Identification of a novel shrimp protein phosphatase and its association with latencey-related ORF427 of white spot syndrome virus

Liqun Lu, Jimmy Kwang*

Animal Health Biotechnology Unit, Temasek Life Science Laboratory, National University of Singapore, Singapore 117604, Singapore

Received 9 August 2004; revised 29 August 2004; accepted 30 August 2004

Available online 13 October 2004

Edited by Gianni Cesareni

Abstract To characterize the role of latency-associated ORF427 of white spot syndrome virus (WSSV), a shrimp cDNA library was constructed to screen interacting proteins of ORF427. Employing the yeast two-hybrid system, a novel shrimp protein phosphatase (named PPs), sharing 93% homology with human protein phosphatase 1, has been identified able to bind ORF427 in yeast. Through co-immunoprecipitation assays, the interaction between PPs and ORF427 was further confirmed both in vitro and in vivo. Interestingly, the novel shrimp protein phosphatase consists of only 199 aa and contains almost all the functional catalytic domains of human protein phosphatase, while it lacks the corresponding C-terminal non-catalytic sequence. Transcription and translation products of the identified cDNA can be detected in both normal and WSSV-infected shrimps; and PPs was found to localize mainly in the lysosome of shrimp cells. To characterize its function, the PPs cDNA was highly expressed in bacteria and the purified protein showed phosphatase activity when tested against pNPP in a standard phosphatase assay. Our results suggest that the identified protein phosphatase, PPs, may represent a novel member of protein phosphatase family and might be involved in the regulation of WSSV's life cycle through interaction with latency-related ORF427 of WSSV.

Keywords: Protein phosphatase; WSSV; ORF427; Latency; Lysosome

1. Introduction

White spot syndrome virus (WSSV) is an important pathogen of the penaeid shrimp with high mortalities. Since the first outbreak of WSSV infection in China in 1993, it has raised major concerns in aquaculture around the world [1]. Even though WSSV represents one of the largest known animal viruses with a 305 kb double-stranded circular DNA genome, there are many well-known technical difficulties that had inhibited further in depth WSSV studies. These include the lack of established shrimp cell lines for in vitro reproduction of the virus and most of the predicted WSSV ORFs (163 of 181 ORFs) encode proteins with little or no homology to any known proteins [2,3]. Recently, three viral transcripts (Orf427, Orf151, and Orf366) and their corresponding DNA sequence have been detected in both specific-pathogen-free (SPF) shrimps and WSSV-infected shrimps through a WSSV-specific DNA microarray study, thus Orf427, Orf151, and Orf366 were determined to be latency-associated genes of WSSV [4]. It suggests that the SPF shrimps are asymptomatic carriers of WSSV. Although little is known about these three ORFs, it is our interest to characterize their roles in the viral life cycle – from latency to lytic replication. For this purpose, a shrimp cDNA library was constructed to screen host proteins that can interact with ORF427; this leads to the discovery of a novel putative shrimp serine/threonine protein phosphatase.

Protein phosphatases and kinases control a variety of physiological events such as cell proliferation, cell cycle, metabolism, fertilization and splicing of RNA [5]. A typical protein kinase catalyzes the phosphorylation of the hydroxyl group on amino acid residues including serine, threonine, and tyrosine; while reverse dephosphorylation was the function of protein phosphatase. According to the substrate specificity, protein phosphatases are classified into two major groups: serine/threonine and tyrosine phosphatases. Three major types of serine/threonine protein phosphatase catalytic subunits have been identified, termed PP-1, PP-2A, and PP-2B, which are classified according to their substrate preferences, mechanisms of activation and sensitivity to inhibitor proteins or naturally occurring toxins [5–7]. Although almost all the catalytic subunits share a common catalytic region of approximately 280 amino acids, they become divergent when comparing their N- and C-terminal non-catalytic domains. These phosphatases are mainly regulated by different holoenzymes in which the same catalytic subunit is complexed to distinct regulatory and targeting subunits [8,9]. The protein phosphatase encoded by coliphage lambda (PPλ) was found to be the equivalent of the minimal catalytic core of serine/threonine protein phosphatases by biochemical criteria [10]. Although the true physiological substrates of most phosphatases remain elusive, certain peptides and small chemicals have been widely applied to test the activity of phosphatases [11,12].

In this report, the physical interaction between the novel shrimp PPs and latency-associated protein ORF427 was investigated; the phosphatase activity of this novel enzyme was primarily tested against pNPP in a standard phosphatase assay; and cellular expression, as well as subcellular localization, of this novel protein phosphatase was further characterized.

* Corresponding author. Fax: +65-68727007.
E-mail address: kwang@tll.org.sg (J. Kwang).

