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Molecular identification of insulin-related peptide receptor and its potential role in regulating development in *Pinctada fucata*☆

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ABSTRACT

The insulin-like family is not only an important regulatory factor for animal growth, development, and metabolism, but also a mediator for initiating growth activity of growth hormone. It plays an important role in transferring transmembrane information and regulating cell function by binding to tyrosine kinase receptors (insulin receptors). To better understand the role of insulin-related peptide receptor (*Pfirr*) on the developmental regulation in *Pinctada fucata*, 5.326 kb encoding cDNAs for *Pfirr* have been cloned and functionally characterized. *Pfirr* displays significant homologies to *Crassostrea gigas*, and exhibits all the typical features of insulin receptors and tyrosine kinase domain structure, both of which are typical for the protein family sharing high similarity to other orthologs. Real-time PCR analyses show that *Pfirr* widely expresses in tissues and developmental stages of *P. fucata*. Expression of *Pfirr* mRNAs at different developmental stages (polar body stage, the trocophore stage and D-shaped larva stage) following treatment with agonist IGF-I(1, 2, 4 and 8 μM/L) and antagonist PQ401 (5, 15, 25, 50 and 100 μM/L) indicated that *Pfirr* may be involved in regulating the development of embryos in *P. fucata*. These results clearly demonstrate *Pfirr* is involved in regulating developmental process in *P. fucata*.

1. Introduction

The insulin-like family, one of the most widely distributed peptide families even among invertebrate species (Renteria et al., 2008), is an important regulatory factor for animal growth (Schlueter et al., 2007), development (Riehle and Brown, 2002) and metabolism (Lardans et al., 2001).

The insulin-like family includes insulin and the insulin-like growth factors (IGFs), which play distinct physiological roles in animals mediated by specifically binding to the Insulin Receptor (IR) or the type-I of Insulin-like Growth Factor Receptor (IGF-IR) (Nakae et al., 2001). The IR, the IGF-IR, and the Insulin Receptor-related Receptor (IRR) (Ebina et al., 1985; Shier and Watt, 1989; Ullrich et al., 1986) form the subclass II of the Receptor Tyrosine Kinase (RTK) superfamily (Hubbard and Till, 2000), sharing a common character of covalently-linked homodimers ($\alpha_2\beta_2$) and several structural domains (Heldin and Ostman, 1996). Vertebrates possess more than one homologous receptor of the IR family, whereas invertebrates possess only a single one—the IRR (Leevers, 2001). This single IRR regulates both growth and metabolism in invertebrates (Kimura et al., 1997; Tatar et al., 2001; Wheeler et al., 2006), whereas two different and specialized receptors combined matching ligands undertake different physiological roles in vertebrates (Kimura et al., 1997; Roovers et al., 1995).

In molluscs, the occurrence of an insulin system was first investigated in gastropod species: in the pond snail, *Lymnaea stagnalis*, seven MIPs (mollusc insulin-like peptide) expressed in the nervous ganglia have been identified and characterized (Li et al., 1992; Smit et al., 1988, 1998). In another model gastropod, the sea hare *Aplysia californica*, insulin is produced restrictedly in the central region of the cerebral ganglia, appeared to be unique, and characterized with an extended A chain compared with other invertebrate and vertebrate insulin (Floyd et al., 1999). In *Anodonta cygnea*, six ligands were identified, purified and characterized using a radioreceptor test system (Shipilov et al., 2005). Four genes coding insulin-related peptides were also identified in the complete sequencing of the *Lottia gigantea* genome (Veenstra, 2010). In bivalve species, insulin-related peptide cDNA was characterized in the Pacific oyster *Crassostrea gigas*, and was found to be expressed as three transcripts with differing lengths of 3′-untranslated region (3′-UTR) in visceral ganglia (Hamano et al., 2005), but the exact number of the genes remains unknown.

The IRR have been identified only in a few molluscs (Gricourt et al., 2006; Lardans et al., 2001; Roovers et al., 1995), with the common character of a typical tyrosine kinase (TK) domain, having high evolutionary conservation with the vertebrates, such as human (Lu et al., 2008), *Gallus gallus* (Basu et al., 2012), *Xenopus laevis* (Klein et al.,

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2002) and *Danio rerio* (Jurczyk et al., 2011). The possible role of IRR in the activating and proliferating processes observed in an embryonic cell line of the snail *Biomphalaria glabrata* has been implicated (Lardans et al., 2001). The affinity has been researched among the insulin-related peptide receptor of the mussel *Anodonta cygnea*, recombinant piscine IGF-I, and porcine insulin. The results indicate that the receptor has similar binding properties with the vertebrates (Leibush and Chistyakova, 2003). The insulin-like receptor (CIR) of Pacific oyster (*C. gigas*) shares 66.5% homology with the tyrosine kinase domain of turbot (*Psetta maxima*), and is expressed in the mantle edge and gonads, which indicates its participation in the formation of its shell and reproduction (Gricourt et al., 2003).

Numerous insulin signaling pathways from invertebrate to vertebrate species have been found to be relatively well conserved, including ligand and its receptors which were mentioned above, to final effectors (Broughton and Partridge, 2009; Claeys et al., 2002; Wu and Brown, 2006). Studies in the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* have revealed that components of the insulin signaling pathway have been highly conserved during evolution (Garofalo, 2002). On the basis of sequence conservation, three potential elements of the oyster insulin pathway were found in *C. gigas*: CgRas, CgPten and CgP70S6K (Jouaux et al., 2012). And in other molluscan species like *Mytilus trossolus* (Ciocan et al., 2006) and *Aplysia californica* (Swanson et al., 1986), Ras has been already identified. This evidence suggests that the conservation of insulin signaling system may extend throughout bivalve species.

