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The comprehensive expression analysis of the G protein-coupled receptor from *Penaeus monodon* indicating it participates in innate immunity and anti-ammonia nitrogen stress

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ABSTRACT

The G protein-coupled receptors (GPCRs) composed a superfamily that played an important role in physiological processes of crustaceans, with multiple functions such as growth and development, acting as a defense against stimulations from external factors. In this paper, one kind of GPCRs were identified from *Penaeus monodon*, called *PmGPCR*, included an open reading frame (ORF) of 1113 bp. Bioinformatic analysis showed that PmGPCR protein had the typical structure of seven transmembrane domains (7TM), especially the special Asp-Arg-Try motif (DRY motif) between the third transmembrane structures (TM3) and the second intracellular loops (IL-2) which can prove that PmGPCR belongs to the rhodopsin-like family. The analyses of phylogenetic tree indicated that the amino acid sequence of *PmGPCR* should be merged into *Procambarus clarkiic* with high identity (98%). Quantitative real-time PCR (q RT-PCR) revealed that *PmGPCR* mRNA was highly expressed in hepatopancreas, abdominal ganglia and lymph, in which it was significantly higher than that of other tissues ($P < 0.05$). In addition, the expression of *PmGPCR* was analyzed during three days post-stimulation with the gram-positive/negative bacteria, the mRNA expression level increased after challenged with gram - positive bacteria in hepatopancreas, lymph and intestines. During the development stages, *PmGPCR* showed significantly higher expression in nauplius, zoea III, mysis III and post larvae stages than that in other development stages. Meanwhile, the highest transcripts expression of *PmGPCR* in abdominal ganglia, hepatopancreas, lymph and intestines respectively appeared at D0, D1, D2 and D3/D4 stages of molting. High or low concentration of ammonia nitrogen up-regulated the expression level of *PmGPCR* at the initial stage in hepatopancreas and gill, and then down-regulated at 48 h. These results indicated *PmGPCR* may mediate the pathways that involved in growth and development process, survival in the adversity, in addition, provided the useful data to research GPCR-mediated physiological and biological process and explain the mechanisms to defense pathogens and anti-stress in shrimp.

1. Introduction

The G protein coupled receptors (GPCR*s*), which also known as the seven-transmembrane signal transducing proteins receptors, are large superfamily of cell surface receptors. GPCRs was founded widespread in cell membrane with critical role in range of physiological processes [1], for example, cellular differentiation, growth and development, inflammatory and immune responses [2], neurotransmission [3,4] and endocytosis exocytosis, also as well as cellular metabolism [5]. The structure of GPCRs was a highly-conserved architecture mainly consisted of seven transmembrane spanning helices that linked by alternate extracellular and intracellular loops. The extracellular portion usually was glycosylated and consisted of a highly variable N-terminus and three extracellular loops (EL-1 to EL-3). Meanwhile, the phosphorylated intracellular portion was consisted of a variable C-terminus and three intracellular loops (IL-1 to IL-3) [6]. Seven transmembrane alpha helix structures (TM 1 ~TM 7) were located between the above, which can be divided into these regions: N terminus-TM1-IL1-TM2-EL1-TM3-IL2-TM4-EL2-TM5-IL3-TM6-EL3-TM7-C terminus [7]. These parts, as marks of GPCRs superfamily, were the basis of the various function

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to be carried out. Based on protein structure and ligand binding properties, GPCRs were classified into four subfamilies: rhodopsin-like, secretin-like, metabotropic glutamic, and atypical [8]. Rhodopsin-like GPCR family was the largest family among four GPCR families and acted as a crucial role in regulating many physiological processes [9], it had a characteristic "DRY" motif (Asp-Arg-Tyr) at the cytosolic end of the third transmembrane domain (TM3). Arg was thought to be critical of forming intramolecular interactions and stimulating receptors be inactivated or activated [10]. Once the GPCRs were activated, a string of extracellular domains transduced extracellular chemical information into the cell [11], combined with downstream signaling molecules, like G protein, β-arrestin protein or second messenger, to simulate the regulation of many physiological activities, like being involved in cAMP pathway or other phosphatidylinositol signal pathways [12].

Just like be mentioned above, the research on the functions of GPCRs was focused on neurotransmission [3,4], survival and stress resistance [13], germ cell migration [14] and developmental regulation [15] in vertebrate. GPCRs have physiological functions of increasing the level of cAMP and regulating the concentration of ion. For example, G protein-coupled estrogen receptor GPER (formly known as GPR30) was participating in initiating multiple cell effects accused by estrogen [16], increasing the concentration of calcium ions, accelerating the rate of phosphorylation [17]. Because of the closely relationships between life-span and stress resistance, GPCRs should also have the ability to indirectly resist various kinds of stress by regulating an associated G protein and its downstream pathway. GPCRs coupled to a stimulatory G protein are also expressed on the plasma membranes of croaker oocytes, participated in the regulation of ovarian development [18]. Recent study showed that the expression level of GPER in multiple immune cells, including B and T cells, macrophages, and neutrophils, suggested that GPER could mediated some estrogenic effects in the immune system [16], it should had a close relationship with the central immune pathway [19]. However, it had been identified that GPCRs were thought to be a transducer in the function of inhibition synthesis, the regulation and mechanism also had got an explanation in part, such as being involved in NF-κB signal pathway by phosphorylating G proteins in *Drosophila* [20], but the function of GPCRs in crustacean was not known enough.

