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WSSV infection activates STAT in shrimp

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Summary
Although the JAK/STAT signaling pathway is usually involved in antiviral defense, a recent study suggested that STAT might be annexed by WSSV (white spot syndrome virus) to enhance the expression of a viral immediate early gene in infected shrimps. In the present study, we clone and report the first full-length cDNA sequence for a crustacean *STAT* from *Penaeus monodon*. Alignment and comparison with the deduced amino acid sequences of other STATs identified several important conserved residues and functional domains, including the DNA binding domain, SH2 domain and C-terminal transactivation domain. Based on these conserved sequences, a phylogenetic analysis suggested that shrimp STAT belongs to the ancient STAT family, while the presence of the functional domains suggested that shrimp STAT might share similar functions and regulating mechanisms with the well-known STATs isolated from model organisms. Real-time PCR showed a decreased transcription level of shrimp STAT after WSSV infection, but a Western blot analysis using anti-phosphorylated STAT antibody showed an increased level of phosphorylated (activated) STAT in the lymphoid organ of shrimp after WSSV infection. We further show that a primary culture of lymphoid organ cells from WSSV-infected shrimp resulted in activated STAT being translocated from the cytoplasm to the nucleus. This report provides experimental evidence that shrimp STAT is activated in response to WSSV infection. Our results support an earlier finding that WSSV does not disrupt JAK/STAT pathway, but on the contrary benefits from STAT activation in the shrimp host.

Introduction

The importance of the JAK/STAT signaling pathway in antiviral immunity has been addressed extensively in mammals [1], and increasing evidence has suggested that this pathway also plays a role in invertebrate organisms during virus infection [2–4]. Activation of the JAK/STAT

Abbreviations: STAT, signal transducers and activators of transcription; WSSV, White Spot Syndrome Virus; LPS, lipopolysaccharide.

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pathway results in the phosphorylation of STATs at a single tyrosine residue near the carboxyl terminus. The tyrosine-phosphorylated STATs then dimerize and translocate to the nucleus, where they transcriptionally activate appropriate genes to mediate various responses, including cell growth, cell differentiation and immune responses [5].

The immune responses mediated by the JAK/STAT pathway have been widely studied in model organisms. Genetic knock-out studies of mice suggested that the mammalian JAK/STAT signaling pathway is predominant in controlling immune responses, especially the antiviral response [6]. In insects, the first evidence of the JAK/STAT pathway being involved in immune responses was in the mosquito *Anopheles gambiae*, whereas in mammals, AgSTAT translocated into the nucleus of the fat body cell upon bacterial infection [7]. When *Drosophila* is challenged by bacteria, the JAK/STAT pathway activates at least two gene families, *TEP* and *TOT*, that are involved in innate immunity [8,9]. The transcriptional profile of Drosophila C Virus-infected *Drosophila* further suggests that the JAK/STAT pathway might also respond to viral infection [10]. Taken together, these data suggest that in addition to the Toll and Imd pathways, the JAK/STAT pathway could be a third evolutionarily conserved pathway that supports innate immunity in insects. Because the JAK/STAT pathway plays a significant antiviral role, it follows that this pathway might be targeted by a virus that is attempting to evade the host's immune responses; and in fact, several viruses have been shown to counter their hosts' antiviral mechanisms by inhibiting the JAK/STAT signaling pathway. For example, the V protein of simian virus targets its host's STAT1 for degradation [11], while the human cytomegalovirus targets JAK1 for degradation [12]. Infection with the varicella-zoster virus inhibits the expression of both STAT1 and JAK2 [13]. Hepatitis C virus also inhibits host immunity by disrupting the crosstalk between the MAPK and JAK/STAT pathways [14].

WSSV also targets STAT, but instead of inhibiting or disrupting its activity, WSSV exploits the host STAT by using it to bind to the promoter region of the WSSV immediate early gene *ie1* and thus enhance *ie*1 transcription [2]. To better understand how the JAK/STAT pathway generally responds to pathogens in shrimp, in the present study, we first cloned and characterized the full-length cDNA sequence of shrimp *STAT* from *Penaeus monodon*. Next, real-time RT-PCR was used to investigate whether the transcription level of *STAT* was changed after *Litopenaeus vannamei* were challenged with WSSV and the immune stimulants LPS and PGN (lipopolysacharide and peptidoglycan). A previous study had used EMSA to show that WSSV infection led to increased levels of activated STAT, so when our RT-PCR results showed that STAT transcription was reduced after challenge with WSSV, we used two other more direct methods (a phosphorylation status analysis and an immunostaining assay) to reconfirm the activation status of STAT in lymphoid organ cells of WSSV-infected shrimp.

