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RESEARCH REPORT

Identification and characterization of a cyclophilin A gene from Chinese shrimp *Fenneropenaeus chinensis*: sequence features and expression profiles

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Abstract

Cyclophilin A (CypA), a key member of the immunophilin family, is the most abundantly expressed one among the 16 identified cyclophilins. Besides acting as an intracellular receptor for cyclosporine A, CypA plays a vital role in various pathological responses such as inflammation and microbial infections. In the present study, the full-length cDNA of *Fenneropenaeus chinensis* CypA (*Fc*CypA) was cloned by RACE technique. The complete sequence of *Fc*CypA cDNA contained a 5' untranslated region (UTR) of 33 bp, a 3' UTR of 341 bp with a polyA tail, and an open reading frame (ORF) of 495 bp encoding a polypeptide of 164 amino acids with the predicted molecular weight of 17.70 kDa. The amino acid sequence of CypA has a typical peptidyl prolyl cis-trans isomerase family tag sequence (YKGSTFHRVIPNFMCQGG) as those of other species. The mRNA transcripts of *Fc*CypA were constitutively expressed in all the tested tissues, including eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach, with the highest expression level in hepatopancreas. The mRNA expression profiles of *Fc*CypA in hemocytes and hepatopancreas could be significantly induced by the stimulation of *Vibrio anguillarum* suspension and white spot syndrome virus (WSSV) stock, which verifies that CypA has an effect on viral and bacterial infection in shrimps. These results indicate that CypA may be involved in the innate immunity, biological immune response and physiological stress response of Chinese shrimps.

Key Words: Cyclophilin A; *Fenneropenaeus chinensis*; innate immunity; *Vibrio anguillarum*; white spot syndrome virus

Introduction

Generally, elementary conformational transitions in protein folding and protein restructuring take place spontaneously (Schiene-Fischer *et al*., 2011). peptidyl prolyl cis-trans isomerase domain (PPIase) basically catalyzes the inter conversion between Cis and Trans isomers of N-terminal amide bond of Proline (Davis *et a*l., 2010), which can be divided into four structurally unrelated families: cyclophilins (Cyps), FK506-binding proteins (FKBPs), parvulins and the recently identified protein Ser/Thr phosphatase 2A (PP2A) activator (PTPA). Cyclophilins have been the subject of intense research because they are cellular targets for the clinically used immunosuppressive drugs cyclosporin A (CsA), an immunosuppressant which was used to suppress rejection after organ transplantations (Lu *et al.*, 2007).

Cyclophilins, also known as immunophilins, are a family of highly conserved multifunctional proteins widely distributed in nature and have been found in mammals, plants, insects, fungi, and bacteria. (Göthel and Marahiel, 1999). So far, a variety of different cyclophilins have been identified, various cyclophilins perform different functions by targeting unique domains of specific organelles (Lee, 2013). 16 different types of cyclophilins have been identified in human, and the main 7 cyclophilin subtypes include cyclophilin A (CypA), cyclophilin B (CypB), cyclophilin C (CypC), cyclophilin Protein D (CypD),

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cyclophilin E (CypE), cyclophilin 40 (Cyp40) and cyclophilin NK (CypNK). Drosophila has 9 cyclophilins, while 8 are found in *Saccharomyces cerevisiae* (Kumari *et al.*, 2013).

Among them, CypA is the most widely distributed, exists in almost all prokaryotic and eukaryotic cells, act in Cytosol and nucleus, has a variety of biological activities, and plays an important role in the immune system (Harikishore and Sup Yoon, 2015). With the increasing research efforts on CypA, its biological role is recognized steadily. It participates in many biological processes, such as protein folding (Fruman *et al*., 1994), cell growth (Obchoei *et al*., 2011), cholesterol metabolism (Smart *et al*., 1996), protein trafficking in cells and regulation of immune function of the body (Hoffmann and Schiene-Fischer, 2014; Li *et al*., 2016). CypA is also a direct inflammatory cell chemotactic substance (Dawar *et al*., 2017). At present, high expression of CypA has been detected in various inflammatory diseases, such as atherosclerosis (Coppinger *et al*., 2004), rheumatoid arthritis (Kim *et al*., 2005), sepsis and inflammatory heart disease (Dear *et al*., 2007; Seizer *et al*., 2013). Additionally, accumulating evidences indicated that CypA plays an important role during viral infection (Chatterji *et al*.,2009), CypA may either promote or inhibit virus replication, depending on the host cell type and the viral species. CypA can promote the secretion of host interferon (INF-β) and inhibit rotavirus replication, thereby reducing the risk of rotavirus infection (He *et al*., 2012). CypA is a component of dynamic actin-rich structures formed during bacterial infection and within cells in general and may be involved in pathogenic processes in several bacteria or membrane translocation of bacterial toxins (Dhanda *et al*., 2018). Studies have reported that CypA may be the potential receptor of MgPa, which mediates the adhesion and invasion of *Mycoplasma genitalium* to human urothelial cells (Deng *et al*., 2018). CypA also affects parasite infection in different ways, for example, TcCyP19, a human CypA, may be part of a complex interaction between parasites and host cells (Perrone *et al*., 2018). These demonstrate the unique role of CypA in the replication and infection of different viruses, pathogens, mycoplasmas and parasites. At present, CypA gene is one of the hotspots of current research, and it is of great significance to use CypA as a molecular target for new drugs, animal and plant stress resistance breeding, etc. The latest research shows that CypA may have immune stress function in some aquatic organisms. However, the basic and applied research of cyclophilin A in aquatic animals is still very limited and the value of this gene has not been fully exploited.

