Molecular cloning and expression of a mammalian homologue of a translationally controlled tumor protein (TCTP) gene from Penaeus monodon shrimp

Phuwadol Bangrak, Potchanapond Graidist, Wilaivan Chotigeat, Amornrat Phongdara*

Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkhla 90110, Thailand

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Abstract

White spot syndrome, caused by white spot syndrome virus (WSSV), is a deadly disease of shrimps, causing a catastrophic loss in shrimp industries worldwide. In order to investigate molecular response of shrimp haemocyte to WSSV infection, we performed subtraction hybridization of mRNAs from healthy and WSSV-infected haemocytes. One of the genes that were severely down-regulated in moribund WSSV-infected haemocyte was translationally controlled tumor protein (TCTP) (or fortilin). Strikingly, while there was a slight difference in the amount of TCTP message between normal and early WSSV-infected shrimps, shrimps that exhibited severe symptoms uniformly had very little TCTP in their haemocyte. Taken together with the fact that TCTP functions as an anti-apoptotic protein in mammals, our data suggest that TCTP in shrimp protects WSSV-infected shrimps from death.

Keywords: Penaeus monodon; TCTP; Fortilin; Anti-apoptotic protein

1. Introduction

The crustacean immune response can be divided into cellular and humoral components. The cellular component comprises phagocytosis and cellular encapsulation of potential pathogens by haemocytes. The humoral component is characterized by temporarily enhanced antimicrobial activity in the cell-free hemolymph. As opposed to vertebrates, invertebrate responses, whether local or systemic, do not involve a clonal amplification of the cells producing given effector molecules and lack specific immunoglobulins and immunological long-term memory (Niere et al., 1999). However, all the mechanisms conserved between invertebrates and vertebrates are related to
innate immunity (Pasquier, 2001). Knowledge of shrimp immunity at the molecular level has been increasing rapidly in recent years, and several genes involved in anti-microbial have been cloned and characterized (Supungul et al., 2002; Roux et al., 2002). EST upon viral infection has been studied and several hundred of sequences have been reported (Rojtinnakorn et al., 2002; Astrofsky et al., 2002). The objective of such studies is to look for homologues of immune responsive molecules and related novel gene products, and use them to predict and test their real functions in the defense mechanisms of shrimp.

In previous work, we used the cDNA subtraction technology to differentially screen the cDNA libraries from normal and white spot syndrome virus (WSSV) infected shrimp (Bangrak et al., 2002). Several hundred positive clones were obtained for sequence analysis. One of the clones, p43S2, showed statistically significant similarity with translationally controlled tumor protein (TCTP). It is therefore referred as Pm-TCTP.

One family of TCTPs was initially described as a growth-related protein in mouse Ehrlich ascites tumor cells and erythroleukemia cells (Bohm et al., 1989; Yenofsky et al., 1983; Chitpatima et al., 1988). Subsequently, TCTPs were found to be present in many cell types throughout the entire animal and plant kingdoms (Chitpatima et al., 1988; Gross et al., 1989; Pay et al., 1992; Bhissuthiban et al., 1998; Sturzenbaum et al., 1998). This protein is also referred to as P23, P21, and Q23 (Gachet et al., 1999). In addition, characterization studies revealed that TCTPs are calcium binding (Kim et al., 2000) and heat stable protein (Niak et al., 2001).

That it is well preserved throughout the entire animal and plant kingdom and the functions mentioned above suggest a crucial cellular role. Interestingly, recent reports (La et al., 2001; Zhang et al., 2002) describe the finding of human TCTP (or newly named fortinlin) as a novel anti-apoptotic protein involved in cell survival. This finding prompted us to study the function of Pm-TCTP in shrimp, starting by following the expression of this gene in WSSV-infected shrimp, and the results suggested the involvement of an anti-apoptosis process. Therefore, we constructed and expressed the recombinant clone in Escherichia coli for further biological tests.

2. Materials and methods

2.1. Cloning of Pm-TCTP

A partial sequence of Pm-TCTP was obtained from the clone p43S2 isolated from the subtraction cDNA library. The subtraction cDNA library was prepared from WSSV-infected haemocyte as described previously (Bangrak et al., 2002). To obtain the total coding sequence of p43S2 cDNA, rapid amplification of cDNA ends (RACE) was performed. Reagents for 3’ RACE were purchased from Life Technologies and reactions were conducted according to the instruction manual (GIBCO BRL). The gene-specific oligonucleotide primers were as follows: GSP-1: 5’-CGA GA T GGC A TG GTT GTT CTC A TG-3’ and GSP-2: 5’-CGA AGA CA T TGA TGG AGA AGA GCG-3’. The obtained PCR fragments were cloned into pGEM-TEasy (Promega Corporation) and sequence analysis performed using the ABI prism 377 apparatus.

