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Molecular characterization and expression profiles of cdc2 and cyclin B during oogenesis and spermatogenesis in green mud crab (*Scylla paramamosain*)

Kunhuang Han [a,b], Yanbin Dai [a], Zhihua Zou [a], Mingjun Fu [a], Yilei Wang [a,*], Ziping Zhang [c,**]

[a] Key Laboratory of Healthy Mariculture for East China Sea, Ministry of Agriculture, Fisheries College, Jimei University, Xiamen 361021, China
[b] Ningde Fufa Fishery Company Limited, Ningde 352103, China
[c] Department of Biological Science, Seton Hall University, NJ 07079, USA

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ABSTRACT

The maturation promoting factor (MPF) is a key regulator of controlling G2/M phase transition in the meiotic maturation of oocyte and spermatocyte in animals, which is a complex of CDC2 (CDK1) and cyclin B. To better understand the molecular mechanism of oocyte and spermatocyte maturation in mud crab (*Scylla paramamosain*), the full length cDNA of *cdc2* (*Sp-cdc2*) and *cyclin B* (*Sp-cyclin B*) were cloned and characterized. The full length cDNA of *Sp-cdc2* gene is of 1593 bp encoding a protein of 299 amino acids. Real-time quantitative PCR analysis revealed that the expression level of *Sp-cdc2* in the ovary was higher than in other tissues ($P<0.01$); and its expression level was not significantly different in different stages of ovary development ($P>0.05$), meanwhile there was higher expression in T3 stage than in T1 and T2 stages ($P<0.05$). The full length cDNA of *Sp-cyclin B* is 1492 bp encoding a protein of 391 amino acids. The real-time PCR results showed that its expression level in the ovary was the highest in all examined tissues ($P<0.01$), and the gonad expression level in O5 stage was significantly higher than in previous 4 stages and the testis ($P<0.05$), and was also significantly higher in T2 stage than in T1 stage ($P<0.05$). *In situ* hybridization analysis showed that the expressions of *Sp-cdc2* and *Sp-cyclin B* transcripts were presented in similar distribution patterns in different developing stages of ovary and testis. The positive signals of *Sp-cdc2 and Sp-cyclin B* mRNA were detected in the oocytoplasm of oogonia and pre-vitellogenic and primary vitellogenic oocytes, while these two genes had higher expression level in the spermatid and secondary spermatocyte following primary spermatocyte. These results suggested that *Sp-cdc2* and *Sp-cyclin B* may play essential roles in the oogenesis and spermatogenesis of the crab.

1. Introduction

In multi-cellular organisms, gametogenesis is an important part of gonad development, which is controlled by several cell cycle regulators, such as cyclins, CDK (cyclin-dependent kinase) and CKI (cyclin-dependent kinase inhibitor). It is well known that both mitoitc and meiotic cell cycles are regulated by the maturation promoting factor (MPF) (Murray and Hunt, 1993), which is a complex of regulatory subunit cyclin B and its catalytic subunit CDK1. The complex controls the G2/M phase transition in eukaryotes by promoting germinal vesicle breakdown (GVBD), chromatin condensation, microtubule spindle formation and progression into metaphase of the second meiotic division, and so on (Banerjee et al., 2000). MPF is maintained in an inactive state (pre-MPF) by inhibitory kinases such as Weel and Mytl by Thr 14 and Tyr15 residues phosphorylation of CDK1. The pre-MPF complex becomes active by directly dephosphorylating Thr14 and Tyr15 residues and phosphorylating Thr 161 residue by cdc25c phosphatase of CDC2 (Nigg, 1995; Basu et al., 2004; Chesnel et al., 2007; Kaushal and Bansal, 2007). The cyclin B contains a conserved motif called the destruction box in its N-terminal region, which serves as a signal for ubiquitination and is necessary for cell cycle-regulated proteolysis to exit from M phase (Glotzer et al., 1991).

The mechanism of MPF activation during oocytes maturation has been well studied in a wide variety of vertebrate animals such as mammalians (Chapman and Wolgemuth, 1992; Hoffmann et al., 2006), amphibians (Nebreda and Ferby, 2000; Karaiskou et al., 2001; Kotani et al., 2001) and fishes (Hirai et al., 1992; Kajiura et al., 1993; Terasaki et al., 2003; Lapasset et al., 2008). The crustacean is a big branch of animal kingdom, and most of decapods are important economic breeding species. However, there are only a few of researches of CDC2 or cyclin B functions on the oogenesis in the crustacean, such *as Eriocheir sinensis* (Fang and Qiu, 2009), *Penaeus monodon* (Qiu et al., 2007; Visudtiphole

Abbreviations: BCIP, (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate; BSA, bovine serum albumin; CDC, cell division cycle; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; GSI, gonadosomatic index; GVBD, germinal vesicle breakdown; MPF, maturation promoting factor; NBT, nitroblue tetrazolium; ORF, open reading frame; PBS, phosphate-buffered saline; SSC, saline sodium citrate buffer.