The Chinese shrimp *F. chinensis* is one of the most commercially important cultured shrimp species in China. It is mainly distributed in the Yellow Sea and Bohai Sea of China, and west and south coast of the Korean Peninsula (Wang *et al*., 2017). With the development of intensive culture and environmental deterioration, various diseases caused by bacteria, viruses and rickettsia-like organisms had frequently occurred in cultured shrimp populations, which resulted in enormous losses to this aquaculture industry. A better understanding of shrimp immune defense mechanisms is necessary for shrimp health management, and the CypA gene plays an important role in microbial infections and body's innate immunity. The main objectives of this study were to (1) clone the full-length cDNA of CypA from *F. chinensis*, (2) investigate the tissue distribution of *Fc*CypA transcripts, and (3) its temporal distribution after microbial challenge.

Table 1 Oligonucleotide primers used in the experiments

Primer	Sequence (5'-3')	Tm (°C)	Information
Fc-CypA-RACE-F1	CTCAGAACATTCCGCCTTAGCCGC	66.2	1st PCR for 3'-RACE (forward)
Fc-CypA-RACE-F2	TATAATCTTTGCTGTATTGGCACTTCAGTG	65.9	2nd PCR for 3'-RACE (forward)
Fc-CypA-RACE-R1	GAATTTGTTGCCGTAGATGGACTTGCC	66.1	1st PCR for 5'-RACE (reverse)
Fc-CypA-RACE-R2	TCACGCGGTGGAAGCACGAGCCCT	68.2	2nd PCR for 5'-RACE (reverse)
Adaptor-oligo(dG)	GGCCACGCGTCGACTAGTACG_{10}HN	-	Anchor primer for 5'-RACE
Adaptor-oligo(dT)	GGCCACGCGTCGACTAGTACT_{18}VN	-	cDNA synthesis for 3'-RACE
Fc-CypA-CDS-F	ATGGGCAATCCCAAAGTCTTTTTCGAC	66.6	Gene specific primer for sequence identification
Fc-CypA-CDS-R	TTACAGCTGGCCGCAGTTGGCGATCATCAC	69.2	Gene specific primer for. sequence identification
Fc-CypA-qRT-F	TTACAAACCTACGCCAACTGAACC	62.6	Gene specific primer for real-time PCR
Fc-CypA-qRT-R	CTCCATGATGATCCTGCCAACTG	64.1	Gene specific primer for real-time PCR
Fc-18S-qRT-F	TATACGCTAGTGGAGCTGGAA	55.7	Internal control for real-time PCR
Fc-18S-qRT-R	GGGGAGGTAGTGACGAAAAAT	57.6	Internal control for real-time PCR
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	64.5	Vector primer for seqvence
RV-M	GAGCGGATAACAATTTCACACAGG	62.9	Vector primer for seqvence

Materials and methods

Experimental animals, microbes challenge and samples collection

The Chinese shrimp used in the present study were obtained from Ruizi Seafood Development Co. Ltd., Qingdao, China White shrimps, with body weight 8 - 12 g, were cultured placed in 640 L cylindrical tanks with 500 L air-pumped circulating seawater at 20 ± 1 °C for two weeks before processing. Hemolymph was extracted from the ventral sinus of at least three untreated shrimps using a sterile syringe preloaded with equal volume of anticoagulant buffer (NaCl 510 mmol L^{-1}, glucose 100 mmol L^{-1}, citric acid 200 mmol L^{-1}, tri-sodium citrate 30 mmol L^{-1} and EDTA·2Na 10 mmol L^{-1}, pH 7.3). Then the hemocytes were collected by centrifugation at 800 g for 10 min at 4 °C. Tissues including eyestalk, gill, gonad, heart, hepatopancreas, intestine (mid gut), muscle, nerve and stomach were collected from at least three untreated shrimps, kept in RNAlater (AM7020, Thermo Fisher Scientific, USA) and stored at -80 °C until RNA isolation.

Approximately 200 shrimps were employed for microbe stimulation assay, and they were randomly divided into three groups which contained about 60 - 70 individuals. The *V. anguillarum* suspension and WSSV stock were prepared according to previous reports (Sha *et al*., 2016; Xia *et al*., 2015). The shrimps were received an injection at the abdominal segment with 100 μL phosphate buffered saline (PBS, pH 7.4, 10010023, Thermo Fisher Scientific, USA), *V. anguillarum* suspension (1×10^4 CFUs μL-1, in PBS) and WSSV stock (1×10^4 copies μL-1, in PBS), respectively. After stimulation, samples were taken from each group at 0 h, 3 h, 6 h, 12 h, 24 h, 36 h and 48 h, with 5 replications at each time point, and each replication was a mixture of 3 individuals. The hemocytes and hepatopancreas from each sample was collected and stored at -80 °C for RNA extraction.

Total RNA isolation and cDNA synthesis

Total RNA of ten tissues was isolated with TRIzol Reagent (15596026, Thermo Fisher Scientific, USA) following manufacturer protocol and a DNase I (RQ1, M6101, Promega, USA) treatment was carried out to eliminate genomic DNA contamination. RNA degradation and contamination were monitored on 1% agarose gels. The concentration and purity of RNA were measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). RNA samples and adaptor primer-oligo (dT) were used as template and primer to synthesize the first strand of cDNA (for gene cloning), which carried out with Promega M-MLV. The reaction mixture was incubated at 42 °C for 1 h, terminated by heating to 95 °C for 5 min, and then stored at -80 °C until analysis as template.