0014-5793/$22.00 © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2004.08.087

2. Materials and methods

2.1. Virus and cells

WSSV DNA was isolated from infected Penaeus monodon with white spot syndrome from Malaysia. An immortal shrimp cell line (PMO) (US Patent No. 6 143 547) derived from Penaeus monodon was employed for localization study. The cell line was maintained in L15 medium.

2.2. Yeast two-hybrid screen

To construct shrimp two-hybrid cDNA library, total RNA from *Litopenaeus vannamei* was extracted by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was prepared by a SMART™ PCR cDNA Synthesis Kit (Clontech) using SMART Oligo II (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′) and CDS Primers [5′-AAGCAGTGGTAT CAACGCAGAGTA-d(T)30-3′] (Clontech). Double-strand cDNA was amplified using SMART PCR primer (5′-AAGCAGTGGTATCAACGCAGAGT-3′) in the SMART approach. Normalization of first-strand cDNA and SMART-amplified cDNA (including cDNA denaturation/reassociation, treatment by duplex-specific nuclease and amplification of normalized fraction by PCR) were performed according to protocols described by Zhulidov et al. [13]. The normalized cDNA was cloned into shuttle expression vector pGADT7 (Clontech) downstream of the GAL4 DNA activation domain and subsequently transformed into *Escherichia coli* (XL1-Blue) cells.

Matchmaker Two-Hybrid System 3 (Clontech) was employed to screen the library described above for novel proteins that interacted with the known bait protein, ORF427. Orf427 was subcloned downstream of the yeast GAL4 DNA binding domain in the pGBKT7 vector (Clontech). Library plasmids were amplified from *E. coli* according to the standard protocol. YeastMAKER™ Yeast Transformation System (Clontech) was used in transforming the DNA-binding construct (pGBKT7-427) and DNA-activation library plasmids (pGADT7-library) into yeast strain AH109, which virtually eliminates false positives by using three reporters – ADE2, HIS3, and MEL1 (or lacZ) – under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes. The transformants were screened using high-stringency media (SD/-Ade/-His/-Leu/-Trp/X-α-gal). Blue colonies appearing within 4 days indicated an interaction between the two-hybrid proteins. Plasmids from the selected colonies were subjected for sequence analysis.

2.3. In vitro translation and co-immunoprecipitation analysis

Coupled transcription–translation reactions were performed using rabbit reticulocyte lysates and (^{35}S)Met-Cys label (Perkin–Elmer Life Sciences) as specified by the TNT manufacturer (Promega Corp.). pGBKT7-427 expresses ORF427 fused with both the GAL4 DNA-binding domain (DNA-BD) and a c-Myc epitope. Transcription and translation of pGBKT7-427 in vitro yield Myc-tagged 427 (427-Myc). pGADT7-PPs expresses the shrimp protein phosphatase fused with both the GAL4 activation domain (AD) and a hemagglutinin (HA) epitope tag. Transcription and translation of pGADT7-T in vitro yields HA-tagged protein phosphatase. For co-immunoprecipitation assay with in vitro translated proteins, 10 μl of ^{35}S-labeled methionine ORF427 and PPs was gently mixed and incubated at room temperature for 2 h with 10 μl of anti-Myc monoclonal antibody or anti-HA polyclonal antibody. Three μl of protein A beads was then added to every 20 μl of the protein mixtures and the reaction tube was rotated at room temperature for 1 h to ensure adequate mixing. The beads were collected by centrifugation at 6000 rpm for 10 s in a microcentrifuge and washed twice with RIPA buffer [1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl (pH 7.4) and 10 mM EDTA, and 0.1% sodium dodecyl sulfate, SDS] followed by once with washing buffer [50 mM Tris–HCl (pH 7.4), and 150 mM NaCl]. The beads were resuspended in 20 μl of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer containing 10% β-mercaptoethanol, boiled for 5 min, and analyzed by SDS–12% PAGE. The gels were dried on filter papers and autoradiographed using a PhosphorImager (model SI; Molecular Dynamics).

For co-immunoprecipitation experiments with proteins expressed in shrimp cells, the ORF427 expression construct was first transiently transfected into PMO cells in 60 mm culture dishes. 36 h later, cells were collected and lysed in RIPA buffer for 30 min at 4 °C. Lysates were centrifuged at $10\,500 \times g$ for 10 min at 4 °C and the supernatant was subjected for immunoprecipitation assay as described above.

2.4. Confocal microscopy

PMO cells were cultured on four-well chamber slides (IWAKI). At 36 h post-transfection, cells were rinsed once with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 25 min at room temperature, and then permeabilized for 5 min in 0.2% Triton X-100 in PBS at room temperature. For detection of EGFP fusion proteins, the slides were directly observed under an inverted Fluorescence microscope (Olympus) and a confocal inverted laser microscope (Zeiss and Leica). For immunofluorescence assays with Lysotracker Red DND99 (Molecular Probe), the slides were labeled with the lysosome-specific dye in accordance with the manufacturer's instructions.

