The first molluscan TRIM9 is involved in the negative regulation of NF- κ B activity in the Hong Kong oyster, *Crassostrea hongkongensis*

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Abstract

TRIM proteins are a group of highly conserved proteins participating in a variety of biological processes such as regulation of development, apoptosis, and innate immunity. However, the functions of these proteins in the mollusk are still poorly understood. In the present study, a *TRIM9* homolog (named *ChTRIM9*) was first identified from a transcriptome library in the Hong Kong oyster *Crassostrea hongkongensis*. The full-length cDNA of *ChTRIM9* is 2928 bp and has a predicted Open Reading Frame (ORF) encoding 721 amino acids, encoding a putative 80.2 kDa protein. SMART analysis indicated that ChTRIM9 contains the three typical TRIM domains, a RING finger, two B-boxes, and a coiled-coil domain in the N-terminal region, whereas the C-terminal region contains a SPRY domain. qRT-PCR analysis revealed a ubiquitous presence of *ChTRIM9*, with the highest expression in the gills. Upon bacterial challenge *in vivo*, the *ChTRIM9* transcripts in hemocytes were significantly down-regulated, indicating its involvement in signal transduction in immune response of oysters. Furthermore, ChTRIM9 was found to be localized mainly in the cytoplasm, and its over-expression inhibited the transcriptional activity of the NF- κ B gene in HEK293T cells, demonstrating its negative role in regulating NF- κ B signaling.

Key words: *Crassostrea hongkongensis*, TRIM9, innate immunity, NF- κ B

Introduction

The large family of tripartite motif (TRIM) containing proteins is phylogenetically conserved throughout the metazoan kingdom [1]. They derive their name from their conserved order N-terminal RBCC motif consisting of a RING domain, one or two B-Box domains and a coiled-coil domain. The order of the domains that compose the motif is also conserved: a RING finger domain precedes the B-box motif(s) and a Coiled-coil (CC) region invariably follows [2, 3]. RING domain functions as a post-translation modification mediator that is involved in ubiquitination, small ubiquitin-like modifier (SUMO) modification and the ubiquitin-like molecule IFN-stimulated protein of 15KDa (ISG15) conjugation. The B-box domain often exists as a duplex, while each adopts a distinct consensus sequence. The coiled-coil domain is localized at the C-terminal posterior to the B-box and is related to oligomerization among proteins and large complex assembly. The main structural differences within the family are the presence of different C-terminal domains, of which 11 distinct motifs have been described and can be present alone or in combination[4]. TRIM are involved in various biological processes, including growth, differentiation, apoptosis, immune signaling pathways[5, 6] and neurological disorders [7].

TRIM9 is a highly conserved member of the TRIM family and is the most abundant member in most cell types. Given its abundance and numerous cellular functions, TRIM9 has been drawn great attention since its discovery [8]. TRIM9 was initially identified in the embryonic and adult nervous system and plays vital roles in various cellular processes [8]. The eminent role of TRIM9 in the nervous system was unveiled as the discovery of its implication in neuronal dysfunction and subsequently the involvement in synaptic vesicle traffic [9]. In addition to its well-known function in the nervous system, TRIM9 was found to play a fundamental role in host defense. Expression or depletion of the

TRIM9 gene significantly affects NF- κ B-induced inflammatory cytokine production, indicating that TRIM9 is a negative regulator of NF- κ B signaling [10]. More recently, TRIM9 was identified as a positive regulator in type I IFN signaling in antiviral immunity, which serves to balance pro-inflammatory responses and the production of type I IFNs[11]. These studies indicate that TRIM9 plays an important role in the regulation of innate immunity. It has been reported that many TRIMs affect the ubiquitination states of several key signaling molecules in NF- κ B and type I IFN signaling via their E3 ligase activities [12]. Therefore, the alterations of TRIMs usually lead to diverse clinical disorders, such as developmental disorders, neurodegenerative diseases, and vulnerability to viral infection[13, 14]. However, the potential roles and functions of TRIMs in mollusks are still poorly understood. Until now, only sequence information of *Crassostrea gigas* (EKC37550.1) *Saccoglossus kowalevskii* (XP_002741117.1) has been reported in mollusks. No experimental and functional evidence of TRIM9 in mollusks has been obtained. Thus, it is of interest to identify the origins and functions of TRIM9 proteins in mollusks. Therefore, in the present study, to better understand the role of TRIM9 in innate immunity of the Hong Kong oyster, *C. hongkongensis*, the first TRIM9 homolog was identified and characterized.