Cloning the full-length cDNA of Fc*CypA*

One EST (BM302619) of 740 bp from the cDNA library was homologous to previously identified CypAs (Zhang *et al.*, 2010), and it was selected for further cloning of CypA cDNA from *F. chinensis* (designated as *Fc*CypA). The 5′-terminal- region of CypA cDNA was determined by 5′-RACE, primers Fc-CypA-RACE-R1 were used for the first PCR and Fc-CypA-RACE-R2 was used for the second PCR (Table 1). The 3'-terminal-region of CypA cDNA was determined by 3'-RACE, which primers Fc-CypA-RACE-F1 were used for the first PCR and Fc-CypA-RACE-F2 was used for the second PCR (Table 1) , which used for the synthesis of single-stranded cDNA by reverse transcription. The PCR products were gel-purified using Monarch DNA Gel Extraction Kit (T1020S, NEB, USA) and cloned into the pMD18-T simple vector (D103A, Takara, Japan). After being transformed into the competent cells of *Escherichia coli* strain DH5α (CB101, Tiangen, China), the positive recombinants were identified through anti-Amp selection and PCR screening with M13-47 and RV-M primers (Table 1). Then three positive clones were picked for sequencing.

Sequence analysis

SignalP 3.0 program was utilized to predict the presence and location of signal peptide (https://www.cbs.dtu.dk/services/SignalP/). The glycoslanation were performed with the(http://www.cbs.dtu.dk/services/NetNGlyc/). The protein motif features were predicted by Simple Modular Architecture Reasearch Tool (SMART) version 7.0 (http://smart.embl-heidelberg.de/) and InterPro (https://www.ebi.ac.uk/interpro/).

Multiple sequences alignment and phylogenetic analysis

Multiple sequence alignment of *Fc*CypA and other reported Cyps was performed with ClustalW multiple alignment program (http://www.ebi.ac.uk/Tools/clustaw2/) and multiple alignment show program (http://www.bioinformatics.org/sms/). An unrooted phylogenic tree was constructed based on the deduced amino acid sequences of *Fc*CypA and other reported Cyps by the neighbour-joining (NJ) algorithm using the MEGA 7.0 software (http://www.megasoftware.net). To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times. The amino acid sequences information for homologous and phylogenetic analysis was shown in Table 2.

Expression analysis of Fc*CypA genes by RT-qPCR*

The mRNA transcriptional levels of *Fc*CypA in different tissues and its temporal expression pattern in hemocytes and hepatopancreas of Chinese shrimps stimulated with *V. anguillarum* suspension and WSSV stock were determined by quantitative real-time PCR using a LineGene K FQD-48A (A4) Fluorescence Quantitative PCR Detection System (Bioer, China). All qPCR reactions were performed with SYBR premix ExTaq (Tli RNaseH plus) (RR420, Takara, Japan) using about 100 ng cDNA template and 0.2 μM of each primer. Amplification conditions were as follows: 94 °C for 10 s followed by 40 cycles of 94 °C for 5 s, 60 °C for 30 s (to acquire fluorescence). The expression of FcCypA was normalized to the expression of 18S rDNA gene for each sample. All the primers for qRT-PCR were designed using PerlPrimer 1.1.21 and listed in Table 1.

Table 2 Species and accession number of CypAs used in the homologous and phylogenetic analysis

Scientific	Query Cover	Identity	E-value	Accession
Fenneropenaeus chinensis	100%	100%	1e-123	ANH58180.1
Penaeus monodon	100%	98.78%	3e-116	AGS46493.1
Penaeus vannamei	100%	95.12%	4e-113	ADY69343.1
Penaeus japonicus	100%	94.51%	9e-113	XP_042880185.1
Scylla paramamosain	100%	86.59%	2e-102	AEN94575.1
Eriocheir sinensis	100%	85.37%	1e-101	ADF32017.1
Danio rerio	100%	77.44%	3e-95	NP_001315353.1
Ictalurus punctatus	100%	78.66%	3e-96	NP_001187167.1
Azumapecten farreri	100%	76.22%	2e-98	AAR11779.1
Argopecten irradians	100%	76.22%	3e-97	ABM92916.1
Xenopus laevis	100%	71.95%	1e-89	AAH97540.1
Gallus gallus	99%	71.95%	1e-88	ACX31829.1
Homo sapiens	99%	75.61%	8e-93	AAU13906.1
Mus musculus	100%	75.00%	7e-93	AAH99478.1

Statistical analysis

All data were given in terms of relative mRNA expression as means ± standard deviation (SD). The expression level of *Fc*CypA mRNA was estimated using the comparative CT method ($2^{-\Delta\Delta Ct}$). Gene expression data were subjected to two-factor analysis of variance (ANOVA) using SPSS Statistic 26.0 software where values of $p < 0.05$ were considered significantly different.

