling oxygen was removed from the tank. When the conditions became hypoxic at 1.65 ppm DO, fish started to die. Then, the tissues were removed, immediately frozen in liquid nitrogen, and stored at − 80 °C until RNA extraction.

2.5. Quantitative RT-PCR

Total RNA was prepared from cells or tissues using TRIzol® reagent (Gibco BRL) following the manufacturer's instructions. One microgram of total RNA was treated with DNase I (New England BioLabs), and cDNA was synthesized using the Advantage RT-for-PCR kit (BD Biosciences, CA). The total RNA concentration was determined, and 1 μg of total RNA was used for reverse transcription. The first-strand cDNA was synthesized using an Advantage® RT-for-PCR kit (BD Sciences). Quantitative real-time PCR was performed using LightCycler® FastStart DNA Master SYBR Green I (Roche) and the following forward and reverse primers: syntenin, forward 5′-AGA ACT GTG CTG GCT GGA GT-3′ and reverse 5′-CCA TCC TTG ACC AGT GAG GT-3′; β-actin, forward 5′-CAG ACT ACC TGA TGA AGA TCC-3′ and reverse 5′-ATT CCA TGC CCA AGA ATG A-3′. Following an initial 10-min *Taq* activation step at 95 °C, LightCycler PCR was performed for 40 cycles under the following cycling conditions: 95 °C for 10 s, 55 °C for 5 s, 72 °C for 15 s, and fluorescent reading. PCR reactions were performed at least in triplicate.

2.6. Cell culture and hypoxia-mimic condition

HINAE flounder embryonic cells, a gift from Takashi Aoki, were maintained in Leibovitz L-15 medium (L-15; GIBCO BRL) with 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL) and 1% (v/v) penicillin–streptomycin (PS; GIBCO BRL) at 20 °C. To mimic hypoxia, cells were incubated in the presence of 200 μM $CoCl_2$ for 24 h (Sigma-Aldrich) (Fang et al., 2008; Choi et al., 2009). When cells were subjected to $CoCl_2$, all of cell medium was replaced with serum-free medium.

3. Results

3.1. Cloning of the P. olivaceus syntenin (PoSyntenin) cDNA

An EST clone of 2074 bp from an olive flounder *P. olivaceus* cDNA library contains the partial cDNA sequence and 3′ untranslated region (UTR) of a syntenin homolog. The full-length CDS along with the 5′-UTR sequence of the PoSyntenin gene was established through RACE using the obtained partial sequence. The PoSyntenin cDNA is 2618 bp long and consists of a 909 bp open reading frame preceded by 158 bp of 5′ UTR and followed by 1551 bp of 3′ UTR (GenBank accession no. GU808360) (Fig. 1).

3.2. Analysis of the nucleotide and deduced amino acid sequences of PoSyntenin

UTRScan program analysis (http://www.ba.itb.cnr.it) revealed that the flounder syntenin mRNA contains the 3′-UTR CU-rich element.