2 Material and methods

2.1 Cloning the full-length ChTRIM9 cDNA and bioinformatics analysis

Partial *TRIM9* sequences were obtained from our *C. hongkongensis* transcriptome database. To obtain the full-length cDNA of *ChTRIM9*, SMART-RACE was used to obtain the whole length mRNA. Briefly, cDNA templates for 5'-RACE and 3'-RACE were prepared by using a SMART-RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's instructions. By using the outer gene-specific

primer(*ChTRIM9*-5RACE1 and *ChTRIM9*-3RACE1) and universal primer A mix (UPM), the first round touchdown PCR was performed as follows: 95°C for 5 min followed by a touch down program in 35 rounds(the annealing temperature reduced from 72°C to 55°C and the elongation time in each round remained 2.3 min), and kept at 4°C after 72°C for 10 min. In the next round, a nested PCR was performed under the general setting program (95°C for 5 min followed by 35-round cycle including 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, eventually kept at 4°C after 72°C for 10 min)by using the inner gene-specific primer(*ChTRIM9*-5RACE2 and *ChTRIM9*-3RACE2) and universal primer for nested (NUP)(Table1S). The expected PCR products were gel-purified by using a gel-purificationkit (MAGEN, China), and then cloned into a pMD19-T vector (Takara, Japan) and sequenced on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, USA) with M13 universal primers.

The *ChTRIM9* sequence was analyzed using the BLAST algorithm at NCBI (<http://www.ncbi.nlm.nih.gov/blast>) and the Expert ProteinAnalysis System (<http://www.expasy.org/>). Multiple sequence alignments were generated using Clustal X (version 1.81), and protein domains were predicted with SMART (<http://smart.emblheidelberg.de>). A neighbor-joining (NJ) phylogenetic tree was constructed by the MEGA4.0 package based on the protein sequences of the known TRIMs (Table 2S). The reliability of branching was tested using bootstrap re-sampling (with 1000 pseudo replicates).

2.2 Animals, tissue collection and bacterial challenge

100 Healthy *C. hongkongensis* adult individuals were collected from Zhanjiang, Guangdong Province, China and cultivated in aerated sea water at 25°C. The oysters were fed with 0.8% *Tetraselmis suecica* and *Isochrysis galbana* for one week before processing. The tissue distribution and bacterial challenge experiments were performed according to our previous work [15]. The oysters were randomly divided into two groups used for bacteria challenge and negative control respectively. Oysters received an injection of 100 µL (approximately 1×10^8 bacterial cells) of *Vibrio alginolyticus* or PBS that were used as the challenged or control groups, respectively. After injection, 5 individuals were randomly sampled at 3, 6, 12, 24, 48 and 72 h post-injection. The hemocytes from both groups were collected and stored at -80°C until RNA extraction.

2.3 RNA isolation, cDNA synthesis and plasmids construction

Isolation of total RNA from tissue was performed with TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. The integrity of isolated RNA was verified by electrophoresis on a 1.0% agarose gel while the concentration and purity were verified by an absorbance ratio of 260nm and 280nm using electron spectrometer (Thermo, USA). Reverse transcription was performed with a PrimerScript RT reagent Kit with gDNA Eraser (Takara, Japan) with the standard amount of 1 µg of template RNA. The predicted ORF was used to construct the pEGFP-N1-ChTRIM9 and pcDNA 3.1a-ChTRIM9 plasmids, which contributed to a subcellular localization and NF-κB dual-luciferase reporter assay, respectively. Briefly, to produce apEGFP-N1-ChTRIM9 and pcDNA 3.1a-ChTRIM9, the cDNA fragment of the ChTRIM9 ORF was amplified and subcloned into the pEGFP-N1 and pcDNA 3.1a vector. After transformation, the positive recombined plasmids were isolated from overnight bacterial cultures using a HiPure Plasmid EF Kit (MAGEN, China) according to the manufacturer's instructions.

The NF- κ B reporter vector had been previously constructed in our laboratory [16], and the Renilla luciferase pRL-Tk vector (Promega, USA) was used as an internal control.