Results

Cloning and characterization of Fc*CypA cDNA*

The full-length cDNA sequence of *Fc*CypA was obtained by 3' RACE technique and deposited in GenBank under the accession number KU361825. It comprised 869 bp, containing a 5' untranslated regions (UTR) of 33 bp, a 3' UTR of 341 bp with a poly A tail and an open reading frame (ORF) of 495 bp encoding a polypeptide of 164 amino acids with the predicted molecular weight of 17.70 kDa and theoretical isoelectric point of 8.55. It has 17 strong basic amino acids (Arg + Lys), 14 strongly acidic amino acids (Asp + Glu). In 3´UTR (untranslated region), the sequence has polyadenylation signal (AATAAA). There was no availability of a signal peptide revealed by SignalP program analysis. The glycosylation prediction showed that *Fc*CypA is characterized by presence of six N-glycosylation sites (N3, N71, N106, N108, N149 and N160). A highly conserved signature sequences of peptidyl-prolyl cis-trans isomerase (48YKGCAFHRVIPNFMCQGG65) and a pro-isomerase domain (from F7 to Q163) was identified in the amino acid sequence of *Fc*CypA (Fig. 1).

Multiple alignment and phylogeny relationship of Fc*CypA*

The deduced amino acid sequence of *Fc*CypA shared significant homology with other reported CypAs, such as 98.78 % identity with *P. monodon*, 95.12 % with *P. vannamei*, 94.51 % with *P. japonicus*, and 85.37 % with *E. sinensis* (Fig. 2). Fourteen amino acid residues critical for the fundamental structure and function of the CypA PPIase activity, including R55, I57, F60, M61, Q63, G72, A101, N102, A103, Q111, F113, W121, L122 and H126, were all conserved in *Fc*CypA (Fig. 2). The construction of NJ phylogenetic tree from multiple tetraspanins was based on the amino acid sequences of 14 CypA and separated into four branches, *Fc*CypA was clustered with its homologue as one of branches, arthropod, other branches teleost, mollusc and vertebrate (avian, amphibian and mammalian). Among them, it is most closely related to several species of shrimps and crabs, followed by fish and shellfish, and the most distant relatives of mammals (Fig. 3).

The tissue distribution of Fc*CypA mRNA*

The mRNA expression level of *Fc*CypA in ten different tissues of *F. chinensis* was quantified by real-time PCR with the 18S rDNA gene as an internal control. The *Fc*CypA mRNA transcripts could be detected in all the tested tissues, and the relative expression level was from high to low in hepatopancreas, hemocytes, gill, nerve, intestine,

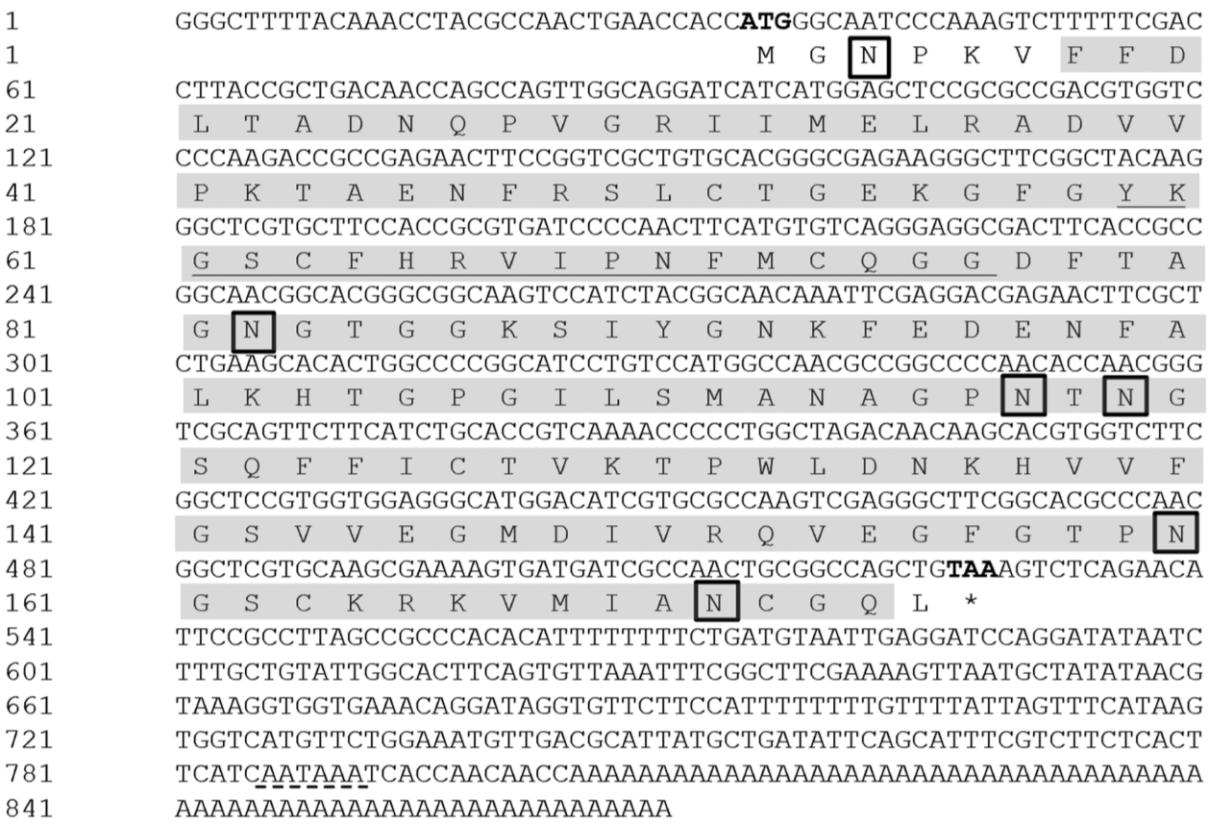

Fig. 1 Nucleotide and deduced amino acid sequence of *Fc*CypA. The nucleotides and amino acids were numbered along the left margin. The function domain was in shade. The start and codon was marked in bold, the asterisk indicated the stop codon and the classical polyadenylation signal in the 3'-UTR is dotted underlined. The Cyp-type peptidylprolyl cis-transisomerase signature was indicated in underline. The black boxes are showing the glycosylation sites.The amino acid sequence of *Fc*CypA has been submitted to GenBank with the accession number KU361825