2.2. Isolation of total RNA

Total RNA was extracted from white spot syndrome virus infected shrimp haemolymph using Trizol reagent (GIBCO BRL).

2.3. Analysis of expression of Pm-TCTP transcripts

To determine the expression of the Pm-TCTP in shrimp, RT-PCR analyses were performed. Primers were designed for each of the detected sequences and synthesized by Life Technologies, USA. Oligonucleotides used as PCR primers were as follows: sense primer 5’-CGG GAT CCT GCG AGG-TCA TCT TCA-3’ and antisense primer 5’-GCG TCG ACT TAT AGC TTC TCC TC-3’. Degenerate β-actin primers were designed according to the conserved amino acid sequences QLMFETF and MKCDVDI (Yeh et al., 1999) and used to amplify a PCR product of 500 bp in the RT-PCR experiments as an internal control of gene expression. Total RNA (1 μg each) from haemocytes of uninfected samples and infected samples was extracted by using the SV total isolation system (Promega Corporation) for use as the template in 50 μl of RT-PCR reaction mixture according to the manufacturer’s instructions (Promega Corporation).
The reaction started at 50 °C for 30 min followed by an initial PCR activation step at 95 °C for 15 min followed by 30 cycles of 94 °C for 1 min and 72 °C for 1 min. The RT-PCR products of each sample were analyzed on a 1.8% agarose gel and visualized by ethidium bromide staining under ultraviolet light. The identity of PCR products was confirmed by cloning into pGEM-TEasy (Promega Corporation) and sequence analysis using the ABI prism 377 apparatus.

2.4. DNA sequencing and data analysis

The DNA sequence was analyzed using BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (PE Applied Biosystems) and an Applied Biosystems 377 sequencer (Perkin-Elmer, Norwalk, CT, USA). Gene database searches were performed through the National Center for Biotechnology Information using the BLAST network service. Sequences alignment was done by using PHYLIP (http://evolution.genetics.washington.edu/phylip.html).

2.5. Construction of Pm-TCTP expression plasmid

The open reading frame (ORF) of Pm-TCTP was amplified by RT-PCR. The forward PCR primer corresponded to the beginning of ORF with the addition of an upstream in-frame BamHI restriction site (5′-CGGGATCCATGAAGGGTTCAG-3′). The reverse primer corresponded to the 3′ end of the coding region flanked by a SalI restriction site (5′-GCGTCGACCTATAGCTTCTC-3′). The PCR parameter was initially 95 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min. The PCR products were cloned in-frame with 6× Histidine tags into the BamHI and SalI restriction sites of the pQE40 (QIAGEN GmbH) to obtain pQE-TCTP. Subsequently, the inserted DNA fragment was sequenced using the ABI prism 377 apparatus to ensure authenticity of the cloned nucleotide sequence.

2.6. Expression and purification of Pm-TCTP

The E. coli strain M15 (pRep4) harboring pQE-TCTP was grown in LB medium containing 100 μg/μl ampicillin and 25 μg/μl kanamycin until the OD600 reached 0.5. One milimolar of IPTG was added to the culture and it was incubated for an additional 3 h. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl and 10 mM imidazole) containing 1 mM PMSF. The protein lysate was purified by using Ni-NTA column, the histidine tagged fusion protein was eluted by elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl and 250 mM imidazole) containing 1 mM PMSF. The eluted protein recovered in fractions was analyzed for purity by 12.5% SDS–PAGE gels.

2.7. 45 Calcium-overlay assay

The calcium overlay assay was performed according to Garrigos et al. (1991). Five micrograms of purified protein and the control protein were separated on 12.5% polyacrylamide gel electrophoresis and blotted on a nitrocellulose membrane. The membrane was briefly rinsed with assay buffer (60 mM KCl, 5 mM MgCl2, 10 mM Imidazole, pH 6.8) and then incubated with the same buffer containing 20 μCi/ml 45CaCl2 (Amersham Bioscience, USA) for 1 h at room temperature with gentle agitation. The radioactive solution was removed and the membrane was washed in 50% ethanol for 20 min, completely air-dried and subsequently placed in film cassette containing autoradiography film (X-ray film from Kodak, USA). The radioactive associated protein signal was developed using a developing machine (Kodak, USA).