2. 4 Cell culture, transient transfection, subcellular localization and dual-luciferase reporter assays

HEK293T cells were cultured in modified Eagle's medium (MEM, Gibco, USA) supplemented with 10% FBS (Fetal bovine serum, Gibco USA) and penicillin and streptomycin in a humidified incubator with 5% CO₂ at 37°C. Prior to transfection, the cells were seeded overnight, and plasmids were transfected by using ViaFectTM Transfection Reagent (Promega, USA) following the manufacturer's instructions. Sub-cellular localization was performed using GFP-fusion protein expression. HEK293T cell culture was performed as described above. The endofree plasmids, including ChTRIM9-GFP, and pEGFP-N1 (negative control), were transfected into HEK293T cells using ViaFectTM Transfection Reagent (Promega, USA). Forty-eight hours after transfection, HEK293T cells were washed with PBS buffer for 5 min, fixed with 4.0% paraformaldehyde for 10 min, and stained with 4, 6-diamidino-2-phenylindole hydrochloride (DAPI). Cells transfected with fluorescent vectors were directly observed under a fluorescent microscope.

For the luciferase reporter assay, cells were co-transfected with 100 ng/well of pRL-TK and 100ng NF- κ B luciferase reporter plasmids. pcDNA3.1 was used as a control. Each experiment was conducted in triplicate. Reporter signal detection was performed by Dual-Luciferase® Reporter Assay System (Promega, USA). Briefly, passive lysis of cells was adopted by adding 1x passive lysis buffer (100 μ L/well) after one wash with PBS and the growth medium was removed. After 20 min incubation in room temperature, the cell lysis was centrifuged at 12000 g for 5 min and the supernatant was collected into the

96-well enzyme labeled board (40 μ L/well). Following the manufacturer's instructions, Luciferase Assay Reagent II and Stop & Glo Reagent were added successively into the 96-well enzyme labeled plate(100 μ L/well). Signal detection was performed on a Microplate Reader Infinite M200 PRO.

2.5 Transcriptional analysis of ChTRIM9 by qRT-PCR and statistical analysis

The transcriptional expression of *ChTRIM9* in various tissues and *V. alginolyticus* challenge were determined by quantitative real-time PCR(qRT-PCR). *GAPDH* was used as an internal control and the primers are listed in Table 1S. The qRT-PCR reactions were carried out using the Light-Cycler 480 II System (Roche, USA) in a 20 μ L volume containing 10 μ L of 2 $\times$ SYBR Green PCR Master Mix (Roche, USA), 1 μ L of 10 μ M primers, 8 μ L of PCR-grade water, and 1 μ L of cDNA template. The PCR cycling program was as follows: initial denature at 95 $^{\circ}$ C for 3 min; followed by 45 amplification cycles at 95 $^{\circ}$ C for 15 s, 55 $^{\circ}$ C for 15 s, 72 $^{\circ}$ C for 10 s, and 85 $^{\circ}$ C for 20 s for signal acquisition in each cycle. A melting curve analysis was performed at the end of each PCR reaction to confirm the specificity of the amplicons. For each sample, experimental and control reactions were run in triplicate. Negative controls were included to assess genomic contamination. The PCR efficiencies for the target and reference genes were approximately equal. All data obtained from qPCR was calculated based on the $2^{-\Delta\Delta CT}$ method[17]and analyzed using SPSS 13.0 software for Windows. Differences were considered significant at $p < 0.05$ and $p < 0.01$.

3 Results

3.1 Bioinformatics analysis of ChTRIM9

The *ChTRIM9* partial sequence was identified after screening our *C. hongkongensis* transcriptome. The full-length cDNA of *ChTRIM9* was obtained and submitted to the

NCBI under GenBank accession number KU885983. The complete cDNA sequence of ChTRIM9 consists of a 71bp 5'UTR, 2166bp Open Reading Frame (ORF) and 691 bp 3'UTR. The deduced molecular weight (Mw) and isoelectric point (pI) are 80.2kDa and 5.38, respectively. A polyadenylation signal (AATAAA) is located upstream of the poly (A) tail. SMART analysis revealed that ChTRIM9 contains a RING finger domain in the N-terminus, two B-BOX domains, a coiled-coil, and a C-terminal SPRY domain (Fig 1S). These molecular features demonstrate that ChTRIM9 has properties that are characteristic of the TRIM9 family and indicate that ChTRIM9 is a newly discovered mollusk protein.

