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Identification and characterization of the cDNAs encoding the two subunits of Chinese mitten crab (*Eriocheir sinensis*) calcineurin: Their implications in stress and immune response

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ABSTRACT

Calcineurin (CN), the only Ca^{2+}/calmodulin-activated serine/threonine protein phosphatase, is a key effector participating in Ca^{2+}-dependent signal transduction pathways in a number of cellular processes under normal, stress and pathological conditions. However, the expression and the relevance of CN in stress and immune response have not been characterized in crustaceans. Here, we identified the cDNAs that encode the two subunits of CN (termed *EsCN-A* and *EsCN-B*, respectively) in Chinese mitten crab *Eriocheir sinensis* and analysed their expression patterns in response to stress and immune challenges. The catalytic subunit EsCN-A is comprised of 511 amino acids with a theoretical molecular mass of 57.5 kDa and shows 80% sequence identity with human beings CN-A alpha isoform, while the regulatory subunit EsCN-B protein is composed of 170 amino acids with an estimated molecular mass of 19.3 kDa and shares 88% sequence identity with human beings CN-B type 1. Tissue distribution analysis reveals that both *EsCN-A* and *EsCN-B* mRNA transcripts are expressed in all tested tissues with the greatest expression in hepatopancreas and the lowest expression in haemocytes. In addition, both *EsCN-A* and *EsCN-B* genes could be significantly up-regulated but with different expression patterns by ambient salinity (15‰ and 30‰ salinities) and pH (pH 6 and 8.5) stresses in gill, hepatopancreas, haemocytes, intestine and muscle. Furthermore, *EsCN-A* and *EsCN-B* were up-regulated by LPS and Poly(I:C) immune stimulations in *E. sinensis* haemocytes *in vitro*. Moreover, *EsCN-A* and *EsCN-B* mRNA were significantly up-regulated in haemocytes, gill, hepatopancreas, intestine and muscle in response to *Edwardsiella tarda* challenge *in vivo*. Finally, we revealed the importance of EsCN in LPS-induced nitric oxide production in *E. sinensis* haemocytes. Together our observations suggest that EsCN, the important downstream effector of CaM-mediated signalling pathway(s), may possess vital roles in stress and immune response in the Chinese mitten crab.

1. Introduction

Calcineurin (CN) is a highly conserved Ca^{2+}/calmodulin (CaM)-dependent serine/threonine phosphatase [1], consisting of a CaM-binding catalytic subunit (A subunit, termed CN-A) which mediates interaction with phosphorylated substrates and a Ca^{2+}-binding regulatory subunit (B subunit, termed CN-B) [2]. CN-A subunit contains four functional motifs including the N-terminal catalytic domain, a CN-B binding helix, a CaM-binding domain, and an autoinhibitory domain; these last three domains together comprise the regulatory region of the A subunit. CN-B has four EF-hand Ca^{2+}-binding motifs and tightly binds CN-A at sub-micromolar concentrations of Ca^{2+}. The phosphatase activity of CN-A is regulated by Ca^{2+} through both CaM and the CN-B subunit [3], both of which are EF-hand Ca^{2+}-binding proteins. Upon binding and interacting with Ca^{2+}/CaM, a conformational shift of CN-A was induced and the autoinhibitory domain is displaced and no longer obstructs the active site which resulted in full phosphatase activation [1].

Calcineurin has broad expression in a variety of tissues and cell types [4] and plays multiple functions under stress and pathological conditions [1,5]. For example, CN has been suggested an

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important role in endoplasmic reticulum stress caused by Ca^{2+} depletion [6]; calcineurin is also involved in oxidant stress that could induce apoptosis in cardiac myocytes [7]. In addition, CN has been identified as an important determinant for high salt stress adaptation [8] and CN-NFATc signalling can regulate the osmotic stress response in mouse kidney [9].

In addition to the involvement in stress response, CN also is one of the most important functional proteins that play essential roles in immune system [10,11]. The role of CN-B in activation of innate immune cells including macrophages, monocytes and dendritic cells has been widely studied [12]. It also has been recognized that CN plays a critical role in programmed cell death of T and B lymphocytes [13] and in regulating T-cell development and activation [14]. Using knockout *CN-A alpha* mice as an animal model, Zhang et al. has reported that CN-A is involved in T-cell response in mice [15]. Recently, it has been evidenced that CN can promote induction of robust innate immune response in *Drosophila* [16].

Our previous studies have indicated that CaM is a stress and immune response gene and CaM-mediated signalling pathways may paly essential roles in stress and immune response in Chinese mitten crab *Eriocheir sinensis* [17]. As a key downstream target of CaM, the presence of CN in nerve tissue of the horseshoe crab, *Limulus Polyphemus*, has been detected biochemically [18]. However, no literature has reported the expression and the relevance of CN in stress and immune response in crustaceans. In the present study, we set out to identify the cDNAs that encode the two subunits of CN (namely *EsCN-A* and *EsCN-B*) and to analyse their expression in response to environmental stress and immune challenges in *E. sinensis*. We for the first time showed the involvement of CN in responsive to ambient stress and immune challenges in crustaceans. Our findings have provided additional evidence for the CaM-regulated calcium signalling pathways in stress and immune response in the Chinese mitten crab.