2.5. Expression of PPs in E. coli and development of polyclonal antiserum

PPs was expressed in *E. coli* as a glutathione *S*-transferase (GST) fusion protein. Briefly, 100 ml of Luria–Bertani medium containing 100 μg ampicillin/ml was inoculated with 1/100 of an overnight culture and grown to an optical density of 0.6 at 600 nm. Protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. The bacteria were collected at $6000 \times g$ for 10 min, and the pellet was resuspended in 5 ml of PBS and sonicated on ice three times for 30 s, each time with 2-s intervals (model W-385; ultrasonics Inc.). Triton X-100 was added to a final concentration of 1% and the proteins were solubilized for 30 min at 4 °C with constant agitation. The insoluble fraction and cell debris were removed by centrifugation at $10\,000 \times g$ for 10 min at 4 °C. The supernatants were incubated with 100 μl of a 50% slurry of glutathione *S*-Sepharose 4B beads (Amersham Pharmacia) for 30 min at 4 °C with constant agitation. The GST fusion proteins bound to the beads were collected by centrifugation at $1000 \times g$, washed five times in PBS containing 1% Triton X-100, and eluted three times with glutathione elution buffer (0.154 g of reduced glutathione dissolved in 50 ml of 50 mM Tris–HCl, pH 8.0). The purified protein was further digested with thrombin to remove the GST-tag from the fusion protein.

Protein purity of the purified protein described above was more than 90% as judged by SDS–PAGE. SPF Guinea pigs were immunized and specific antisera were prepared using standard procedures [14]. The specificity of the serum was determined by immunoblotting following the standard procedure [14].

2.6. Phosphatase assay

To analyze the generic phosphatase activity of PPs, pNPP (Calbiochem) was used as a substrate. Phosphatase activity at various enzyme concentrations (0, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, and 5 μg) of bacterially purified protein PPs was examined in 100 μl of phosphatase buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 20 mM $MnCl_2$, and 25 mM pNPP. The reaction tubes were incubated at 30 °C for 1 h and terminated by the addition of 0.25 M NaOH. Absorbance was determined at 405 nm using a spectrophotometer [7,10].

3. Results

3.1. Identification of a novel protein phosphatase cDNA through library screening

Since straightforward random sequencing of clones from standard cDNA libraries is inefficient for discovering rare transcripts, SMART prepared amplified cDNA was normalized using a DSN normalization method. Samples from the SMART prepared cDNA and the normalized cDNA were analyzed in 1% agarose gel (Fig. 1A). The resulted normalized cDNA was then used for library preparation. Amplified normalized cDNA was ligated into pGADT7 vector and used for *E. coli* transformation. The library was arrayed in 30 plates (approximately 50 000 colonies per plate) and about 1 500 000 independent clones were obtained. Therefore, the library contains approximately 1 125 000 inserts containing independent clones. 40 randomly picked clones were tested by PCR with SMART PCR primer. The inserts were found in 75% of the tested colonies (Fig. 1B).

To understand better the role of the latency-related ORF427 during WSSV's life cycle, we were interested to study its interacting partner in shrimp cells. For this purpose, Orf427 was cloned into another vector pGBKT7 to express ORF427 in the yeast cell to serve as bait. After co-transformation of the bait with the library into the AH109 cell, a total of 11 positive colonies were identified on the most stringent selection plate (SD/-Ade/-His/-Leu/-Trp/X-α-gal). Both the bait plasmid and a library plasmid could be isolated from these colonies. DNA sequencing revealed that 10 of the 11 colonies contained an identical library plasmid, named pGADT7-PPs, bearing a full-length cDNA (GenBank Accession No. AY606063) that shares 83% homology with *Neurospora crassa* protein phosphatase-1 (ppp-1) gene (GenBank Accession No. AF124149.1). Through NCBI Blast analysis, PPs was found to share the highest homology at amino acid level (91%) with human protein phosphatase 1 γ subunit; while λ protein phosphatase 1 (PPλ), the minimum protein phosphatase known so far, had a closer size to PPs.

Protein phosphatases from many species have been characterized, and multiple alignment of the primary amino acid sequences of known Ser/Thr phosphatases has revealed a highly conserved central region of about 250 residues that contained three invariant stretches of amino acids, viz. GDXHG, GDXVDRG, and RGNHE, separated by 25–30 residues (X representing any amino acid) [11]. Thus, it will be interesting to identify whether the conservative functional domains described above exist in shrimp PPs. For this purpose, a pairwise comparison between PPs, human protein phosphatase 1 γ subunit (327 aa) and PPλ (221 aa) was done with software DNAstar. The comparison revealed that, like lambda protein phosphatase, PPs contains all the three conserved catalytic domains. Compared with PPλ, PPs shares higher homology with human protein phosphatase. Interestingly, PPs totally lacks the C-terminal sequence of human PP1 and has high identity at N-terminus; while PPλ only shares certain homologies at N-terminus with human PP1 (figure not shown).