A multiple sequence alignment showed a high conservation between ChTRIM9 and its orthologs (Fig2S). ChTRIM9 has 44.7-98.2% identity with its homologs, ranging from mollusks to mammals. In addition, phylogenetic analysis of the TRIM9 gene family shows that these sequences are divided into two major groups (invertebrates and vertebrates), with ChTRIM9 belonging to the invertebrate group. The relationships displayed in the phylogenetic tree are generally in agreement with those of traditional taxonomy (Fig3S).

3.2 Tissue distribution and temporal expression of ChTRIM9 following bacterial challenge

qRT-PCR analysis with various tissues (including hemocytes, digestive gland, heart, gill, mantle, adductor muscle, labial palp, and gonad) showed that *ChTRIM9* is ubiquitously expressed in all examined tissues. It was expressed at higher levels in the gill, digestive gland, and labial palp. In contrast, expression of *ChTRIM9* was lower in heart and hemocytes (Fig 1A). Expression of *ChTRIM9* in hemocytes after *V. alginolyticus* challenge was investigated (Fig 1B). The expression level significantly reduced after 6 h post-challenge then recovered to the equal level between bacterial and PBS groups.

3.3 Subcellular localization of *ChTRIM9*

To investigate the intracellular localization of ChTRIM9, subcellular localization of ChTRIM9 was examined with the introduction of the pEGFP-N₁-ChTRIM9 plasmid into HEK293T cells by transient transfection. The pEGFP-N₁-ChTRIM9 transfected cells exhibited a punctate GFP fluorescence pattern in the cytoplasm under a fluorescent microscope. In contrast, the cells with the negative control plasmid pEGFP-N₁ showed a uniform presence of green fluorescence throughout the cells (Fig 2).

3.4 NF- κ B dual-luciferase reporter assays

For the determination of ChTRIM9's involvement in the regulation of NF- κ B signaling, dual-luciferase reporter assays were performed. HEK293T cells were transfected with either a plasmid expressing pcDNA3.1-ChTRIM9 or a pcDNA3.1 empty plasmid (Fig 3). The results showed that expression of recombinant ChTRIM9 significantly ($p < 0.05$) suppressed NF- κ B-luciferase activity, indicating its negative regulatory role in oysters.

Discussion

In the present study we reported the identification and characterization of *ChTRIM9* in Hong Kong oysters using a combination of a homology search in the transcriptome and SMART-RACE methods. ChTRIM9 possesses a RING finger domain in the N-terminus, two B-BOX domains, and a coiled-coil domain, which is required for a protein to be defined as a TRIM family member. In addition, ChTRIM9 contains a C-terminal SPRY domain. A multiple alignment and phylogenetic analysis also confirmed that ChTRIM9 shares a high degree of identity with its orthologs in mollusks and vertebrates, consistent with that in human and zebra fish TRIM9 proteins.

The expression pattern analysis indicated that *ChTRIM9* mRNA was constitutively

expressed in all examined tissues, with the highest expression in gill tissues and the lowest in heart tissues. Previously, a ubiquitous expression level was also observed in the mouse. However, in contrast to *ChTRIM9*, the expression of the mouse counterpart was mainly confined to the central nervous system [8]. In oyster, the gill represents the main interface between the organism and the surrounding external environment and is the first line of defense against potential pathogens. The present study contributes to a better understanding of the complex molecular mechanisms of host defenses in mollusks. The predominantly expressed *ChTRIM9* in the gill suggests that ChTRIM9 might be involved in the innate immune response or tissue metabolism. Increasingly, more reports have demonstrated the crucial role of TRIM9 in the innate immune responses of mammals. However, few studies have reported on the functions of TRIM9 in the immune defense responses of invertebrates, and especially marine mollusks. Therefore, we characterized the expression pattern of *ChTRIM9* in hemocytes in response to stimulation with *V.Alginolyticus*. During bacterial challenge, *ChTRIM9* mRNA in hemocytes showed a significant decrease at 6 h bacterial challenge compared to the control group, indicating that ChTRIM9 was probably involved in the interaction between oyster and bacteria, and played an important role in the signal transduction in the immune response of oysters. In agreement with this observation, arecent proteomic technique demonstrated that the repressed level of TRIM9 is also observed in the brain infected with rabies virus[18].Its repressed effect in immune response implies the vigorous immune control role of ChTRIM9. As we known, the out of control immune response and allergic reaction can also do harm to the body and thus the drastic reaction by immune weapons must to be strictly regulated so as to work in the right time and right place. ChTRIM9 is very likely to be one of the most important modulators in immune reaction control.

