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Calreticulin is a microbial-binding molecule with phagocytosis-enhancing capacity

Q2 Xuemei Liu, Na Xu, Shicui Zhang*

Laboratory for Evolution & Development, Institute of Evolution & Marine Biodiversity and Department of Marine Biology, Ocean University of China, Qingdao 266003, China

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ABSTRACT

Calreticulin (CRT) is a highly conserved calcium-binding protein mainly involved in directing proper conformation of proteins and controlling calcium level. Accumulating data also show that CRT is emerging as an immune-relevant molecule. In this study, we demonstrated that the CRT gene from the amphioxus *Branchiostoma japonicum*, named *Bjcrt*, consisted of a signal peptide, three domains (N-, P-, C-domains) and an ER retrieval signal sequence (KDEL), which appears to be the ancient form of vertebrate CRTs, and *Bjcrt* was expressed in a tissue-specific manner, with the most abundant expression in the notochord. We also demonstrated for the first time that the recombinant BjCRT (rBjCRT) was able to bind the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Staphylococcus aureus*. Moreover, both BjCRT as well as human recombinant calreticulin were able to promote the phagocytosis of *E. coli* and *S. aureus* by sea bass macrophages. These results indicate that CRT is a microbial-binding molecule and possesses an ability to enhance phagocytosis, a novel function assigned to CRT, reenforcing the notion that CRT is an immune-relevant molecule associated with host immune responses.

1. Introduction

Calreticulin (CRT), first isolated from endoplasmic reticulum (ER) of rabbit skeletal muscle [1], is a 46-kDa protein which resides in the lumen of the ER. It consists of three distinct structural and functional domains: a globular N-domain, an extended P-domain and an acidic C-domain. The N-domain contains an N-terminal cleavable signal sequence that directs it to the ER, and the double cysteine residues that are involved in the disulfide bond formation. The N-terminal domain is highly conserved between species and plays a role in chaperone activity. The C-domain is the least conserved among CRTs, but includes a highly conserved motif KDEL (Lys-Asp-Glu-Leu) involved in ER retention/retrieval. The C-terminal domain has a large number of negatively charged residues and is responsible for Ca^{2+} buffering, with relatively low affinity to Ca^{2+} (Kd = 2 mM) but high Ca^{2+}-binding capacity (25 mol of Ca per mol of protein) [2]. Between the N- and C-domains of CRT is the proline-rich P-domain, which binds Ca^{2+} with a relatively high affinity (Kd = 1 μM) but low capacity (1 mol of Ca^{2+} per mol of protein) [3]. The CRT P-domain contains pairs of triple repeats A, PXXIXDP-DAXKPEDWDE, and B, GXWXPPXIXNPXYX [4].

In addition to being an ER luminal protein, CRT is also localized to cytoplasm, cell surface and extracellular matrix [5–9]. Within the lumen of the ER, CRT, working together with other ER-resident chaperones such as calnexin and Erp57 [10,11], ensures proper folding of proteins and glycoproteins [12], mainly via its lectin-binding site [13]; prevents protein aggregation; and is engaged in protein quality control through identifying and banning misfolded proteins from the ER for ubiquitin-mediated destruction [14]. Another critical role of CRT is in the regulation of intracellular Ca^{2+} homeostasis, influencing a variety of cellular functions, including cell signaling and cellular stress responses [15]. Besides, CRT also mediates diverse and important biological processes from the non-ER compartments. For example, CRT has been shown to be required for cell adhesion, phagocytosis of apoptotic cells, wound healing, cancer and autoimmunity [4,15–18] as well as oxidative stress responses caused by hydrogen peroxide, hypoxic injury and iron overload in mammals [19–21].

Accumulating data also suggest that CRT may play an important role in immune responses. In mammalian species, CRT was shown to act as a receptor for C1q, mannose-binding lectins, and ficolins [22,23]; in catfish, CRT was markedly up-regulated following infection with *Edwardsiella ictaluri*, a bacterial pathogen that causes enteric septicemia [24–26]. Similarly, CRT was also related to

* Corresponding author. Room 205, Ke Xue Guan, 5 Yushan Road, Ocean University of China, Qingdao 266003, China. Tel.: +86 532 82032787.
E-mail address: sczhang@ouc.edu.cn (S. Zhang).

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immune responses in invertebrate species. For example, in insects, CRT was found to be an early stage capsulation protein involved in non-self recognition in cellular defense reaction [27–30]; in shrimps, CRT was considerably increased when challenged by white spot syndrome virus (WSSV) at 6 h post-challenge [31,32]. Apparently, CRT is emerging as an immune-relevant molecule, and we anticipate that novel functions will continue to unfold.

Amphioxus or lancelet, a basal chordate and a sister group of vertebrates, has a vertebrate-like body plan including dorsal neural tube, notochord, segmented somites and pharyngeal gill slits, but it is less complex than vertebrates, having a genome uncomplicated by extensive genomic duplication [33]. Its genetic sequence information and expression pattern have been widely used for interspecies genome comparison, developmental homology analysis and comparative immunology study [34–37]. However, little information is available about CRT to date in this evolutionarily important organism. The aims of this study were thus to identify the CRT gene of the amphioxus *Branchiostoma japonicum*, designated as *Bjcrt*, to examine its expression pattern and to investigate its bioactivity.