Materials and methods

Cloning of full-length shrimp *STAT* cDNA

Specimens of adult *P. monodon* (30–40 g) were collected from a culture pond at the Biotechnology Division of the Fisheries Research Institute in southern Taiwan and kept in a 70 l tank maintained at 25–28 °C. Pleopods were excised from two or three of these shrimp, immediately frozen and stored in liquid nitrogen until used. These frozen pleopods were then homogenized in 6 ml Trizol reagent (Invitrogen, USA) and subjected to RNA extraction. An aliquot of 10 μg of RNA was treated with 200 U of RNase-free DNase I at 37 °C for 30 min and then extracted with phenol-chloroform. The DNase-treated total RNA was denatured by heating at 85 °C for 10 min in 10 μl DEPC-water containing 100 pmol oligo(dT) anchor primer. The first-strand cDNA was synthesized by SuperscriptII reverse transcriptase (Invitrogen, USA) at 37 °C for 1 h, followed by heating at 95 °C for 5 min to stop the reaction. A partial *STAT* cDNA fragment was then cloned using the forward and reverse VNM-STAT primers (5′-GAGTCAGTGATGGATGAGAAA-3′ and 5′-GTCGGAGAAACG-GAGCAAGAA-3′; designed from a *L. vannamei* EST clone whose 5′ end had a high homology to insect STATs). The resulting PCR product was subcloned into pGEM-T easy vector (Promega, USA) and sequenced.

The 5′/3′ regions of the shrimp *STAT* transcript were determined by 5′/3′ RACE using a commercial 5′/3′-RACE kit (Roche, Germany). For 5′ RACE, the appropriate gene-specific primer, stat-5′RACE-rp5 (5′-CACTAATGATTGAGA-CCCGCAC-3′), was used for cDNA synthesis. Before being subjected to PCR, a polyA sequence was added to the cDNA products with terminal transferase (TdT) in the presence of dATP. The first step of the PCR for shrimp *STAT* was performed using the gene-specific primer stat-5′RACE-rp6 (5′-CTTTGAATGTGGGAAAGGTGAGC-3′) and an oligo (dT) anchor primer (5′-GACCACGCGTATCGATGTCGACTTTTTTT-TTTTTTTTTV-3′). This was followed by nested PCR with the gene-specific primer stat-5′RACE-rp7 (5′-TCATTTGCCGT-TTCCCGTGTAC-3′) and the anchor primer (5′-GACCACGCG-TATCGATGTCGAC-3′). The cDNA synthesized from the oligo(dT) anchor primer was subjected to 3′ RACE PCR. Similarly, 3′RACE was performed with two rounds of PCR, first with the gene-specific primer stat-3′RACE-fp (5′-GTG-GAAGGATGGCTCCATCATG-3′) and an oligo (dT) anchor primer and then with gene-specific primer stat-3′RACE-fp1 (5′-ATGCTTAAGAATTCCAAGTCAGG-3′) and an anchor primer. The resulting 5′/3′ RACE PCR products were cloned into pGEM-T vector and sequenced.

Shrimp STAT sequence analysis and phylogenetic analysis

The complete coding sequence of shrimp *STAT* was compared with the GenBank database using the program BlastX, and the cDNA sequence were deposited in the NCBI database (AY327491). Ten STAT amino acid sequences were retrieved from GenBank, and were used for sequence alignment and phylogenetic analysis. Multiple sequence alignment was done using the program ClustalX and edited with Genedoc software. Phylogenetic analysis was based on the sequence of the STATs' conserved central region from the DNA binding domain to the SH2 domain (aa340–aa692 in shrimp STAT), and was performed with Mega4.0 software using the Neighbor-Joining algorithm. One thousand bootstrap replicates were generated to test the robustness of the trees.