gonad, heart, stomach, eyestalk, muscle. The highest mRNA expression level was found in hepatopancreas, which was 309.28-fold ($p < 0.05$) of that in muscle, followed by hemocytes, gill and nerve, which were 15.34-fold, 4.60-fold and 2.87-fold of that in muscle ($p < 0.05$), respectively (Fig. 4).

The temporal expression of Fc*CypA mRNA after microbe challenge*

The *Fc*CypA mRNA expression levels of hemocytes and hepatopancreas were all up-regulated post the two kinds of microbe stimulation. The *Fc*CypA mRNA expression level of hemocytes was significantly up-regulated at 6 h post *V. anguillarum* stimulation (4.25-fold compared with the origin level, $p < 0.05$), and the highest level was observed at 12 h (13.42-fold, $p < 0.05$, Fig. 5a). While after stimulated with WSSV stock, the *Fc*CypA mRNA expression level of hemocytes significantly increased at 3 h post stimulation (2.30-fold, $p < 0.05$) and reached the peak at 24 h (3.94-fold, $p < 0.05$) and then decreased to the origin level at 36 h (Fig. 5a). In the hepatopancreas group, the mRNA transcripts of *Fc*CypA significantly increased at 6 h post *V. anguillarum* stimulation (2.71-fold, $p < 0.05$) and reached the maximum level at 12 h (3.61-fold, $p < 0.05$) and then down to the normal level at 24 h (Fig. 5b). While the mRNA expression level of *Fc*CypA was reached the peak at 6 h (6.08-fold, $p < 0.05$) post WSSV stimulation (6.21-fold, $p < 0.05$) and then down to the normal level at 48 h (Fig. 5b).

Discussion

Cyclophilin A is a highly conserved important protein that widely exists in prokaryotes to higher mammals. The two most important biological functions are currently known: one is PPIase activity, which can catalyze the process of protein folding and transport and play the role of molecular chaperone. The second is the immunosuppressive function, which can mediate in vivo. It is involved in a variety of physiological and biochemical reactions such as tumor proliferation, inflammation, virus infection, cell apoptosis, and various physical and biological environmental stress responses.

In the present study, the full-length cDNA sequence of *Fc*CypA was obtained by 3' RACE technique. The full length of the sequence is 869 bp, including the 5'-end untranslated region (UTR) of 33