3.2. *In vitro interaction between ORF427 of WSSV and shrimp protein phosphatase, PPs*

To confirm the interaction between the shrimp protein phosphatase and ORF427 of WSSV, the isolated library plasmid, pGADT7-PPs, was co-transformed into AH109 yeast cell with the bait plasmid pGBKT7-427, while empty vectors served as controls. The controls could only grow on medium supplemented with Adenine and Histidine, while yeast cells transformed with pGADT7-PPs and pGBKT7-427 efficiently grew on the most stringent selection plate and showed dark blue color (figure not shown).

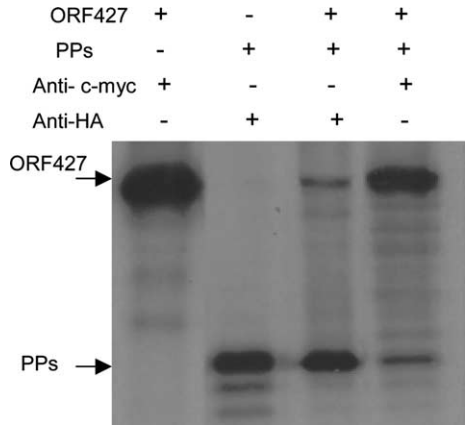

Fig. 2. Interaction between in vitro translated PPs and ORF427. C-myc-tagged ORF427 and HA-tagged PPs were in vitro translated as ^{35}S-labeled proteins. These two proteins, individually or togetherly, were subjected for immunoprecipitation analysis with monoclonal antibody anti-myc or polyclonal anti-HA. After separation of the immunoprecipitated samples through 12% SDS–PAGE, the gel was dried and submitted for X-ray autoradiography.

After confirming the interaction in yeast between ORF427 and shrimp PPs, we proceed to investigate the in vitro interaction employing co-immunoprecipitation assay. Both Orf427 and shrimp PPs cDNA were in vitro translated into ^{35}S-labeled proteins. Since ORF427 was tagged with c-myc epitope and PPs was tagged with HA epitope in our system, monoclonal anti-myc and polyclonal anti-HA antibodies were used in the immunoprecipitation assays. Fig. 2 shows that, from a mixture of in vitro translated PPs and ORF427, PPs could be co-immunoprecipitated down when using anti-myc toward the c-myc-tagged ORF427; and ORF427 could be co-immunoprecipitated down when using anti-HA toward HA-tagged PPs. The in vitro co-immunoprecipitation assay further

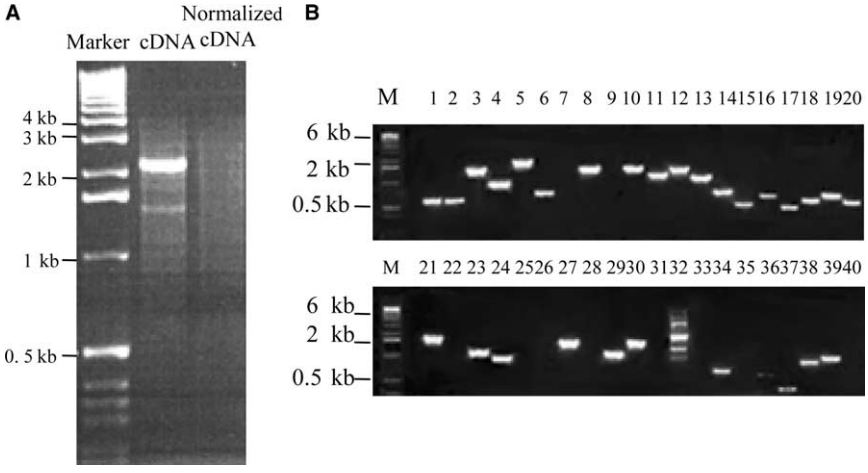

Fig. 1. Quality control of the shrimp cDNA library. (A) Agarose gel electrophoresis of SMART-amplified cDNA before and after normalization; (B) agarose gel electrophoresis of PCR products obtained from 40 randomly picked clones.