The black tiger shrimp, *Penaeus monodon*, is one of the important species of shrimp in the southern of China, however there are many problems caused by pathogens-infection disease and environmental stresses which have threatened the shrimp culture industry, causing the serious economic losses [21,22]. Research in its mechanism on innate immunity to defense pathogen and anti-environment stress is becoming an urgent task that benefit to improve the immunity of shrimp in the future. In this study, we identified a G protein coupled receptor gene (*PmGPCR*) from the hepatopancreas transcriptome of *P. monodon*, and systematically analyzed the expression profile during the development stage, under the immune challenge and the ammonia nitrogen stress. Our results will provide a foundation to further research the function of G protein coupled receptor and its family in tiger shrimp.

2. Materials and methods

2.1. Amplification the full-length cDNA of PmGPCR

A 1331 bp EST of a *GPCR* analogue was identified by a transcriptome of black tiger shrimp hepatopancreas tissues (data not shown). The ORF sequences were verified by blast comparison and common PCR, the special primer could be found in Table 1. The 3′ end of mRNA was obtained by rapid amplification of cDNA ends (RACE) methods. For 3′ RACE-PCR, PCR reaction was performed initially with GPCR-rF1 (Table 1) and adaptor primer, followed by semi-nested PCR with GPCR-rF2 (Table 1) and adaptor primer. The PCR profile was as follows: 94 °C, 5 min, one cycle; 94 °C, 45 s; 61 °C, 30 s; 72 s C, 45 s; 35 cycles; 72 °C, 10 min, one cycle. The PCR products were gel-purified, sequenced, and the resulted sequences were subjected to analyze.

Table 1
PCR primers used in the experiment.

Primer	Primer sequence(5′-3′)	Purpose
GPCR-rF1	CAACCCCAGAAGCATCAGCAGAACC	3′RACE
GPCR-rR1	CTGGTGGCTATCGTCGTGAGTTTCC	
GPCR-F	CGAATAATCCCAGAGCCT	ORF Validation
GPCR-R	TGTTTTGAATGTCCCTTTT	
GPCR- GSP1	CAACCAGATGAGGAGCAAG	q RT-PCR
GPCR- GSP2	CAGAGTAGTCGCCAGGAA	
EF-1α-F	AAGCCAGGTATGGTTGTCAACTTT	Reference gene [30]
EF-1α-R	CGTGGTGCATCTCCACAGACT	

2.2. Bioinformatics analysis

Sequence was analyzed by ORF-Finder and BLAST program at the National Center for Biotechnology Information server (NCBI) (http://www.ncbi.nlm.nih.gov/). Primer 5.0 was used to predict the amino acid sequence; the deduced amino acid sequence and the protein physicochemical properties were predicted by ProtParam software (http://web.expasy.org/protparam/); the analysis of protein domains was doing by using SMART4.0 online program (http://smart.embl-heidelberg.de/); using CBS Prediction Servers online programs—SingalP4.1, to predict signal peptide in protein sequences(https://psort.hgc.jp/); the NetPhos2.0 program can predict protein phosphorylation sites(http://www.cbs.dtu.dk/services/NetPhos/); using GOR to predict the secondary structure(https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html); using SWISS-MODEL online analysis software to predict the tertiary structure of the protein and viewed by PyMOL software(https://swissmodel.expasy.org/); multiple sequence alignment and phylogenetic tree were constructed by Clustal X, BioEdit and MEGA 6.0 software.

2.3. Experimental animals

Healthy black tiger shrimps (*P. monodon*), with average weight of 18–21 g, were obtained from the Experimental Base of South China Sea Fisheries Research Institute in Shenzhen city (Guangdong Province, China). They were temporarily kept in filtered aerated seawater at 25 ± 1 °C for 7 days that were fed with normal commercial bait during acclimatization until 24 hs before treatment.

For tissue expression profile analysis, fourteen kinds of tissues, including eyestalk, gill, brain, heart, hepatopancreas, abdominal ganglia, thoracic ganglia, lymph, intestines, stomach, ovary, testis, epidermis and muscle, was collected and stored in RNAlater® RNA Stabilization Solution (Invitrogen, USA).

2.4. Collection of molting stages and developmental stages of samples

The shrimps were collected on the basis of molting stages which were judged by the performance of setogenesis and epidermal in the inner uropod near the telson tip [23]. Five kinds of tissues, including hepatopancreas, thoracic ganglia, abdominal ganglia, lymph, intestines and gills, were collected from shrimps at the different process, such as postmolt (stage A and stage B), intermolt (stage C), premolt (stage D0 ~ D3/D4) and ecdysis (stage E) [23–25].

The different developmental stages were also collected from breeding process which included the stage of oosperm, nauplius, zoea I, zoea II, zoea III, mysis I, mysis II, mysis III and post larvae [26,27].

2.5. In vivo challenge of P. monodon with bacteria

Bacteria were prepared according to the following procedure: *Vibrio harveyi* and *Streptococcus agalactiae* were cultured in marine agar 2216E