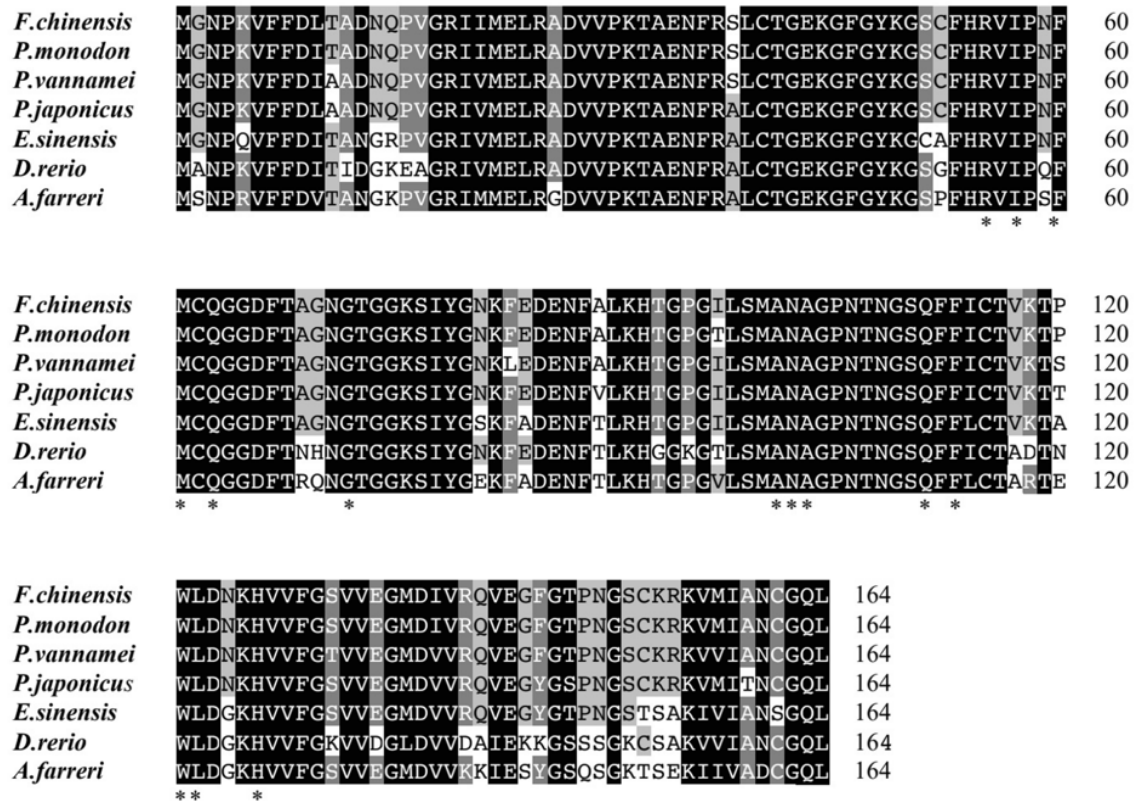

Fig. 2 Multiple alignment of *Fc*CypA with other CypAs deposited in GenBank. Thesame amino acid residues were shaded inblack and the similar amino acids wereshaded in grey. Gaps were indicated bydashes to improve the alignment. The sequences and their accession numbers are as follows: *Fenneropenaeus chinensis* (ANH58180.1), *Penaeus monodon* (AGS46493.1), *Penaeus vannamei* (ADY69343.1), *Penaeus japonicus* (XP_042880185.1), *Eriocheir sinensis* (ADF32017.1), *Danio rerio* (NP_001315353.1), *Azumapecten farreri* (AAR11779.1)

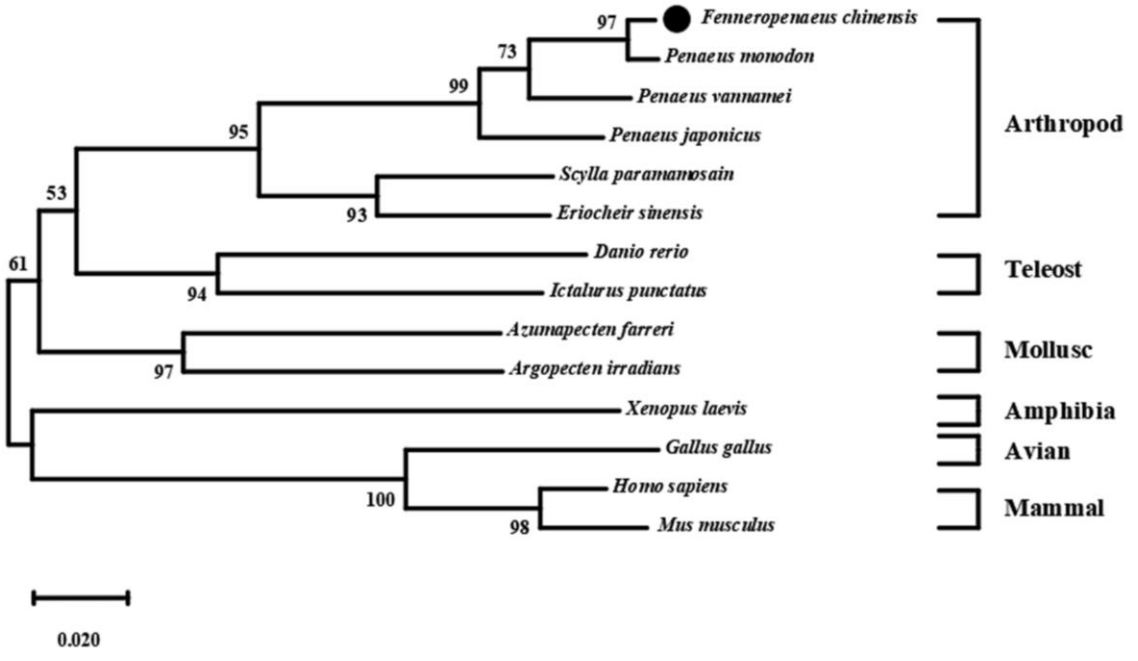

Fig. 3 Neighbor-Joining (NJ) phylogenic tree of *Fc*CypA constructed based on the protein sequences from different organisms. To derive confidence value for the phylogeny analysis, bootstrap trials were 1000 replicates. The black round indicated the tetraspanin protein of *Fc*CypA The numbers at the forks indicated the bootstrap value

