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Knockdown of a novel G-protein pathway suppressor 2 (*GPS2*) leads to shrimp mortality by exuvial entrapment during ecdysis☆

Pakkakul Sangsuriya[a,b,c], Kornsunee Phiwsaiya[b,c], Benjamart Pratoomthai[d], Thanawat Sriphaijit[b,e], Piti Amparyup[a,c], Boonsirm Withyachumnarnkul[b,f,g], Saengchan Senapin[b,c,*]

[a] *Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand*
[b] *Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Mahidol University, Rama VI Rd., Bangkok 10400, Thailand*
[c] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathum Thani 12120, Thailand*
[d] *Anatomy Unit, Department of Biomedical Science, Faculty of Science, Rangsit University, Pathum Thani 12120, Thailand*
[e] *Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand*
[f] *Shrimp Genetic Improvement Center, Surat Thani 84100, Thailand*
[g] *Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

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ABSTRACT

A novel G-protein pathway suppressor 2 (*GPS2*) has been identified from hemocytes of the whiteleg shrimp *Penaeus vannamei* (*Pv*) and appears to play a role in ecdysis. The full-length of *PvGPS2* cDNA consisted of a 1230-bp open reading frame, encoding 409 deduced amino acids with significant sequence homology to *GPS2* sequences of crustaceans and insects. RT-PCR revealed that *PvGPS2* was expressed in all *P. vannamei* tissues examined, but that expression was molt stage specific in eyestalk tissue. Relative expression was higher in the period before molting (i.e., intermolt and pre-molt stages) than in the post-molt stage. When double-stranded RNA (dsRNA)-mediated RNA interference was employed to inhibit *PvGPS2* formation in shrimp, it led to significant mortality due to unsuccessful separation of new cuticle from old cuticle (exuvial entrapment) during ecdysis.

1. Introduction

Ecdysis or molting is essential for growth and development in ecdysozoan animals, including nematodes and arthropods. During molt cycles, new cuticle is formed under the old and then the old exoskeleton is shed (moltcast) and replaced by the new one. This occurs several to many times during the life cycle in order to achieve metamorphosis and growth. The molt process depends on hormonal regulation involving changes in gene expression in signaling cascades (see review in [1]). It is also influenced by environmental conditions, physiological states and developmental stages [2,3]. In shrimp, the majority of research on ecdysis has focused on the regulatory hormones molt-inhibiting hormone (MIH) and molt-stimulating ecdysteroid hormone secreted by the X- and Y-organs, respectively (see review in [1]). Besides hormone-related genes, there is increasing evidence for involvement of other genes such as cathepsin-L [4], hemocyanin [5], and selenophosphate synthetase [6].

G-protein pathway suppressor 2 (*GPS2*) is a small 37-kDa protein that is ubiquitously expressed in different tissues and cell types [7,8]. It was initially identified as a G-protein suppressor that interfered with JNK1 activation in the RAS/MAPK pathway [7,9]. Several reports have suggested roles of *GPS2* in transcriptional regulation as both co-activator and co-repressor via its ability to interact with various cellular and viral regulatory proteins [10–13]. *GPS2* has also been found in the complex formed between nuclear receptor co-repressor and histone deacetylase (N-CoR-HDAC3

☆ Footnote: The GenBank accession numbers for *PvGPS2* cDNA sequence reported in this paper is JX185300.

* Corresponding author. Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Mahidol University, Rama VI Rd., Bangkok 10400, Thailand. Tel.: +66 2 2015889; fax: +66 2 3547344.
E-mail addresses: saengchan@biotec.or.th, senapin2010@gmail.com (S. Senapin).

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complex) [9]. In addition to transcriptional roles, *GPS2* has recently been reported to be involved in inflammatory responses and adipogenesis [14,15].

In this study, a novel shrimp *GPS2* cDNA sequence was identified and characterized from *Penaeus vannamei*. Its expression pattern was investigated in various shrimp tissues and at different molting stages. In addition, use of RNA interference (RNAi) revealed that it has an essential role in ecdysis (molting).

2. Materials and methods

2.1. Experimental shrimp

Specific pathogen free *P. vannamei* (5–6 g body weight) were kindly provided by Charoen Pokphand Company. The animals were acclimatized in a wet laboratory in 1000 L aquaria containing continuously aerated artificial seawater at 15 ppt and 26–28 °C for 3 days before the experiments began.

2.2. RNA preparation

Hemolymph was withdrawn from the ventral sinus cavity of each shrimp using a sterile 1-ml syringe with a 25-gauge needle containing an equal volume of anticoagulant AC1 solution [16]. Hemolymph from three shrimp was pooled and centrifuged at 800 × g for 10 min at 4 °C to obtain a hemocyte pellet. Selected tissues, including the intestine, heart, gills, muscle, eyestalks, pleopods, and lymphoid organ from three shrimp were also collected and washed three times with diethyl pyrocarbonate (DEPC)-treated TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8). Hemocytes and tissue samples were homogenized in Trizol reagent (Invitrogen) and total RNA was prepared following the manufacturer's instructions. RNA quantity and quality were assessed by measuring absorbance at 260 and 280 nm.