Different TRIMs have been reported to localize to various sub-cellular

compartments[2, 19]. In most cases the TRIM proteins identify different discrete nuclear and/or cytoplasmic sub-cellular structures[20]. In this study, fluorescence microscopy showed that ChTRIM9 was localized in the cytoplasm, suggesting that ChTRIM9 is a cytoplasm-localized protein under normal circumstances, which is in good agreement with other reports[20, 21]. These results indicated that ChTRIM9 mainly functions within the cytoplasm instead of the nuclei.

Previous evidence demonstrated that TRIMs can modulate various stages of NF- κ B activation post PRR activation. TRIM27 has been shown to inhibit NF- κ B signaling by interacting with and repressing IKKs[22]. More recently, a role for TRIM30a in the negative regulation of NF- κ B downstream of TLR signaling has been described[23]. To determine whether ChTRIM9 has a similar role in the regulation of the NF- κ B signaling pathway, we performed dual luciferase reporter assays in HEK293T cells. Our previous study confirmed that this cell line is appropriate for investigation of the functional properties of Hong Kong oysters. The result showed that over-expression of ChTRIM9 could significantly down-regulate NF- κ B reporter gene activity compared to the empty vector pcDNA3.1, indicating its negative regulation of NF- κ B in oysters. This part illustrated the way to restrict the immune response of ChTRIM9 under normal circumstance. While it's necessary to make a deeper understanding about the detail mechanism of NF- κ B signal targeting negative control and perhaps there existing the other target proteins and signal pathways, further study is carried out to find the answers.

In conclusion, an oyster *TRIM9* homolog was identified from *C. hongkongensis*. Multiple sequence alignment and phylogenetic analysis indicated that the ChTRIM9 has typical characteristic features of other TRIM family proteins. Tissue-specific expression analysis showed that *ChTRIM9* was constitutively expressed in all examined tissues, with highest expression in the gills. Moreover, its transcript level could be significantly

down-regulated by bacterial challenge. In addition, ChTRIM9 is mainly distributed in the cytoplasm, and it is a negative regulator of NF- κ B signaling in oysters. The present study contributes to a better understanding of the complex molecular mechanisms of host defenses in mollusks.

Acknowledgements

This work was supported by the Joint Funds of NSFC-Guangdong of China (U1201215), National Science Foundation of China (No.31502193), Guangdong Natural Science Foundation (No.2014A030310402) and Science and Technology Planning Project of Guangdong Province, China (No.2014B030301064).

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Figure caption

Fig1. Expression analysis of *ChTRIM9* by qRT-PCR. A. The tissue distribution of *ChTRIM9* in different tissues. *GAPDH* was used as a reference gene. Vertical bars represent the mean $\pm$ SD (N=3). B. The expression of *ChTRIM9* after bacterial challenge in hemocytes. Each bar represents the mean $\pm$ SD of the normalized expression levels of replicates (N=5). An asterisk indicates a significant difference at $p < 0.05$ and two asterisks indicate a significant difference at $p < 0.01$ between the challenged and control groups at the same time point.

Fig2. The sub-cellular localization of ChTRIM9 in HEK293T cells. The location of the nucleus was shown by DAPI staining (blue). The green fluorescence signal was distributed in both cytoplasm and nucleus in the control cells transfected with pEGFP-N₁, while the ChTRIM9 was mainly localized in the cytoplasm.

Fig3. The relative value in a NF- κ B dual-luciferase reporter assay of ChTRIM9. The data are means of three replicates of a single transfection experiment after normalization for Renilla luciferase activity. The values are shown as means $\pm$ SD (N=3). The significance is shown by asterisks (* represents $p < 0.05$).