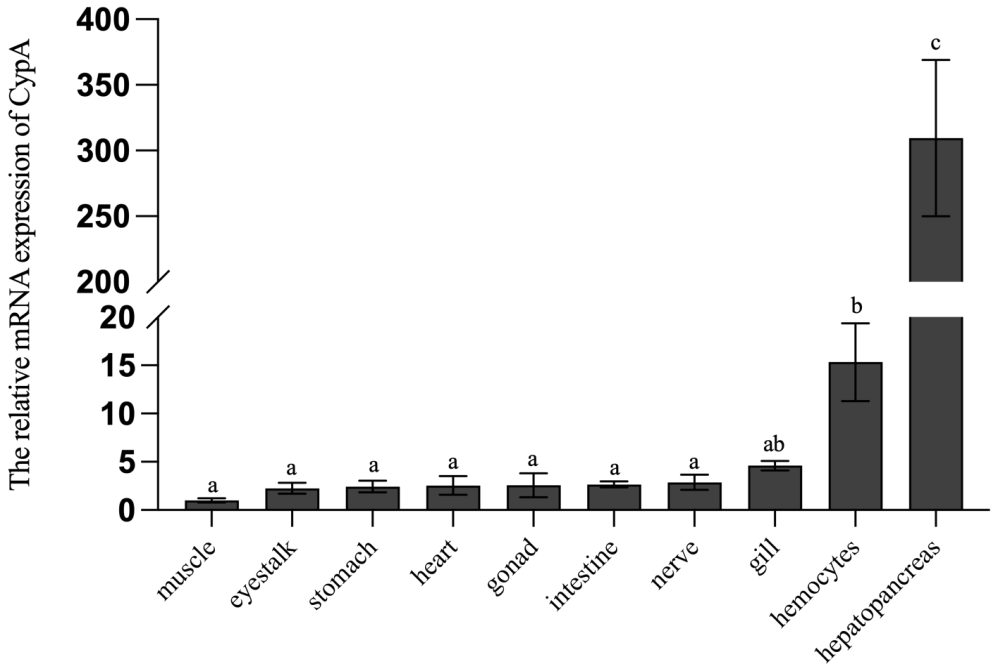

Fig. 4 Relative expression of *Fc*CypA gene in ten tissues. The mRNA transcripts levels in muscle, eyestalk, stomach, heart, gonad, intestine, nerve, gill, hemocytes and hepatopancreas of three untreated shrimps were normalized to that of muscle. The 18S rDNA gene was used as an internal control to calibrate the cDNA template for each sample. Each vertical bar represents the mean ± SD (n = 5), and Different letters indicate significant differences ($p < 0.05$)

bp and the 3'-end untranslated region (UTR) of 341 bp. The length of the open reading frame is 495 bp, which can encode 164 amino acid residues. The 3'UTR region contains typical polyadenylation tailed signal sequences AATAAA and PolyA tail. The primary sequence structural features of the CypA gene of *F. chinensis* were basically the same as those of other species. The BLAST protein alignment showed that there were 14 highly conserved amino acid residues in the amino acid sequence of CypA of *F. chinensis.* Like CypA of other known species, PPIase was found in the encoded amino acid sequence, and no signal peptide sequence was detected at its N-terminus. This site is very conserved in both invertebrates and vertebrates, and it is located between the 48th and 65th bases (YKGSTFHRVIPNFMCQGG). Sequence analysis found that it has 8 amino acid residues, which is the binding site of cyclosporin A and the active center of PPIase. In the evolutionary analysis, the phylogenetic tree analysis results showed that it is most closely related to several species of shrimps and crabs, followed by fish and shellfish, and mammals are the most distant, which is in line with the evolutionary sequence of animals and has a high degree of conservatism. We obtained a new CypA gene from *F. chinensis*, and analyzed its sequence characteristics, laying a foundation for further study of its functional characteristics.

In this study, the distribution and expression of *Fc*CypA in different tissues were detected, and it was found that it was expressed in various tissues, with the highest expression in hepatopancreas, followed by hemocytes, gill, nerve, intestine, gonad, heart, stomach and eyestalk, and the lowest expression in muscle. This is consistent with the widespread tissue distribution of cyclophilin A in other animals (Tu *et al*., 2003; Qiu *et al*., 2009; Chen *et al*., 2011; Wang *et al*., 2013; Muhammad *et al*., 2017). The ubiquity of *Fc*CypA transcripts in different tissues indicated that it could be potentially involved in some important physiological processes and played important roles in the basal metabolism of shrimp cells. Muhammad *et al*. found that the tissues with CypA expression from high to low in *Litopenaeus vannamei* were muscle, gill, lymphoid organ and hepatopancreas in order (Muhammad *et al*., 2017). In previous reports, the expression level of CypA was found to be highest expressed in hepatopancreas and haemolymph of shrimp *Penaeus monodon* (Qiu *et al*., 2009), while in gonad and gill of scallop *Chlamys farreri* (Song *et al.*, 2009). There are also some studies on CypA transcriptional expression in fish, mollusks and amphibians, overall, no consistent pattern of CypA gene expression was found among tissues in different species (Dorfman *et al*., 1997; Tu *et al.*, 2003; Massé *et al*., 2004). In the present study, *Fc*CypA was highest expressed in hepatopancreas, and it was suspected that *Fc*CypA might play an important role in the immune defence system of shrimp, as hepatopancreas was regarded as the main immune center in crustaceans and many