3. Results

3.1. Cloning and sequence analysis of Pm-TCTP

Pm-TCTP was obtained from the WSSV-infected subtraction cDNA library. Full-length cDNA of Pm-TCTP was generated using the RACE technique. On complete sequencing, it was found that the Pm-TCTP cDNA clone was a 702 bp in length. The completed sequence was deposited in GenBank with the accession number AY186580.
The predicted 168 amino acid polypeptide had a calculated molecular mass of 19.2 kDa and a predicted pI of 4.5. Sequence analysis with the BLASTX algorithm of this deduced amino acid sequence had the highest similarity to *Anopheles gambiae* and *Drosophila melanogaster* TCTP at 73% (4e-41) and 74% (8e-41), respectively. In addition, we found the Pm-TCTP also showed similarity to human TCTP or fortilin (a novel anti-apoptotic protein) at 63% (5e-25).

A Clustal X alignment using PHYLIP of Pm-TCTP with TCTP homologue proteins of human, animals, hydra, parasites and yeast is shown in Fig. 1.

3.2. Expression of Pm-TCTP transcripts in infected samples

To investigate whether the gene encoding Pm-TCTP was indeed inducible by viral infection, we performed RT-PCR analyses and semi-quantitative assay on total RNA extracts from normal and WSSV-injected shrimp using specific primers designed from the p43S2 sequences. RT-PCR of the β-actin gene was used as an internal control in the semi-quantitative analysis and to insure that the RT-PCR for each sample contained the same amount of total RNA and that the RNA was...
intact. The purity of the isolated RNA was analyzed for DNA contamination by using RT-PCR in the presence (+RT) and absence (−RT) of reverse transcriptase. The Pm-TCTP was observed in six individuals each of normal shrimps (N), early WSSV-infected shrimps (I), and moribund WSSV-infected shrimps (D) as shown in Fig. 2A. The amount of Pm-TCTP were measured semi-quantitatively by comparing with the amount of the β-actin in the same samples (Fig. 2B). Interestingly, the level of Pm-TCTP expression decreased when shrimp showed the mortality characteristic (D), compared to the β-actin gene in the same samples and others.

3.3. Expression and purification of recombinant Pm-TCTP

Pm-TCTP cloned in pQE40 was expressed as fusion proteins in E. coli. The recombinant Pm-TCTP protein was purified in a Ni-NTA column and separated

![Graph showing expression of Pm-TCTP specific mRNA in response to viral infection and expression of β-actin as an internal control. Total RNA was isolated from haemocytes of 6 individuals of Penaeus monodon uninfected and infected with WSSV. RNA isolation, RT-PCR and the primer sequences were as described in Section 2. N1–N6: normal (uninfected samples), I1–I6: infected (24 h WSSV post-injection samples), D1–D6: moribund (WSSV-infected samples shows mortality fate). (B) The ratio of Pm-TCTP to β-actin was calculated from the image using Scion Image software. The data represent the average results obtained from six samples in each sample set.](image-url)
Fig. 3. 45Ca-overlay assay of purified recombinant Pm-TCTP. The purified protein (5 μg) was resolved by 12.5% SDS-PAGE and the proteins were transferred to nitrocellulose membrane and probed with radioactive 45CaCl2 as described in Section 2. (A) Coomassie blue staining of 12.5% polyacrylamide gel; (B) X-ray film after autoradiography. M: molecular marker, lane 1: purified recombinant Pm-TCTP, lane 2: purified recombinant Pm-syntenin as negative control.

on 12.5% SDS–PAGE. It showed the molecular mass very close to the predicted mass of 25 kDa with the histidine tag.

3.4. Binding of Pm-TCTP to calcium

To analyze the calcium binding properties of Pm-TCTP, a 45Ca-overlay assay was carried out as described by Rao et al. (2002). Five microgram of recombinant Pm-TCTP was separated on 12.5% SDS gel and transferred to a nylon membrane. The same amount of recombinant, Pm-syntenin, a shrimp unrelated to TCTP (Bangrak et al., 2002) was included in the gel as negative control. After incubated with buffer containing 20 μCi/ml 45Ca solution and washing with 50% ethanol, the result was visualized by autoradiography as illustrated in Fig. 3. The result shows a strong dark band on the recombinant Pm-TCTP (B, lane 1) while there was no signal from the other recombinant protein (B, lane 2), the negative control. This result concludes that our Pm-TCTP does indeed bind to calcium.