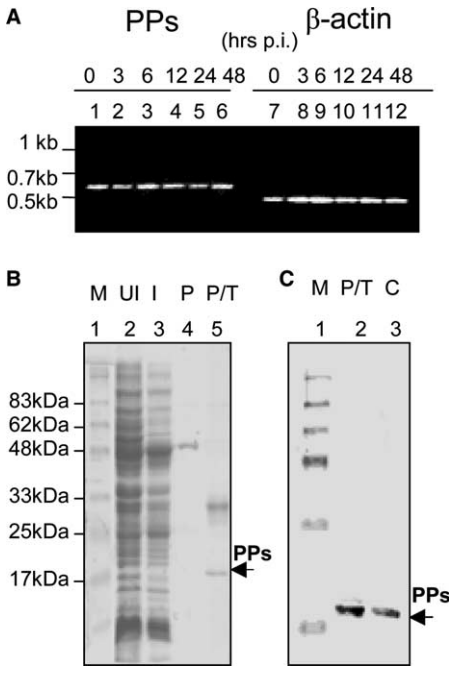

Fig. 3. Detection of the transcription and translation products encoded by the PPs cDNA in shrimp cells. (A) RT-PCR assay for the PPs transcripts in normal and WSSV-infected shrimps. Total RNA was extracted from normal shrimp and WSSV-infected shrimps that were sampled at different time points after infection as indicated above the figure. RT-PCR assay was performed according to standard protocol using primers specific to PPs cDNA. Primers specific to β-actin gene were employed to detect its mRNA from the same shrimp samples, which served here as a control. (B) SDS–PAGE analysis of the expressed and purified PPs from *E. coli*. The PPs cDNA was cloned into the pGEX4T-3 vector and transformed into DH5α; GST-PPs fusion protein was expressed after induction with 1 mM IPTG for 3 h. Purification of the expressed PPs and its cleavage by thrombin to release the GST tag were performed according to standard protocols. (1) Protein marker; (2) uninduced cellular extract; (3) induced cellular extract; (4) purified PPs; (5) purified protein was cleaved by thrombin protease. (C) Western blotting analysis for detection of the endogenic PPs in shrimp cells. Polyclonal antibody toward PPs was raised using the bacterially expressed PPs as antigen from Guinea pigs. (1) Protein marker; (2) PPs released from GST-fusion protein by thrombin as described in (B); (3) total shrimp cellular extracts sampled from normal shrimp.

confirmed the physical interaction between ORF427 and shrimp PPs.

3.3. Detection of endogenic PPs and its association with ORF427 in shrimp PMO cells

To investigate whether the identified shrimp protein phosphatase was present in normal shrimps or WSSV-infected shrimps, RT-PCR was performed to detect the transcription of PPs mRNA with specific PPs primers. Fig. 3A shows that PPs mRNA was transcripted in both normal shrimp and WSSV-infected shrimps, which were sampled at different time points after infection.

To facilitate the study of this novel protein phosphatase, we cloned the PPs cDNA into pGEX4T3 vector. PPs was successfully expressed in *E. coli* as a GST-fusion protein. The soluble protein was purified and injected into Guinea pigs to raise antibody specific to shrimp PPs. Western blotting assay was performed with this antibody to detect the translated protein in shrimp cells. As shown in Fig. 3B, a protein at the expected size could be successfully detected from the shrimp cells.

To determine whether the endogenic PPs in the shrimp cells can form complex with ORF427, co-immunoprecipitation assays were performed with cell lysates containing both the endogenic PPs and transiently expressed ORF427, which was tagged with EGFP (Fig. 4A). Western blotting revealed that ORF427-EGFP, not EGFP control, could co-precipitate with PPs when the immunoprecipitation assay was performed with polyclonal anti-PPs antibody described above (Fig. 4C); and when the assay was tested with monoclonal anti-GFP antibody (Clontech), PPs could co-precipitate with ORF427-EGFP, but not with EGFP control (Fig. 4B).

3.4. Subcellular localization of the novel shrimp protein phosphatase

To study the localization of the identified shrimp protein phosphatase, plasmid pEGFP-PPs, which expressed PPs with an EGFP tag at the C terminus, was transfected into shrimp PMO cells. Thirty-six hours later, transient expression of EGFP-tagged PPs was observed directly under the invert fluorescence microscope. The fusion protein seemed to be amassed in some organelles of the cytoplasm. Through staining with different organelle-specific fluroscence probes, the PPs-dominated organelles were found able to be stained with Lyso-tracker Red DND99 under confocal invert microscope (Fig. 5).

3.5. Preliminary functional assay of PPs towards pNPP

The purified protein phosphatase from *E. coli* was tested against pNPP in a standard phosphatase assay. The assay was performed according to the method described in the study of PPλ [10]. As expected, phosphatase activity can be detected in the assay for PPs (Fig. 6A). Compared with the alkaline phosphatase CIP that served as a positive control here, the phosphatase activity of PPs was low. In an effort to study the effect of ions on its activity, ions were found not necessary in the phosphatase assay, since none of the ions tested (including Ca^{2+}, Mg^{2+}, Co^{2+} and Mn^{2+}) could significantly increase its activity towards pNPP (Fig. 6B).