2. Materials and methods

2.1. Cloning and sequencing of Bjcrt cDNA

Total RNAs were extracted with Trizol (TaKaRa) from adult amphioxus *B. japonicum* collected during the breeding season (mid-June to mid-July) in the vicinity of Qingdao, China, and digested with RNase-free DNase (TaKaRa) to eliminate the genomic contamination. The first-strand cDNA was synthesized with reverse transcription system (Promega) using oligo d(T) primer. To amplify *Bjcrt* cDNA fragment, polymerase chain reaction (PCR) was performed using the first-strand cDNA as template, in a total volume of 20 μl PCR mixture containing 1 × PCR buffer (TaKaRa), 0.5 unit of EX Taq DNA polymerase (TaKaRa) and 0.4 μM of the CRT gene-specific primers P1 and P2 (Supplementary S1), which were designed on the basis of the CRT sequence found in the Florida amphioxus *Branchiostoma floridae* genome database (http://genome.jgi-psf.org//Brafl1/Brafl1.home.html). PCR was carried out at 94 °C for 5 min, followed by 33 cycles at 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR products were gel-purified using DNA gel extraction kit (AXYGEN), ligated into the T/A cloning vector pGEM-T easy (Promega) at 4 °C overnight, and transformed into Top10 competent cells (TIANGEN). The positive clones were selected and sequenced with ABI PRISM 3730 DNA sequencer. The sequences were searched in GenBank with BLASTx for comparative analysis.

After determination of the partial cDNA sequence, rapid amplification of cDNA ends (RACE) was employed to obtain the full-length cDNA. The gene-specific primer pairs P3 and P4 (Supplementary S1) were used in RACE reactions for the cloning of 3′-end cDNAs. The 3′-RACE-Ready cDNA was synthesized from total RNAs using the BD SMARTTM RACE cDNA amplification kit (Clontech) according to the instructions. The 3′-RACE products were gel-purified, sub-cloned, sequenced and assembled.

2.2. Sequence analysis

The cDNA sequence assembled was analyzed for coding probability with the SeqBuilder program of the LASERGENE software suite (DNASTAR). Sequence comparison against the GenBank protein database was performed using the BLAST network server at the NCBI. Protein domains were analyzed using the SMART program (http://smart.embl-heidelberg.de/). The SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. Multiple protein sequences were aligned using the MegAlign program of the LASERGENE software suite (DNASTAR). Isoelectric point and charge at pH7.0 were determined using EditSeq from the LASERGENE package. The potential N-linked glycosylation sites were predicted with the NetGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/).

Phylogenetic tree was constructed by the neighbor-joining method within the package MEGA 4.0 software package using 1000 bootstrap replicates. *Dictyostelium discoideum*, a cellular slime mold, was used as the outgroup [38]. The exon–intron organization of CRT genes were derived from NCBI database (http://www.ncbi.nlm.nih.gov/) and Ensembl database (http://www.ensembl.org).

2.3. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to examine the expression of *Bjcrt* in the different tissues of *B. japonicum*, including hepatic cecum, hind-gut, gill, muscle, notochord, testis and ovary from different individuals. Total RNAs were prepared with Trizol from both the whole animals as well as the different tissues. After digestion with RNase-free DNase to eliminate the genomic contamination, cDNAs were synthesized with reverse transcription system using oligo d(T) primer, and used as templates. The gene-specific primers P5 and P6 (Supplementary S1) were used to amplify a product of 153 bp. The *β-actin* gene was chosen as the reference for internal standardization. The PCR primers specific for *Bjcrt* (P5 and P6) and *β-actin* (P7 and P8) genes were designed using primer 5.0 program (Supplementary S1). The amplification efficiency of each primer set was assessed using the cDNAs from the whole animals serially diluted 2-fold (data not shown).

After qualification of the cDNA template and primers, qRT-PCR was performed on ABI 7500 real-time PCR system (Applied Biosystems, USA) as described by Wang et al. [37]. Reaction of each sample was performed in triplicate. Dissociation analysis was performed at the end of each PCR reaction to confirm the amplification specificity. After the PCR program, data were analyzed with ABI 7500 SDS software (Applied Biosystems), and quantified with the comparative Ct method ($2^{-\Delta\Delta Ct}$) based on Ct values for *Bjcrt* and *β-actin* in order to calculate the relative mRNA expression level [39].

2.4. Construction of Bjcrt expression vector

The complete cDNA region encoding mature *Bjcrt* with the signal peptide deleted was amplified by PCR with the upstream primer P9 (NdeI site is underlined) and downstream primer P10 (XhoI site is underlined) (Supplementary S1). The reaction was carried out under the following condition: initial denaturation at 94 °C for 5 min, followed by 33 cycles each of denaturation at 94 °C for 30 s, annealing for 30 s at 62 °C, and extension at 72 °C for 1 min, and an additional extension at 72 °C for 7 min. The PCR product was digested with NdeI and XhoI and sub-cloned into the plasmid expression vector pET28a (Novagen) previously cut with the same restriction enzymes. The recombinant plasmid was verified by sequencing, and named *pET28a/Bjcrt*.

2.5. Expression and purification of BjCRT

The cells of *Escherichia coli* BL21 were transformed with the recombinant plasmid *pET28a/Bjcrt* and cultured overnight in LB broth containing kanamycin (50 μg/ml). Expression and purification of the recombinant protein was performed as described by Fan et al. [40] with a slight modification. The culture was diluted 1:100 with LB broth and subjected to further incubation at 37 °C until OD600 reached about 1.0. The expression of BjCRT was induced by addition of isopropyl β-d-thiogalactoside (IPTG) to the culture at a final concentration of 0.1 mM. After further incubation at 21 °C for 6 h, the

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