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Short communication

Molecular cloning and characterization of two isoforms of cyclophilin A gene from *Venerupis philippinarum*

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ABSTRACT

Cyclophilin A (CypA) is a ubiquitously distributed intracellular protein belonging to the immunophilin family, which is recognized as the cell receptor for the potent immunosuppressive drug cyclosporine A. In the present study, two isoforms of cyclophilin A gene (named as VpCypA1 and VpCypA2) were isolated and characterized from *Venerupis philippinarum* by RACE approaches. Both VpCypA1 and VpCypA2 possessed all conserved features critical for the fundamental structure and function of CypA, indicating that the two isoforms of cyclophilin A should be new members of CypA family. The expression of VpCypA2 mRNA in haemocytes was significantly up-regulated and the highest expression level was detected at 96 h post-infection with 7.7-fold increase compared with that in the blank group. On the contrary, the relative expression level of VpCypA1 mRNA was down-regulated rapidly at 6 h post-infection and reached 0.4-fold of the control group. They exhibited different expression profile and identical effect of immune modulation, which might suggest the two VpCypA isoforms exert their function in a manner of synergy. These results provide valuable information for further exploring the roles of cyclophilin A in the immune responses of *V. philippinarum*.

1. Introduction

Cyclophilins (Cyps), which contain a single conserved peptidyl-prolyl cis-trans isomerases (PPIase) domain [1,2], are widely distributed in organisms as constitutive proteins. Due to their PPIase activity, Cyps carry out a wide range of functions, such as protein folding [3,4], receptor complex stabilization [5], apoptosis participation [6] and receptor signaling [7]. Various Cyps have been found in diverse organisms ranging from bacteria to humans [8–12]. In mammals, five classic Cyp isoforms (cyclophilin A, B, C, D and cyclophilin 40) have been reported with size ranging from 18 to 40 kDa [13–15].

Cyclophilin A (CypA) is a ubiquitously distributed intracellular protein belonging to the immunophilin family [16] and recognized as the host cell receptor for the potent immunosuppressive drug cyclosporin A [17]. CypA–CsA complex can bind intracellular proteins and play important roles in the immune system of mammals [18]. For example, CypA–CsA complex can bind and inhibit calcineurin in T-lymphocytes, which blocks the T cell signal transduction to achieve immunosuppression [19]. Although CypA was originally believed to exist solely as an intracellular protein, later studies have revealed that it can be secreted by cells in response to inflammatory stimuli [20]. The clinical importance of Cyps has been implicated in diverse pathological conditions, such as HIV [21], hepatitis B and C viral infection [22], atherosclerosis [23], vascular diseases [24] and Rheumatoid Arthritis pathogenesis [25]. Recent studies also revealed that CypA could modulate HIV-1 capsid disassembly and that changes in capsid stability could influence HIV-1 sensitivity to the inhibition of CypA binding [26]. In addition, some results suggested that CypA played an important role in the innate immune system of some aquatic animals. For example, CypA was deduced to be involved in the early infection of *Edwardsiella ictaluri* in channel catfish *Ictalurus punctatus* [27]. In shrimp *Penaeus monodon*, the expression of CypA in the hepatopancreas was up regulated after stimulated with lipopolysaccharide, indicating that CypA was involved in the defense response against the bacterial infections [28].

The Manila clam, *Venerupis philippinarum*, is an important marine bivalve for commercial fisheries, accounting for about 80% of mudflat fishery production in China (China Bureau of Fisheries, 2004). However, clam culture in china has been severely plagued by pathogenic microorganisms, which has caused serious economic losses [29]. Therefore, understanding the immune responses of clams against pathogen challenge has become essential. Previous studies

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on CypA mainly focused on its structure analysis [5–7,30–32]. Rare knowledge is available about the molecular characteristic and expression patterns, especially in invertebrates. In the present study, two full-length cDNAs of CypAs were cloned from *V. philippinarum*, and the expression profiles of VpCypA1 and VpCypA2 transcript characterization after bacterial challenge were also investigated, hopefully shedding light on the roles of CypAs in the immune responses of marine invertebrate.

2. Materials and methods

2.1. Clams and challenge

The clams *V. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from local culturing farm (Bohai Sea, Yantai, China) and acclimatized for 10 days before processing. The seawater was aerated continuously, and salinity and temperature maintained at 32 psu and 25 °C throughout the experiment. Clams were fed with *Chlorella vulgaris* Beij daily and the seawater was renewed daily. After the acclimatization, the clams were randomly divided into six flat-bottomed rectangular tanks and each tanks contained 50 individuals.

For *Listonella anguillarum* challenge experiment, one tank served as control. The other five tanks were challenged with high density of *L. anguillarum* with final concentration of 10^7 CFU mL^{-1}. Four individuals were randomly sampled at 6, 12, 24, 48 and 96 h respectively. The haemolymph from the control and the infected groups was collected using a syringe and centrifuged at 2000 × *g*, 4 °C for 10 min to harvest the haemocytes.