* Corresponding author. Tel.: +86 592 6182723; fax: +86 592 6181420.
** Corresponding author. Tel.: +1 973 761 9055.
E-mail addresses: ylwang@jmu.edu.cn (Y. Wang), ziping.zhang@shu.edu (Z. Zhang).

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et al., 2009) *Marsupenaeus japonicus* (Qiu and Yamano, 2005) and *Macrobrachium nipponense* (Wu, 2009). These studies indicate that MPF activation plays a crucial role during oocyte maturation. Similarly, it is reasonable to assume that MPF also plays an important role in spermatogenesis. To date, however, fewer detailed studies have been conducted on the role of MPF during crustacean spermatogenesis. Meanwhile, how CDC2 and cyclin B work together in oogenesis and spermatogenesis in the crustacean is unclear, especially at molecular level. On the other hand, successful artificial control of gonad maturation of the commercially important species by understanding molecular regulation mechanism of gametogenesis will contribute to a sustainable cultivation and increase in natural resources.

In the present study, the full length cDNAs of *Sp-cdc2* and *Sp-cyclin B* were identified and their differentiated expressions were examined during oogenesis and spermatogenesis in mud crab (*Scylla paramamosain*), one of important economic breeding crabs around the southeast coast of China. The cloning and characterization of *Sp-cdc2* and *Sp-cyclin B* transcripts will provide us useful information to further investigate molecular mechanism of spermatozoan and oocyte maturation in crabs.

2. Materials and methods

2.1. Animals and tissue collection

S. paramamosain (Crustacea: Decapoda: Portunidae) at different stages of ovary and testis development were obtained from a crab farm in Zhangpu, Zhangzhou, Fujian, China. Various tissues including testis, ovary, heart, muscle, hepatopancreas, gill, eye, brain, intestine and haemocytes were immediately frozen in liquid nitrogen and stored at −80 °C for total RNA extraction. According to external morphology, color, gonadosomatic index (GSI) and histological feature, ovarian development was classified into five stages: proliferation (stage I, GSI = 0.57 ± 0.47), pre-vitellogenesis (stage II, GSI = 2.19 ± 0.21), primary vitellogenesis (stage III, GSI = 3.68 ± 0.20), secondary vitellogenesis (stage IV, GSI = 7.81 ± 0.94), and tertiary vitellogenesis (stage V, GSI = 10.49 ± 0.49). The male crabs were grouped into three stages: stage I (GSI = 0.07 ± 0.01), stage II (GSI = 0.21 ± 0.04), and stage III (GSI = 0.34 ± 0.03). Five crabs at each developmental stage were used for the experiments. For *in situ* hybridization and histological observations, ovaries and testes at different developmental stages were fixed in 4% paraformaldehyde at 4 °C overnight, then stored in methanol at −20 °C after washing with PBS four times at room temperature.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was isolated from tissues as described in the previous studies (Zhang et al., 2003), and then treated with RNase-free DNase I at 37 °C for 30 min to remove potential trace amount of contaminated genomic DNA. cDNA was synthesized from 2 μg of total RNA by M-MLV reverse transcriptase (Promega, USA) at 42 °C for 90 min with oligo-dT-adaptor primer according to protocol of SMART-RACE cDNA Amplification Kit. For real-time PCR, cDNA was synthesized by M-MLV reverse transcriptase at 37 °C for 60 min with random primer.