During sequence analysis of the selected positive colonies from library screening plates, a colony bearing a site mutation on one of the conserved motifs of PPs cDNA was identified: a "R" was mutated into "H" in the "GDXVDRG" motif. Since the "R" is involved in phosphate binding and mutation of this site will cause loss of function for human protein phosphatase, we were interested to study the effect of this mutation on the activity of PPs. The mutated PPs cDNA was cloned into pGEX4T3 vector and expressed as a GST-fusion protein. Phosphatase activity of the purified protein was tested against the pNPP substrate. As expected, the mutated PPs showed lower phosphatase activity (Fig. 7).

4. Discussion

For better understanding of the establishment of viral latency in asymptomatic carriers, we employed a GAL4 based yeast two-hybrid system to identify host proteins that can interact with one major latency-related protein of WSSV, ORF427. Hopefully, the identified protein could shed light on

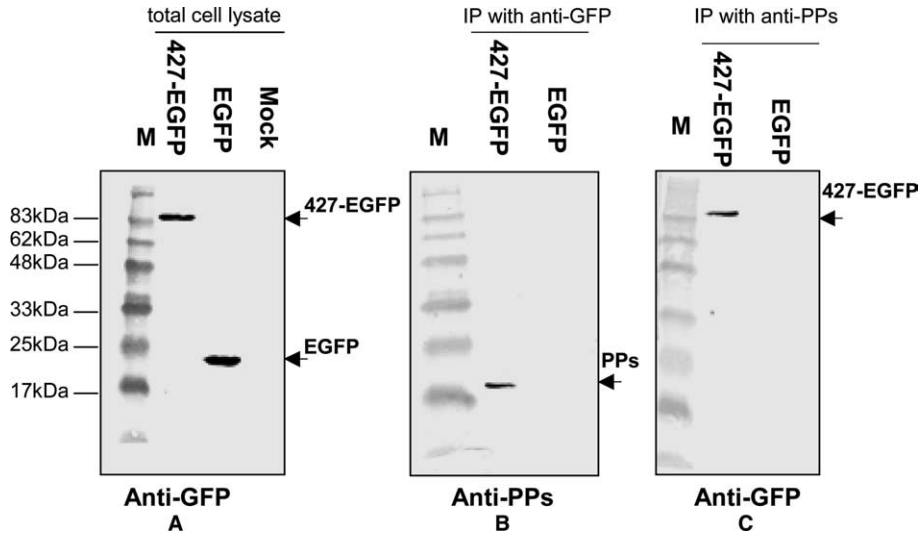

Fig. 4. Association of the endogenic PPs with ORF427 in shrimp cells. (A) Transient expression of ORF427 in PMO cells. Orf427 was cloned into pEGFP-N1 vector to be expressed as a fusion protein with EGFP tagged at the C-terminus. The expression construct, as well as the vector, was transfected into PMO cells. Thirty-six hours after transfection, the cell was harvested in lysis buffer. Total cell lysates were tested against the monoclonal anti-EGFP to detect the expression of ORF427. (B) PPs co-immunoprecipitates with 427-EGFP. Lysates, containing endogenic PPs and 427-EGFP or EGFP, were immunoprecipitated with monoclonal anti-GFP. The immunoprecipitated samples were reacted with polyclonal anti-PPs to detect whether PPs can specifically co-precipitate with 427-EGFP. (C) 427-EGFP co-immunoprecipitates with PPs. Lysates, containing endogenic PPs and 427-EGFP or EGFP, were immunoprecipitated with polyclonal anti-PPs. The immunoprecipitated samples were reacted with monoclonal anti-GFP to detect whether 427-EGFP can specifically co-precipitate with PPs.

the role of the latency-related gene in regulating the life cycle of WSSV. In this study, a novel protein phosphatase, named PPs, was found able to interact with ORF427 in yeast; the interaction was subsequently confirmed through co-immunoprecipitation assays. These are the first data to show that protein phosphatase may be involved in the latent-lytic life cycle of WSSV through interacting with ORF427. Correlated with our findings, protein phosphatase has been implicated in the latent-lytic life cycle for some other model viruses. For example, dephosphorylation of eIF-2α mediated by protein phosphatase 1 was found necessary, although not sufficient, for viral replication of HSV-1 [15]; mutation of Lambda phage protein phosphatase might cause the failure for the phage's reactivation from latent cycle to the lytic cycle [7]. Most recently, nuclear protein phosphatase-1 was reported able to regulate HIV-1 transcription both in vitro and in vivo [16]. In mammalian systems, one catalytic subunit of PP1 dephosphorylates a number of physiological substrates. This seemingly paradoxical situation was resolved by the discovery of multiple regulatory subunits that target the same catalytic subunit to various subcellular locations and, therefore, distinct substrates. In addition, these regulatory subunits often change the specific activity of a protein phosphatase toward different substrates [9,21]. Thus, it is possible that ORF427 may serve as a regulator for shrimp PPs. Next, we will further characterize the functional interference of PPs by ORF427. Hopefully, further clarification of the natural substrates of shrimp PPs might present a novel target for anti-WSSV strategy.

