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Identification and functional characterization of an Rbx1 in an invertebrate *Haliotis diversicolor supertexta*

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ABSTRACT

Rbx1 (RING box1) is an evolutionarily conserved RING-H2 finger protein and belongs to the RING-finger family of Ubiquitin ligase E3, which determines the substrate specificity of ubiquitination and regulates a variety of biological processes. We report here the identification and functional characterization of an Rbx1 homologue in abalone, which we named ab-Rbx1. Ab-Rbx1 contains conserved cysteine/histidine residues which are the characteristics of Rbx proteins. Phylogenetic tree analysis further demonstrated that ab-Rbx1 belongs to the Rbx1 family other than Rbx2 family. Real-time PCR analysis revealed that ab-Rbx1 was ubiquitously expressed in all examined tissues of abalone and the expression level of ab-Rbx1 was significantly induced by mitogenic situation. Immunohistochemical and immunofluorescent staining showed that the ab-Rbx1 was expressed predominantly in epithelial cells and localized both in the cytoplasmic and nuclear compartment. Ubiquitination assay demonstrated that ab-Rbx1 had ubiquitin ligase activity and could auto-ubiquitinated itself. These results suggest that ab-Rbx1 is an Rbx1 homologue and may be indirectly involved in the immune response of abalone through ubiquitination.

1. Introduction

Ubiquitin/proteasome system which is one of the major protein degradation pathways (Sun et al., 2001) is thought to mediate selectively the degradation of proteins and directly or indirectly involved in many biological processes, such as cell cycle progression, the response to stress, antigen processing, signal transduction, transcriptional regulation, DNA repair, apoptosis, and organelle biogenesis (Weissman, 2001; Glickman and Ciechanover, 2002). In addition, ubiquitin-mediated protein degradation has been implicated in the control of cellular processes affected by various pathological conditions, especially neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and diseases associated with the expansion of polyglutamine tracts in key proteins (Vanleeuwen et al., 1998; Cummings et al., 1999; Kitada et al., 1998; Kalchman et al., 1996). Moreover, recent reports showed that the protein activity and subcellular localization can be altered through monoubiquitination or polyubiquitination (Pickart, 2001; Hicke et al., 2005). Ubiquitination of a target protein involves multistep enzymatic reaction catalyzed by a cascade of enzymes, including ubiquitin-activating enzyme (also known as E1), ubiquitin-conjugating enzyme (also known as E2) and ubiquitin ligase (also known as E3).

Ubiquitin ligase E3 determines the substrate specificity of ubiquitination and regulates a variety of biological processes. Increasing amounts of evidence strongly suggested that the abnormal regulation of some E3 ligases were involved in cancer development and E3 ubiquitin ligases were potential cancer drug targets and prognostic biomarkers (Sun, 2006). Based on the structure properties, E3 ubiquitin ligases have been generally classified into three families including HECT family, RING-finger family and U-Box family (Hatakeyama and Nakayama, 2003). Among them, RING-finger family was first described in the early 1990s, when there is nothing known about its role in ubiquitination, but as a functional module thought to mediate protein–protein or protein–nucleotide interactions (Lovering et al., 1993; Freemont et al., 1991). RING fingers which are the characteristics of RING-finger family include eight metal-binding residues that coordinate two zinc ions, arranged in an interleaved pattern (Freemont, 2000). Members of the RING-finger family of E3 enzymes are structurally classified as RING-H2 protein (C3H2C3 type which contains histidines at positions 4 and 5) and RING-HC protein (C3HC4 type which contains only one histidine at position 4). RING finger proteins have showed a variety of biochemical properties, including DNA binding (Lovering et al., 1993), RNA binding (Lai et al., 1998), protein binding (Ohta et al., 1999a; Wu et al., 1996) and metal-ion binding (Roehm and Berg, 1997; von Arnim and Deng, 1993). Recently, it was reported

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that TRIM25 RING-finger E3 ubiquitin ligase was essential for RIG-I-mediated antiviral activity, which represents a new class of antiviral regulatory pathway involved in innate immunity (Gack et al., 2007).

RING box1 (Rbx1, also known as ROC1 and Hrt1) is an evolutionarily conserved RING-H2 finger protein. Rbx1 belongs to the Rbx family which also contains another member Rbx2 (also known as SAG, ROC2 and Hrt2) and is homologues to the APC11 subunit of the anaphase-promoting complex/cyclosome (Zachariae et al., 1998). Rbx1 was first described in purification of the endogenous VHL complex from rat liver and was initially identified as a subunit of the multiprotein SCF and VHL tumor suppressor ubiquitin ligase complexes (Kamura et al., 1999; Skowyra et al., 1999). Later, it was found that Rbx1 binds to either one of the cullins members (cullin1–5) in both two-hybrid and coimmunoprecipitation assays (Ohta et al., 1999a). In addition, overexpression of Rbx1 can protect cells from apoptosis induced by the redox agent (Sun et al., 2001).