2.2. Cloning the full-length cDNA of VpCypA1 and VpCypA2

Two ESTs which shared homology to the previously identified CypA, were identified from the *V. philippinarum* haemocytes cDNA library. The 5′ and 3′ ends of VpCypA1 and VpCypA2 were obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech, USA) according to manufacturer's recommendations. The PCR products were gel-purified and subcloned into pMD18-T simple vector (Takara, Japan). After transformed into the competent cells of *Escherichia coli* Top10F′, three positive clones were sequenced on an ABI3730 Automated Sequencer (Applied Biosystem).

2.3. Sequence analysis

The cDNA and amino acid sequences of VpCypA1 and VpCypA2 were analyzed by using the BLAST algorithm at NCBI website (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The protein domain features were determined by using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). The percentage of similarity and identity of VpCypA1 and VpCypA2 with CypA proteins from other organisms was calculated by the Identity and Similarity Analysis program (http://www.biosoft.net/sms/index.html). Multiple alignments of VpCypA1 and VpCypA2 were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment Show program (http://www.bio-soft.net/sms/index.html).

2.4. Phylogenetic analysis

The deduced amino acid sequences of VpCypA1 and VpCypA2 were aligned with the corresponding cyclophilin sequences from various organisms using the Clustal X software. Based on this alignment, a phylogenetic tree was constructed with MEGA 4.0

Table 1
Primers used in the present study.

Primer Sequence	(5′–3′)	Sequence information
P1(forward)	CTCCCTTGAGAAGAGCTACGA	Real time β-actin primer
P2(reverse)	GATACCAGCAGATTCCATACCC	Real time β-actin primer
P3(forward)	AGGAGTCGGTTCCCAAAGTG	Real time VpCypA1 primer
P4(reverse)	TATTGCATCCGGGGGTAGGT	Real time VpCypA1 primer
P5(forward)	CTGGAGGCAAGAGCATTTATGG	Real time VpCypA2 primer
P6(reverse)	TCGTGACTACATCCATACCCTC	Real time VpCypA2 primer

A

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1                M  V  F  H  V  R  I  A  H  I  L  R  T  Q  G  V  R
1    AACGAAACCTCATGGTCTTCCACGTGCGAATCGCTCATATTTTGCGAACACAAGGAGTGC
18     Y  S  S  S  A  K  M  P  I  Q  T  F  F  D  I  T  I  G  G  K
61   GCTATTCATCATCTGCAAAAATGCCGATTCAGACATTCTTCGACATAACAATTGGTGGCA
38     P  A  G  R  I  V  M  E  L  N  N  E  V  V  P  K  T  A  E  N
121  AACCAGCTGGCAGAATTGTCATGGAGCTGAACAATGAAGTTGTTCCAAAAACTGCTGAAA
58     F  R  A  L  C  T  G  E  K  G  F  G  F  K  N  S  P  F  H  R
181  ATTTCCGTGCCTTGTGCACCGGAGAAAAAGGATTTGGATTTAAGAATTCCCCATTCCACC
78     V  I  P  G  F  M  C  Q  G  G  D  F  T  K  R  N  G  T  G  G
241  GTGTAATTCCAGGCTTCATGTGCCAAGGTGGAGATTTCACTAAACGAAATGGCACCGGAG
98     K  S  I  Y  G  N  K  F  A  D  E  N  F  I  L  K  H  D  G  E
301  GCAAAAGCATATATGGGAACAAATTTGCAGATGAAAACTTCATTCTCAAGCACGATGGTG
118    G  I  L  S  M  T  N  A  G  P  N  T  N  G  S  Q  F  F  L  C
361  AAGGAATTCTGTCCATGACAAATGCAGGTCCAAATACAAATGGATCACAGTTTTTCTTGT
138    T  A  K  T  P  W  L  D  G  K  H  V  V  F  G  R  V  T  S  G
421  GTACAGCAAAAACACCATGGTTGGATGGTAAACATGTTGTATTTGGTAGAGTTACAAGTG
158    M  D  V  V  K  K  I  E  G  V  G  S  Q  S  G  K  T  S  Q  A
481  GTATGGATGTTGTGAAGAAAATTGAAGGAGTCGGTTCCCAAAGTGGCAAAACTAGCCAGG
178    V  E  V  V  N  C  G  E  L  *
541  CTGTGGAGGTTGTTAACTGTGGAGAATTATAAATTTATTGTCTTCGAAGGATGTAGGACT
601  CACTAGGAGCAGTATTGTAAGCAAGAAAACTTTCATTGCCTACAATTTTTTTGCAATCAA
661  AATTTGCTATAAAATTGATCCAAATAATTGTTGGTATTTTATCTTTTAATTATATCTCAT
721  CTTTGCTAACAATACCTACCCCCGGATGCAATACCTTTTTTACTTACTTTTGTTTTTATA
781  AAAATTTATTATGGAATTCTATTCGTGTATCACGCGTTTTACAATAAATGACTCTTTACT
841  TGACCAAAAACAACTAATTAATCAAGTGAACGAAAAAAACATAAAATCAAGTTAAACGCT
901  TGTTGATTGATACTGTGAAAAATGAAATCCTGAAATATATATAATATAATTCTATTGATT
961  CATTTGGTACAATTCATTAAATTGCACCCTTCTTTTCTCTGTTTGTACTTTAATCCACCC
1021 AAAAAAGGAAATAAAAAAAAAGGAACAAAAAAAAAAAAAAAAAAAAAAAAAA
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B