Studies in invertebrate species have shown the potential involvement of insulin-related peptide and its receptor in many activities such as cell proliferation and developmental processes. Molluscan insulin-related neuropeptide promoted neurite outgrowth in dissociated neuronal cell cultures of *Lymnaea stagnalis* (Kits et al., 1990). Human insulin could stimulate proliferation and differentiation of *Drosophila* embryonic neural cells (Pimentel et al., 1996). In the mollusc *Haliotis tuberculata*, porcine insulin promoted the growth of primary cultures of hemocytes (Lebel et al., 1996). The possible implication of insulin-like receptor (BgIR) in the activating and proliferating processes was observed in *B. glabrata* embryonic cells during their coculture with *Schistosoma mansoni* larvae (Lardans et al., 2001). The expression of maternal CIR during the embryonic and early larval development also suggests that insulin-like peptide is involved in organogenesis (Gricourt et al., 2006).

P. fucata, a marine bivalve mollusc, cultivated in China and worldwide, has a very high economic value in pearl production. Its development process has gone through several ecologically important transitions from planktonic to the creeping stage and to the last adult, including the synergy of the soft part and shell growth. At present, there are no reports on the existence and the role of insulin-related peptide receptor in *P. fucata* (*Pfirr*). To evaluate the functional role of *Pfirr* in development regulation, firstly, the cDNA sequences encoding *Pfirr* were cloned and characterized; secondly, the tissue distribution and the expression profiles of *Pfirr* were examined at developmental stages; thirdly, mRNAs expression of *Pfirr* at different developmental stages were measured following treatment with antagonist PQ401 (the inhibitor of IGF-IR, a diaryl urea compound) and agonist IGF-I.

2. Materials and methods

2.1. Animals and chemicals

The *P. fucata* (body weight 7.21 ± 0.57 g, shell height 38.67 ± 1.38 mm) were obtained from Marine Biology Research Station at Daya Bay of Chinese Academy of Sciences (Shenzhen city, Guangdong, P.R. China) in November, 2011. The oysters were cultivated in floating net cages in the sea under natural conditions. Tissues were dissected, and frozen immediately in liquid nitrogen, then stored at −80 °C until RNA extraction. All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Chinese Academy of Sciences.

Two-year-old breeders came from one cultured population at Daya Bay in China. Gametes were obtained by dissecting the gonads and were passed through a 100 μm screen to remove the large tissue debris. The eggs were fertilized with sperm in filtered seawater containing 0.006% (V/V) of ammonia water at temperatures 24 ~ 25 °C. About two hours after fertilization, the fertilized eggs developed to the 2–4 cell stage.

The inhibitor of IGF-IR, a diaryl urea compound (PQ401) was purchased from Enzo Life Sciences (Farmingdale, USA). Recombinant human IGF-I was purchased from Shanghai Prime Gene Bio-Tech. Co. Ltd. (Shanghai City, China). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO).

2.2. Cloning and sequence analysis of Pfirr

Total RNA from *P. fucata* tissues were prepared using Trizol reagent (Invitrogen). One microgram of isolated RNA was used to synthesize first-strand cDNA using the ReverTra Ace-α First-strand cDNA Synthesis Kit (TOYOBO, Japan).

A fragment cDNA of *Pfirr* was found in the transcriptome of *P. fucata*. To amplify this cDNA fragments, specific PCR primers were designed by using Primer Premier 5.00 (Palo Alto, CA) as presented in Table 1. Full-length cDNA sequences were obtained by the 5′- and 3′-rapid amplification of cDNA ends (RACE) using BD SMART RACE cDNA Amplification Kit (Clontech, USA) (Table 1).

For all PCR reactions in the present study, amplifications were performed as follows: denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 15 s, 52–58 °C for 15 s and 72 °C for 1–1.5 min. The reaction was ended by a further extension of 10 min at 72 °C. The amplification products were purified using the E.Z.N.A. Gel Extraction Kit (Omega BioTek, USA) and subcloned into the pTZ57R/T vector (Fermentas, USA). Three different individual positive clones were sequenced on an ABI 3700 sequencer (Applied Biosystems).

Predictions of export-directing signal sequences, transmembrane regions and domains involved in signal transduction processes were done as described (Schultz et al., 1998) using the simple modular architecture research tool (SMART) available under http://smart.embl-heidelberg.de. Predictions of serine/threonine phosphorylation sites were performed as described (Blom et al., 1999) using the NetPhos 2.0 prediction server available under http://www.cbs.dtu.dk/services/NetPhos. Multiple sequence alignments of amino acids were performed with ClustalX (1.81). Protein phylogenetic analysis was conducted with MEGA4 using the neighbor-joining method.

2.3. Tissue distribution of Pfirr in P. fucata

The tissue expression pattern of *Pfirr* mRNA in the various tissues was analyzed by real-time PCR. Total RNA was isolated from 6 tissues, including mantle, digestive gland, adductor muscle, heart, ovary during the previtellogenic arrest stage and testis at multiplicative stage (n = 3).

Quantitative real-time PCR was performed on a Roche LightCycler 480 real time PCR system using SYBR® *Premix Ex Taq*™ (TAKARA, Japan) according to the manufacturer's protocol. Real-time PCR conditions were as follows: denaturation at 94 °C for 1 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 15 s and 72 °C for 60 s. *Pfirr* transcript levels were normalized against *gapdh* transcripts levels. *Gadph* was expressed equally in all the tested tissues. Real-time PCR primers for *Pfirr* and *gapdh* were present in Table 1. The efficiency of primers for *Pfirr* was 1.963 and that for *gapdh* 1.901.

2.4. Expression of Pfirr mRNA at the developmental stages of P. fucata

For analyzing the developmental expression patterns of *Pfirr*, six developmental stages: fertilized eggs, embryos at polar body stage,