It remains intriguing, since the standard pNPP assay detected comparably low phosphatase activity of the bacterially purified enzyme. It might partially be due to the limited knowledge on the reaction condition and substrate specificity of this enzyme. The lack of non-catalytic region within PPs might suggest that it was less strictly regulated compared with its human homolog; in other words, the activity of PPs might be more limited to certain specific substrates to maintain a balance between phosphorylation and dephosphorylation of proteins in the regulation of cellular activities. Further investigation is needed to address the substrate requirement for the shrimp PPs. Like its lambda homolog, PPs only contains

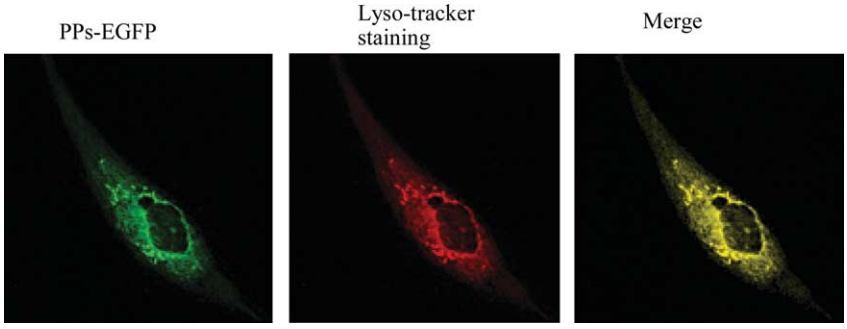

Fig. 5. Localization of PPs in the lysosome of shrimp PMO cells. PMO cells were transfected with plasmid expressing PPs-EGFP fusion protein. At 36 h after transfection, the cells were fixed and stained with lysotracker Red DND99, and observed under the confocal microscope with specific filter to detect the EGFP and Red DND99.

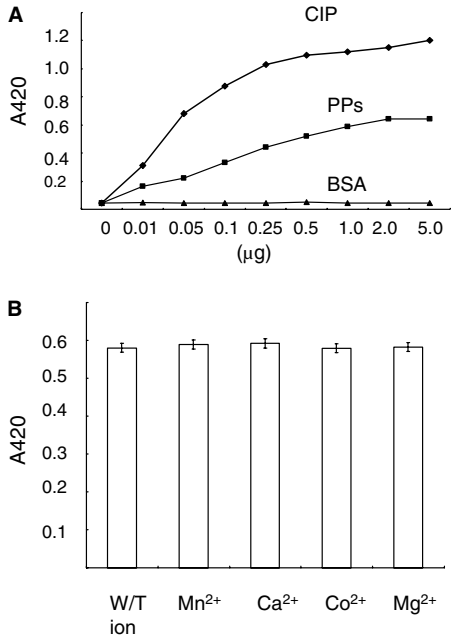

Fig. 6. Phosphatase activity of PPs towards pNPP. (A) Phosphatase activity was measured at varying concentrations of PPs and a fixed amount of pNPP (25 mM) in the presence of 20 mM $MnCl_2$. Data are presented as mean values from four independent assays. Alkaline phosphatase (CIP) and purified BSA (BioLabs) served as positive and negative controls, respectively. (B) Phosphatase activity was measured at fixed concentrations of PPS (1 μg) and pNPP (25 mM) in the presence of different ions. The concentration of the ions was fixed at 20 nm; reaction mixture without any of the mentioned ions served as control here.

amino acid ligands for one metal ion (ASP63, His65, and ASP91) as human protein phosphatase 1; while it lacks a His247 in the amino acid ligands for another metal ion (Asn91, Asn123, His172, and His247) [7]. We do not know whether some other unique PPs residue may serve the function of

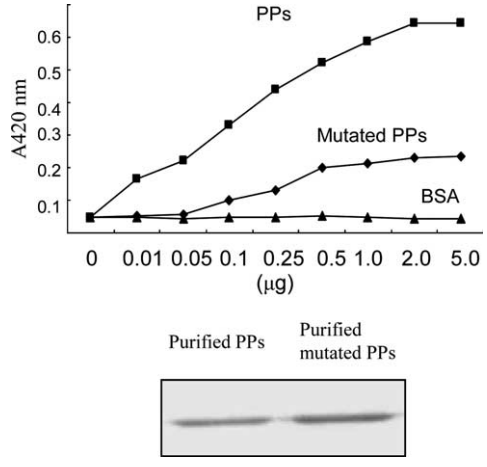

Fig. 7. Mutated PPs shows significantly lower phosphatase activity toward pNPP in the standard protein phosphatase assay. Phosphatase activity was measured at varying concentrations of PPs and a fixed amount of pNPP (25 mM) in the presence of 20 mM $MnCl_2$. Data are presented as mean values from four independent assays. BSA served as negative control.