2.3. Identification of PvGPS2 cDNA sequence

To obtained the full-length *PvGPS2* cDNA sequence, a specific pair of primers was designed corresponding to *Penaeus monodon GPS2* (*PmGPS2*) ORF (GenBank accession number JN714124). The primers, Pv-GPS2-F (5′- ATG GTA AAG TTT GCC GAG GCT GAG -3′) and Pv-GPS2-R (5′- TCA GTA AAA CGA ACG GGT GTA GTT -3′), were synthesized by Bio Basic Inc. (Canada). RT-PCR reactions of 25 μl contained 100 ng of *P. vannamei* hemocyte RNA template, 200 nM of each primer, 1 μl of SuperScript One-Step RT/Platinum Taq Mix (Invitrogen) and 1 × reaction buffer. The RT reaction was carried out at 50 °C for 30 min. The PCR protocol consisted of denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min. Amplified products were cloned into pDrive cloning vector (Qiagen). Recombinant plasmids were sequenced by 1st BASE Pte Ltd. (Malaysia).

2.4. DNA sequence analysis

DNA and protein analysis were carried out using the EXPASY web server (http://au.expasy.org/). The homology search was carried out with BLAST program in the NCBI database (http://www. ncbi. nlm. nih. gov/BLAST/). Multiple sequence alignments were conducted by ClustalW [17] and a Neighbor-Joining (NJ) tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA 5) software [18]. An analysis for protein domains was accomplished by InterPro database [http://www.ebi.ac.uk/InterProScan/, [19] and SMART [http://smart.embl- heidelberg.de/, [20]. Primers for gene expression were designed using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/) primer3_(www.cgi). Potential phosphorylation sites were predicted by NetPhos in EXPASY.

2.5. Tissue expression analysis

RT-PCR was employed to evaluate the expression profiles of *PvGPS2* in different tissues. A partial fragment of 513 bp of *PvGPS2* was amplified using the primer pair Pv-GPS2-F2 (5′-GTA AAG TTT GCC GAG GCT GA-3′) and Pv-GPS2-R2 (5′-TGC CTG TGG TAC TTT GTA TG-3′). A partial sequence of 397 bp of *P. vannamei β-actin* (*ACT*) cDNA sequence (GenBank accession no. AF300705) amplified using primers Pv-Actin-F, 5′-TGA CGG CCA GGT GAT CAC CA-3′ and Pv-Actin-R, 5′-GAA GCA CTT CCT GTG AAC AA-3′ was used as a control. RT-PCR reactions were carried out in a 25 μl reaction solution containing of 100 ng RNA template, 200 nM of each forward and reverse primer, 0.5 μl of SuperScript One-Step RT/Platinum Taq mix (Invitrogen), and 1 × reaction buffer. The reaction protocol was performed as described above except that 30 amplification cycles were used. RT-PCR products were analyzed by agarose gel electrophoresis.

2.6. Transcriptional profile of PvGPS2 at different molt stages

The molt stages of *P. vannamei* were identified based on the degree of setae development previously described [21]. The distal portion of the uropod from each shrimp was observed under a light microscope. Shrimp used in this study were divided into four molt stages: intermolt, early pre-molt, late pre-molt, and post-molt. Eyestalks from individual shrimp (2–3 shrimp) from each molt stage were then subjected for RNA extraction and examined for *PvGPS2* transcriptional expression by RT-PCR assay as described in the section "Tissue expression analysis".

2.7. Knockdown of PvGPS2

Knockdown of *PvGPS2* expression in shrimp was carried out by injection of a long dsRNA duplex. Based on RNAi capacity prediction [22], a 273-bp fragment of *PvGPS2* was selected for dsRNA production. The fragment was amplified by RT-PCR using primers Pv-GPS2-RNAi-F (5′- GAA AGA CCA AAG ATG AGT CG-3′) and Pv-GPS2-RNAi-R (5′- CTT TCT GGT TGC ATC CTC AT-3′) with *P. vannamei* hemocyte RNA as the template. The amplicon obtained was then cloned into pDrive vector (QIAGEN). Recombinant plasmids with opposite orientation of respective fragments were linearized with *Hind*III restriction enzyme to be used as sense and antisense templates. Single stranded RNAs were then generated by *in vitro* transcription using T7 RNA polymerase (Promega) as previously described [23]. Equivalent amounts of sense and antisense RNA were mixed and annealed by incubation at 37 °C for 1 h. After DNA removal and purification, the dsRNA integrity was monitored and verified by agarose electrophoresis and appropriate nuclease treatment. The *in vitro* synthesized dsRNA was subsequently quantified by spectrophotometer and freshly dissolved in 150 mM NaCl solution prior to use. A non-related dsRNA used in control experiments was designed from the capsid protein coding region of infectious myonecrosis virus (IMNV) and prepared in the same manner [23].

For experimental shrimp injection, *P. vannamei* of average 5–6 g body weight were divided into 3 groups of 8–13 individuals each. At day 0, Group I was injected with 40 μl of *PvGPS2* dsRNA (12.5 μg/animal) at the lateral area of the fourth abdominal segment, Group II was injected with 40 μl of IMNV dsRNA (12.5 μg/animal), and Group III was injected with 40 μl of 150 mM NaCl solution. Shrimp mortality was monitored daily. To ensure specificity of gene knockdown, *PvGPS2* transcripts in gill tissues were monitored by