4. Discussion

White spot disease or white spot syndrome virus is associated with remarkably high mortality rate in penaeid shrimps and has caused a catastrophic loss to shrimp industries worldwide. According to China’s Xinhua news agency, the epidemic of disease caused the export value to decrease by 40–50% from the normal level or drop for several million US$. White spot disease found in black tiger shrimp is caused by Baculo virus. The disease was originally reported in China in 1993, rapidly spread to many shrimp farming countries throughout Asia.

Despite exhaustive investigation, little is known about the pathophysiology of white spot diseases, except for the fact that haemocytes play a critical role in host-defense mechanism against shrimp viral infection, including WSSV (Holmblad and Söderhäll, 1999; Bachere et al., 1995; Bachere, 2000; Johansson et al., 2000 and and Söderhäll and Cerenius, 1992; Rojinknakorn et al., 2002). Our laboratory has focused on the molecular responses of haemocytes, a critical component of crustacean cellular immunity, to WSSV infection. We have performed the subtraction hybridization of mRNAs from haemocytes from healthy and WSSV-infected shrimps (Bangrak et al., 2002).

TCTP was one of the genes that were seem to slightly up-regulated in early WSSV-infected shrimps. In the current work, we have shown that the severe systemic illness of the WSSV-infected shrimp correlates with the loss of TCTP messages. The WSSV-induced loss of TCTP message in Penaeus monodon has not been reported in literature. TCTP was initially identified as a tumor-related protein in mouse ascetic tumor and mouse erythroleukemic cells (Yenofsky et al., 1983; Chitpatima et al., 1988). Expression of TCTP is not exclusively restricted to tumor cells, it has been found subsequently in a variety normal human cells, and homologues have been recognized in a number of other species, including nematodes, amphibians, plants and yeast (Sanchez et al., 1997). Recently TCTP has attracted the attention of an increasing number of researchers due to the fact that TCTP levels are highly regulated in response to a wide range of extracellular stimuli. A series of recent reports highlighted the importance of TCTP for cell cycle progression, malignant transformation and anti-apoptotic activity.

We propose that TCTP may be the key factor to allow WSSV-infected haemocytes to survive. It is intriguing that the message of TCTP is severely diminished in shrimps showing severe systemic signs of viral infection when one considers that recent
reports suggest TCTP is an anti-apoptotic protein. First of all, it is possible that viral proteins negatively regulate the transcription of TCTP. The loss of TCTP from haemocytes in turn would cause the death of haemocytes and the loss of host defense, allowing further propagation of the virus and resulting eventually in the death of the shrimp. Secondly, it is also possible that viral infection in haemocytes would prompt the programmed cell death pathway, leading to the down-regulation of survival genes, including TCTP. In fact, the work of Flegel and Pasharawipas (1998) supports the link between viral infection and the activation of cell death pathway. Finally, it is possible that TCTP protects virally infected haemocytes and keeps shrimps less ill. The final possibility is likely because the presence of virus in haemocyte by itself did not make shrimps ill (Fig. 2) while the presence of virus, when combined with the loss of TCTP, made them severely ill.

In the current work, we have shown that shrimp TCTP binds to Ca$^{2+}$. Although the binding of human TCTP to Ca$^{2+}$ has been reported, the binding of shrimp TCTP to Ca$^{2+}$ has not been reported. The binding of TCTP to Ca$^{2+}$ across the species, taken together with the increasing body of evidence that TCTP is an anti-apoptotic protein, suggest that TCTP functions as an anti-apoptotic protein through Ca$^{2+}$ scavenging in the cells (Xu et al., 1999; Li et al., 2001). It is tempting to speculate that TCTP protects virus-infected haemocytes from dying, thus keeping shrimps, despite infection, healthy.

The loss of TCTP in severely symptomatic shrimps infected with WSSV and the presence of TCTP in asymptomatic but WSSV-infected shrimp suggest that TCTP is one of the key genes to protect virally infected haemocytes from death. Our current finding may lead to the strategies to keep shrimps healthy even when they are infected with WSSV.

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