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1    GCTTACATCNAAAAGAATTCGACAAAAAGTACAACCCAACATGGCATTGCATTGTGGGAA
61   GAAACTTTGGCAGCTACGTCACTCATGAAACCAAGCATTTTATTTACTTCTACCTGGGAC
121  AAGTAGCCATCCTCCTATTTAAATCGGGGTGAACAAAAAGTGACAAGGAAAAAAGGAAAA
1                M  A  N  P  K  C  Y  F  D  I  T  I  G  G  K  P
181  TAAGAAAAGCAATCATGGCAAACCCAAAATGCTACTTTGATATTACTATTGGTGGAAAGC
17     A  G  R  I  V  M  E  L  R  A  D  V  V  P  K  T  A  E  N  F
241  CAGCTGGTAGAATTGTCATGGAGTTGAGAGCTGATGTTGTACCTAAAACTGCAGAAAATT
37     R  A  L  C  T  G  D  K  G  F  G  F  K  G  S  K  F  H  R  V
301  TCCGTGCATTGTGCACTGGGGATAAGGGATTTGGTTTCAAGGGCAGCAAATTCCACCGTG
57     I  P  G  F  M  C  Q  G  G  D  F  T  R  G  N  G  T  G  G  K
361  TAATTCCTGGTTTCATGTGCCAAGGTGGTGATTTTACTCGTGGTAACGGTACTGGAGGCA
77     S  I  Y  G  E  K  F  A  D  E  N  F  N  L  K  H  T  G  S  G
421  AGAGCATTTATGGAGAGAAATTTGCTGATGAGAACTTCAATCTGAAGCATACTGGATCAG
97     I  L  S  M  A  N  A  G  P  N  T  N  G  S  Q  F  F  L  C  T
481  GTATTCTTCAATGGCAAATGCTGGTCCTAACACAAATGGGTCGCAGTTTTCTTGTGTA
117    A  K  T  T  W  L  D  G  K  H  V  V  F  G  S  V  V  E  G  M
541  CAGCAAAGACCACATGGCTTGATGGGAAACATGTAGTGTTTGGTTCAGTAGTTGAGGGTA
137    D  V  V  T  K  I  E  S  V  G  S  D  S  G  K  T  K  V  D  V
601  TGGATGTAGTCACGAAAATAGAAAGTGTAGGAAGTGATAGTGGAAAGACAAAAGTGGATG
157    G  I  A  D  C  G  Q  L  *
661  TTGGTATTGCTGATTGTGGACAGCTATAAAGCCTACAATGGTCATTGTGTGACTATCCAT
721  CATAAACAATTTATGTTCTTACATTTTCTGGATCAAAATATGACTTATTTAAATTACTAA
781  TAGCTTGTTAAACAGCTTTTTTGAATTTGATTTTGAAATCTTTTCAATATTTGAGATAAT
841  CGCGTCAACAAATTTATTATTCCTTACATCACACTATTTATAATTTTTTATAGATAATTT
901  CATGTCAGAGGTGATTTTCTTTTTTCTAGGAATTCATTTGGATTATGTATTCTTTTTCAA
961  GGAAAACTGTCTGCTTCTGAAATAAATGTATGATCTTAAAAAAAAAAAAAAAAAAAAAAA
1021 AAAAA
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Fig. 1. A. Nucleotide and deduced amino acid sequences of the CypA1 gene from *Venerupis philippinarum*. Nucleotide and deduced amino acid residues are numbered on the left. The signature of peptidyl-prolyl cis-trans isomerase is shadowed. The start and stop codons are marked in bold and the classical polyadenylation signal in the 3′-UTR is boxed. The conserved amino acids residues are underlined. B. Nucleotide and deduced amino acid sequences of the CypA2 gene from *V. philippinarum*. Nucleotide and deduced amino acid residues are numbered on the left. The signature of peptidyl-prolyl cis-trans isomerase is shadowed. The start and stop codons are marked in bold and the classical polyadenylation signal in the 3′-UTR is boxed. The conserved amino acids residues are underlined.