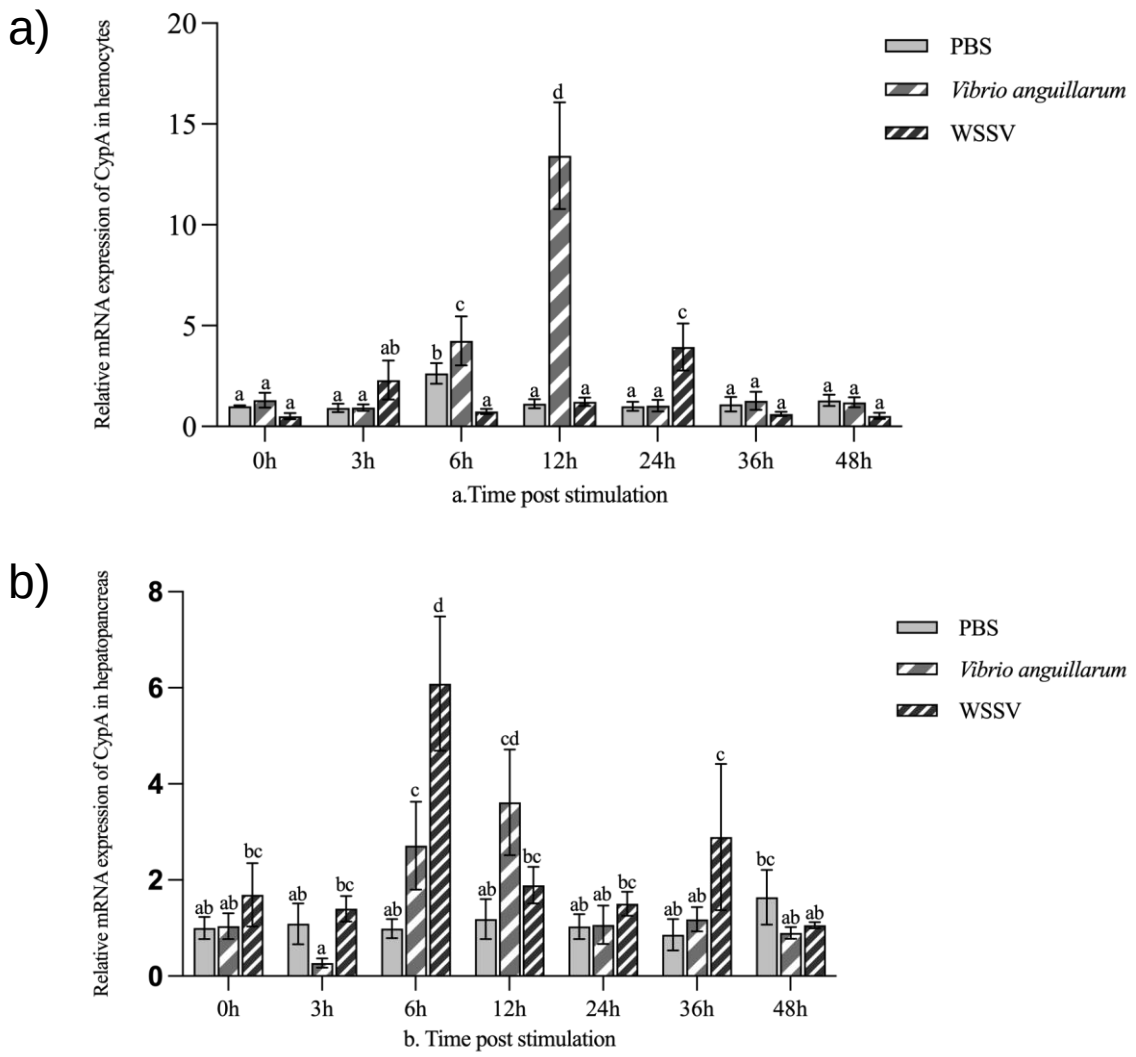

Fig. 5 Relative expression of *Fc*CypA gene in hemocytes(a) and hepatopancreas(b) after microbe challenge *V. anguillarum* suspension and WSSV stock. Sampling times at 0, 6, 12, 24, 36, 7 and 48 hr.18S rDNA gene was used as an internal control to calibrate the cDNA template for all the samples. Each vertical bar represents the mean ± SD (n = 5), and Different letters indicate significant differences ($p < 0.05$)

immune-related genes were high expressed in this tissue (Gai *et al*., 2009; Lu *et al*., 2012). The higher expression level of *Fc*CypA is hemocytes，which also play an important role in the immune defense system of animals, especially for invertebrates that have no adaptive immune system (Yang *et al*., 2015; Koiwai *et al*., 2018). It further proved the importance of CypA gene in innate immunity in *F. chinensis*.

Many studies have demonstrated that CypA is involved in a variety of pathological states, including infection and inflammation. In order to further explore the function of *Fc*CypA, we infected Chinese shrimps with *V. anguillarum* suspension and WSSV stock and measured the relative expression levels of mRNA in the two tissues. In other studies, the relative mRNA expression of CypA of *P. monodon* was up regulated after stimulated by LPS (Qiu *et al*., 2009), significant upregulation of relative mRNA expression levels of *Eriocheir sinensis* CypA was observed throughout the fungal challenge (Wang *et al.*, 2013), slightenhancement of CypA protein was observed in the liver, spleen, body kidney and head kidney of *Pelteobagrus fulvidraco* infected with *Edwardsiella ictalurid* (Dong *et al*., 2015), after bacterial challenge, the expression level of CypA of *C. farreri* was almost unchanged in haemocytes, but up-regulated in gonad and increased to the peak at 4 h post-injection (Song *et al.*, 2009). These results indicated that CypA gene plays an important role in the innate immune system of aquatic organisms. In this study, under the infection of *V. anguillarum* suspension and WSSV stock, the relative mRNA expression of CpyA gene of *F. chinensis* in hepatopancreas was significantly up-regulated between 6 h and 12 h, and gradually returned to the initial expression level from 12 h to 48 h. Similarly,

the *Fc*CypA mRNA expression highest level of hemocytes was observed at 12 h and 24 h after the stimulation of *V. anguillarum* suspension and WSSV stock, respectively. It demonstrates that the mRNA expression profiles of *Fc*CypA in hemocytes and hepatopancreas could be significantly induced by the stimulation of *V.anguillarum* suspension and WSSV stock, further validating the important role of CypA in viral and bacterial infections in shrimps. All these results indicated that *Fc*CypA was a typical CypA member and was potentially involved in the body's innate immunity, biological immune response and physical stress response in Chinese shrimps.

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