2. Materials and methods

2.1. Animals and maintenance

Chinese mitten crabs *E. sinensis* were purchased from a local farm in Qilihai, Tianjin, China and transported to the laboratory. Crabs were cultured with filtered aerated tap water at 19 ± 1 °C for 2 weeks before experimentation to acclimate laboratory conditions. Animals were carefully examined to ensure that all crabs used in experiments were in intermolt stage. Only healthy crabs without any pathological signs were used in the experiments.

2.2. RNA preparation and cDNA synthesis

Total RNA from different tissues of *E. sinensis* was isolated by TRIzol reagent (Invitrogen) as specified by the manufacturer and the integrity of RNA was evaluated by electrophoresis on a 1.2% formaldehyde-denatured agarose gel stained with ethidium bromide on a GelDoc™ XR system (Bio-Rad). Total RNA was quantified spectrophotometrically at wavelengths of 260 and 280 nm with a NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific) and subjected to DNase I (Invitrogen, amplification grade) treatment to remove genomic DNA contamination according to the manufacturer's protocol. cDNAs were prepared using SuperScript III reverse transcriptase (Invitrogen) with oligo$(dT)_{18}$ as primer according to the manufacturer's instructions.

2.3. Isolation of EsCN cDNA

Through data mining the transcriptome database of combined *E. sinensis* eyestalk, Y-organ and hepatopancreas tissues cDNA library [19], two EST clones (termed *EsCN-A* and *EsCN-B*, respectively) encoding proteins with homology to mammalian CN-A and CN-B proteins were retrieved. To validate these sequences, *EsCN-A* and *EsCN-B* gene were amplified with a two-round of nested RT-PCR using the cDNAs synthesized from hepatopancreas tissue of *E. sinensis* as template. PCR products were separated by electrophoresis in ethidium bromide-impregnated 1.2% agarose gels. Identity of PCR products were confirmed by cloning and sequencing. Three independent clones were selected and sequenced on both strands, which showed 100% sequence identity.

2.4. Sequence analyses

Nucleotide sequences were searched against GenBank database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) using BlastX algorithm to identify their coding proteins. The deduced amino acid sequence was analysed with the Expert Protein Analysis System (http://www.expasy.org/) to identify the conserved protein domain. Multiple sequence alignments were performed using ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw/).

2.5. Tissue expression of EsCN mRNA transcripts in E. sinensis

Tissues including haemocytes, brain, gill, muscle, stomach, heart, hepatopancreas, ganglion and intestine from 6 healthy crabs (average 100 ± 10 g) were separated and collected and each kind of tissue was equally pooled to minimize individual variation. After dissection, tissues were frozen immediately in liquid nitrogen and stored at −80 °C. Aliquots (1 μg) of total RNA from each type of tissue were transcribed into single-strand cDNAs as described previously. As a control, RNA samples were also subjected to the first-strand cDNA preparation protocol without reverse transcriptase and no PCR products were amplified (data not shown). The tissue expression of *EsCN-A* and *EsCN-B* mRNA transcripts in healthy *E. sinensis* was determined by quantitative real-time PCR.

2.6. Salinity and pH stress challenge experiments

Salinity and pH stress challenge experiments design was detailed in our previous study [17]. The expression changes of *EsCN-A* and *EsCN-B* genes under salinity and pH stress conditions compared with their respective expression in the control group (tap water), were measured by quantitative real-time PCR (see *section 2.9*).

2.7. Haemocytes preparation, cell culture and immune stimulation

Chinese mitten crab (mean weight 20 ± 3 g) haemocytes were prepared as described previously [20]. Pooled crab haemocytes were cultured at 21 °C in a 24-well plate (2.5×10^6 cell/well) with culture medium supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin antibiotics. To test the involvement of *EsCN* in *E. sinensis* innate immune response, overnight cultured Chinese mitten crab haemocytes were stimulated with 30 μg/ml Poly(I:C) (Sigma–Aldrich, catalog No. P1530) or LPS from *Escherichia coli* serotype O55:B5 (Sigma–Aldrich, catalog No. L2880), two of the best characterized activators of innate immunity. Total RNA were purified from the cells with a RNeasy Mini Kit (Qiagen) after 0, 2, 4, 6, 8, 12 and 24 h of LPS or Poly(I:C) application and cDNA synthesis was performed as described above. The expression changes of *EsCN-A* and *EsCN-B* genes in response to LPS and Poly(I:C) challenges were determined by quantitative real-time PCR.