2.3. cDNA cloning for Sp-cdc2 and Sp-cyclin B

Based on the expressed sequence tags (EST) database from our previous results (Zou et al., 2011), a full-length cDNA of *Sp-cdc2* with complete coding regions and partial cDNA sequence of *Sp-cyclin B* were obtained. The open reading frame (ORF) of *Sp-cdc2* was confirmed by head-to-toe PCR amplification, and the missing 5′ and 3′ sequence of *Sp-cyclin B* was amplified by 5′ RACE and 3′ RACE methods. All the primers used in this experiment were listed in Table 1. PCR analyses were performed in a final volume of 25 μL containing 0.5 μL of the cDNAs, 2.5 μL of 10× PCR buffer, 0.5 μL of 10 mM dNTPs, 0.5 μL GSP and adaptor, and 0.5 μL Taq polymerase (200 U/μL). The PCR programs were carried out at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 65 °C (5′ RACE)/68 °C (3′ RACE) for 30 s, 72 °C for 2 min and a final extension step at 72 °C for 10 min. The PCR products were resolved by electrophoresis on 1% agarose gel. The fragments of interest were excised and then purified by a Gel Extraction Kit (Generay, China). The purified fragments were then cloned into pMD19-T vectors (Takara, Japan), propagated in *Escherichia coli* (JM109) competent cells. The plasmids isolated from positive clones were sequenced.

2.4. Quantitative real-time PCR

For quantitative real time PCR (qRT-PCR), an amount of cDNA derived from 25 ng of input RNA was used in each reaction. Reactions were performed with the SYBR Green PCR Master Mix (Applied Biosystems, USA), and analyzed in the ABI 7500 real time system. The cycling conditions for *Sp-cdc2*, *Sp-cyclin B* and 18S rRNA were as follows: 95 °C, 1 min, followed by 40 cycles (95 °C, 15 s; 60 °C, 1 min). Melting curves were also plotted (60–90 °C) in order to make sure that a single PCR product was amplified for each pair of primers. To identify the efficiency of amplification about both target genes and internal gene, a 10-fold dilution of the cDNA were used as templates to test amplification of different sets of primers of *Sp-cdc2*, *Sp-cyclin B* and 18S rRNA. The comparative threshold cycle (CT) method was used to calculate the relative concentrations. This method involves obtaining CT values for *Sp-cdc2* and *Sp-cyclin B*, normalizing to a reference gene, 18S rRNA (GenBank accession no. FJ646616); and comparing the relative expression level among different developing stages of testis and ovary and different tissues of female crabs. Experiments were performed routinely with more than three of each stage and tissue with values presented as $2^{\triangle\triangle CT}$ for the expression levels of *Sp-cdc2* and *Sp-cyclin B* normalized with 18S rRNA (△CT = CT of *Sp-cdc2*/*Sp-cyclin B* minus CT of 18S rRNA, △△CT = △CT of test sample minus △CT of calibrator sample). Data were expressed as mean and standard error of the mean (SEM) unless otherwise stated. Three separate individuals at least at each time were tested, each assayed in triplicate. Statistical analysis of the normalized CT values was performed with Student's *t*-test using SPSS. Differences were considered significant at $P<0.05$ or most significant at $P<0.01$ (two-tailed test).

2.5. Bioinformatics analysis of sequences

Nucleotide and predicted amino acid sequence data were compiled and aligned with sequences in GenBank using the BLAST and FASTA algorithms (http://www.ncbi.nlm.nih.gov) to determine gene identity. Isoelectric point and molecular weight predictions were carried out at (http://cn.expasy.org/tools/pi_tool.html). Sp-CDC2 and Sp-cyclin B amino acid signatures proposed by the prosite database (http://

Table 1
Oligonucleotide primers used.

Gene (accession no.)	Primer	Sequence
Sp-cdc2 (FJ015041)	Head primer	5′ GAACGGTGCCTCACGAGTCCC 3′
	Toe primer	5′ TGAAGCCCCTGTGTGACCTCC 3′
	qRT-PCR sense primer	5′ AGAATGAGGAGGAGGGTGTG 3′
	qRT-PCR antisense primer	5′ TTGAGGTCCATGTTGAGGAA 3′
Sp-cyclin B (FJ595022)	5′RACE outer primer	5′ CGAGGCGAGAGAGATTCCTGTTG 3′
	5′RACE inner primer	5′ TGGAGTGTGGGGCCCTGGAAC 3′
	3′RACE outer primer	5′ TGACTCCCAGGATGCCAGCAA 3′
	3′RACE inner primer	5′ TGCACTACCATTACCTGGAGGGG 3′
	qRT-PCR sense primer	5′ GGGAGTGACGGCGATGTT 3′
	qRT-PCR antisense primer	5′ TCAGGAAGTCCAGAGGCAGTG 3′
18S rRNA (AY181979)	qRT-PCR sense primer	5′ ATGATAGGGATTGGGGTTTGC 3′
	qRT-PCR antisense primer	5′ AAGAGTGCCAGTCCGAAGG 3′