His247 to make PPs bind two ion metals as human PP1. In our in vitro assay toward pNPP, Mg^{2+}, Ca^{2+}, or Mn^{2+} was found not able to increase the phosphatase activity for PPs; but these do not necessarily mean that ions were not playing a role in regulating the function of PPs in vivo.

Crystallographic analysis of eukaryotic Ser/Thr phosphatase PP1, which contains 327 aa, has revealed the structure of the catalytic site and indicated possible roles for a number of invariant residues in catalysis and/or metal ion binding [17–19]. Interestingly, the identified shrimp protein phosphatase has only 199 aa and lacks the C-terminal non-catalytic domains in its human counterpart. The known minimal protein phosphatase so far is the protein phosphatase encoded by coliphage lambda (PPλ) of 221 aa, which lacks both the N and C-terminal non-catalytic domains in its human counterpart [19]. Multiple alignment of the primary sequences of known Ser/Thr phosphatases has revealed a highly conserved central region of about 250 residues that contained three invariant stretches of amino acids, viz. GDXHG, GDXVDRG, and RGNHE [7,19,20]. Since PPs contains all these three invariant domains, PPs may represent a minimum size of protein phosphatases.

Acknowledgements: We thank Mr. Wong Lin Sheng (Genomax, Singapore) for his technical assistance in preparing the shrimp cDNA library. This study was supported by grants from the Temasek Life Sciences Laboratory of Singapore.

References

[1] Yang, F., Wang, W., Chen, R.Z. and Xu, X. (1997) J. Virol. Methods 67, 1–4.
[2] van Hulten, M.C., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R.K. and Vlak, J.M. (2001) Virology 286, 7–22.
[3] Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X. and Xu, X. (2001) J. Virol. 75, 11811–11820.
[4] Khadijah, S., Neo, S.Y., Hossain, M.S., Miller, L.D., Mathavan, S. and Kwang, J. (2003) J. Virol. 77, 10162–10167.
[5] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[6] Barford, D. (1996) Trends Biochem. Sci. 21, 407–412.
[7] Cohen, P.T. and Cohen, P. (1989) Biochem. J. 260, 931–934.
[8] Luan, S. (2003) Annu. Rev. Plant Mol. Biol. 54, 63–92.
[9] Luan, S. (2000) Adv. Bot. Res. 32, 67–107.
[10] Ansai, T., Dupuy, L.C. and Barik, S. (1996) J. Biol. Chem. 271, 24401–24407.
[11] Barik, S. (1993) Proc. Natl. Acad. Sci. USA 90, 10633–10637.
[12] Zhou, B. and Zhang, Z.Y. (2003) Methods Enzymol. 366, 34–43.
[13] Zhulidov, P.A., Bogdanova, E.A., Shcheglov, A.S., Vagner, L.L., Khaspekov, G.L., Kozhemyako, V.B., Matz, M.V., Meleshkevitch, E., Moroz, L.L., Lukyanov, S.A. and Shagin, D.A. (2004) Nucleic Acids Res. 32 (3), e37.
[14] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[15] Cheng, G., Yang, K. and He, B. (2003) J. Virol. 77, 10154–10161.
[16] Ammosova, T., Jerebtsova, M., Beullens, M., Voloshin, Y., Ray, P.E., Kumar, A., Bollen, M. and Nekhai, S. (2003) J. Biol. Chem. 278, 32189–32194.
[17] Goldberg, J., Huang, H.B., Kwon, Y.G., Greengard, P., Nairn, A.C. and Kuriyan, J. (1995) Nature 376, 745–753.
[18] Barton, G.J., Cohen, P.T. and Barford, D. (1994) Eur. J. Biochem. 220, 225–237.
[19] Zhuo, S., Clemens, J.C., Hakes, D.J., Barford, D. and Dixon, J.E. (1993) J. Biol. Chem. 268, 17754–17761.
[20] Zhuo, S., Clemens, J.C., Stone, R.L. and Dixon, J.E. (1994) J. Biol. Chem. 269, 26234–26238.
[21] Damer, C.K., Partridge, J., Pearson, W.R. and Haystead, T.A. (1998) J. Biol. Chem. 273, 24396–24405.