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Characterization and function of CREB homologue from *Crassostrea ariakensis* stimulated by rickettsia-like organism

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Summary
The cAMP response element-binding protein (CREB) is a transcription factor that plays important roles in cellular growth, proliferation and survival. Here, we report that a homologue of CREB transcription factor, *Ca*-CREB, was identified and functionally characterized in oyster, *Crassostrea ariakensis*. The full-length cDNA consists of 1397 bp with an ORF encoding a 39.3 kDa protein. Amino acid sequence analysis revealed that *Ca*-CREB shares conserved signature motifs with other CREB proteins. *Ca*-CREB was ubiquitously and constitutively expressed in oyster, and the expression level in hemocytes was higher than that in other tissues. The expression level of *Ca*-CREB was not modified after RLO stimulation, while tumor necrosis factor-alpha (TNF-α) expression was increased obviously, which was revealed by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Electrophoretic mobility shift assay (EMSA) and Western blotting showed that recombinant CREB proteins specifically bind the consensus CREB binding site, and DNA-binding activity and phosphorylation of *Ca*-CREB were induced by RLO. These results suggest that *Ca*-CREB is a CREB homologue and may be involved in immune responses against RLO.
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Introduction

The cAMP response element-binding protein (CREB) is a stimuli-induced transcription factor [1] and shares similarities with activation transcription factor in the leucine zipper (basic Leucine zipper domain (bZIP)) domain. Two major domains, the glutamine-rich domain and the

Abbreviations: PBST, phosphate-buffered saline containing 0.1% Tween20; DIG, digoxigenin; NLS, nuclear localization signal; HRP, horseradish peroxidase; DAB, 3,3′-diaminobenzidine; bZIP, basic Leucine zipper domain.

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kinase-induced domain (KID), are responsible for basal transcription activity and signal-induced activation, respectively [2]. When phosphorylated by kinases including PKA, PKC, MAPK and ERK, CREB binds as a homodimer or heterodimer to cAMP response element (CRE), a conserved 5′-TGACGTCA-3′ sequence, via its bZIP and recruits the coactivator CREB-binding protein [3] or TORC (transducer of regulated CREB activity) [4] to activate transcription.

CREB is expressed in all brain cells, as well as many other tissues, and plays important roles in regulation of biological functions including memory [5], apoptosis [6], metabolism [7], oncogenesis [8], proliferation and cell survival [9] and immune responses [1,10]. Many members of the CREB family have been identified among invertebrates (*Aplysia*, *Lymnaea* and *Drosphila*) and vertebrates (fish, frog and mammals), and they are highly homologous in their C-terminal DNA binding and phosphorylation domains [11]. In mammals and invertebrates, there are multiple isoforms of CREB because of extensive splicing of mRNAs [12,13]. A vast variety of stimuli [14], including growth factors (EGF, FGF, IGF and PDGF), hormone, inflammatory cytokines (IL-1, IL-2, IL-3, TGF and tumor necrosis factor (TNF)), bacteria and viral components (LPS, dsRNA, HBVpX, HIV Tat protein and petidoglycan) and environmental factors (ethanol, UV, anoxia, saline and starvation) can induce the activation of CREB through the signaling pathways involving PKA, PKC, AKT, CaMK, MSK and MAPK [9,14,15]. Once CREB is activated, CREB binds as dimers to the CRE found within the regulatory region in numerous genes and turns on the transcription of target genes, including *bcl-2* [16], *c-fos* [17,18], cyclin D2 [19], *IL-1* β [20], MHC Class I gene [21], *smac* [22], and other genes involved in the growth, survival and memory of cells [23]. Currently, CREB targets more than 4000 genes, or up to 6000 loci in numerous physiological events [24,25]. Although tremendous progress has been made in discovering the functional effectors and regulatory mechanisms involved in the CREB signaling pathway, many processes still require further investigation [14].

Crassostrea ariakensis is one of the economically important oysters cultured in China, which suffered from morbidity and mortality caused by RLO in recent years [26,27]. However, molecular mechanisms of immunology in the oysters have not been clearly characterized until recently. Better understanding of invertebrate immune systems may allow us to effectively control those bivalves which are vectors of infectious pathogens such as the hepatitis virus, Norwalk-like virus and *vibrios* [28,29] and provide necessary protection for those valuable species. Prevention and control of diseases is now the priority for the development of aquaculture. Study of oyster immunity will contribute to the prevention and control of RLO disease and in the selection of disease-resistant oysters. In the present study, for the first time, we identify a CREB homologue from *C. ariakensis* in the marine bivalves. Its expression pattern, biological activity and activation mechanism were analyzed. The results suggested that *Ca*-CREB might play a role in the immune responses to rickettsia-like organism. In addition, studying the *Ca*-CREB will be helpful to shed light on the phylogenetic relationship with various invertebrates and vertebrates.

Figure 1 The map of Yangxi County where the samples were collected. Oysters aged 2–3 years were sampled from Hailing Bay in Yangxi County of Guangdong Province.

Material and methods

Isolation and purification of rickettsia-like organisms

Moribund and healthy oysters aged 2–3 years were collected from Yangxi county of Guangdong Province (Figure 1). The individual size was about $8 \times 5.2 \times 11.8$ (length $\times$ width $\times$ height) on average. Isolation and purification of rickettsia-like organisms were carried out with renografin density gradient centrifugation [30]. Briefly, 10 RLO-infected oysters were washed with PBS (53.9 mM Na_2HPO_4, 12.8 mM KH_2PO_4, 72.61 mM NaCl, pH 7.4). Infected tissues, including mantle, gills and digestive gland, were collected from infected oysters, suspended in PBS and homogenized with a homogenizer (Shanghai Chubo). The homogenates were centrifuged at 11,000*g* for 40 min at 4 °C. The pellets were collected and resuspended in PBS, and then centrifuged at 700*g* for 20 min at 4 °C. The pellets were discarded and the supernatants were saved. The supernatants were layered on a solution of 23% sucrose/10% renografin and centrifuged at 25,000*g* for 40 min at 4 °C. The pellets were resuspended in PBS and centrifuged at 11,000*g* for 40 min at 4 °C. The pellets were saved and suspended in PBS, and then the suspension was layered in renografin discontinuous gradients (16%, 22%, 28%, 32%, v/v, prepared by diluting 76% renografin with PBS) and centrifugated at 95,000*g* for 40 min at 4 °C. The suspension at the interface between 22% and 28% renografin gradients was collected and diluted with 9 volumes of PBS. After centrifugation at 25,000*g* for 1 h at 4 °C, the purified RLOs were collected and small parts of purified RLO suspension were negatively stained on copper net for TEM observation as previously [30]. The others were used for experiment.

2.2. Preparation of hemocyte monolayers and experimental bacterial challenge

Healthy oysters were fed in seawater at 15 °C for a week before experiment. Hemocyte monolayers were prepared reference to Lacoste et al. [31] and Canesi et al. [32]. A total of 5–10 oysters were collected and washed with

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