Because of its significance, Rbx1 homologues have been cloned and characterized in human (Ohta et al., 1999b), mouse (Kamura et al., 1999), *Caenorhabditis elegans* (Sasagawa et al., 2003) and *Arabidopsis* (Lechner et al., 2002). But there is nothing known about the Rbx1 in molluscs. Abalone is one of the most important molluscs species for commercial production in the world, including Australia, China, Japan, Korea, Mexico, South Africa, and the United States (Gordon and Cook, 2001). In this report, an Rbx1 homologue was firstly described in abalone based on sequence and structure analyses, examination on expression pattern, determination of intracellular localization and characterization of biological activity.

2. Materials and methods

2.1. Animals and immune challenge

Healthy abalones (*H. diversicolor supertexta*) 3 years of age were collected from an abalone farm in Xiamen (Fujian, China) and kept in artificial seawater with a cycling system at 23 °C (Jiang and Wu, 2007). A minimum of 5 individuals was used in each experimental condition. Abalones were challenged, by injecting into the foot muscle, 50 μL (1 μg/μL diluted in sterile saline: 0.9% sodium chloride) *Escherichia coli* lipopolysaccharide (LPS). In addition, abalones challenged with 50 μL autoclaved 0.9% sodium chloride were used as control. Hemocytes were collected at different times (0, 6, 12, 18, and 24 h) post injection by centrifugation (700 × *g*, 10 min, 4 °C).

2.2. Preparation and screening of abalone cDNA library

Total RNA was isolated from challenged-abalone hemocytes using TRIzol reagent (Invitrogen, USA). Abalone cDNA library was constructed by a SMART™ cDNA library construction kit (Clontech, USA) according to the manufacture's instruction with a few modifications. Briefly, the isolated RNA was reverse-transcribed to synthesize first strand cDNA at 42 °C for 1 h. Then first strand cDNA was mixed with 5′ PCR primer and other PCR reagents at 95 °C for 30 s followed by 20 cycles of 95 °C for 10 s, 68 °C for 6 min to synthesize double-strand DNA. After digestion and purification, double-strand DNA was ligated to TriplEx2 Vectors. Then the resulting ligation reaction was packed using the Lambda DNA Packaging System (Promega, USA) following the manufacturer's instructions. Screening of abalone cDNA library was based on polymerase chain reaction (PCR) with the pTriplEx2 sequencing primers shown in Table 1. Positive plasmid clones were grown in liquid cultures and induced to a high copy number for direct sequencing. Finally, the cDNA sequences were analyzed by NCBI blast and a novel gene showed high similarity with Rbx1 was found.

2.3. Sequence analysis and phylogenetic construction

Sequence analysis was carried out by BLAST software (www.ncbi.nlm.nih.gov/blast). Deduced amino acid sequences were aligned using ClustalW (http://npsa-pbil.ibcp.fr) software. Domain prediction was performed with SMART (http://smart.embl-heidelberg.de) software (Schultz et al., 1998). A phylogenetic tree was constructed based on full-length amino acid sequences using the neighbor-joining method (Saitou and Nei, 1987) and was drawn using MEGA version 3.1 (Kumar et al., 2004).

2.4. Preparation and treatment of hemocytes

Hemocytes were prepared according to the method previously reported (Lacoste et al., 2002). Hemocytes were collected to obtain 15–20 ml samples and adjusted to10^6 cell/ml by the addition of modified Hank's balanced salt solution (MHBSS). 1 ml of cell suspension (10^6 cells) was dispensed into 35 mm petri dishes together with 1 ml of MHBSS. Cells were allowed to attach for 20 min and carefully rinsed with MHBSS. Hemocytes were then maintained at 15 °C in IMDM (Gibco), adjusted to ambient salinity (31 ppm), containing 5% heat inactivated horse serum, 5% heat inactivated fetal bovine serum, penicillin G (50 units/ml) and streptomycin (50 μg/ml). Hemocytes incubated with PHA (100 μg/ml) or equal volume of PBS for different periods of time (0, 30, 60, 90, and 120 min) were used for real-time PCR analysis.

2.5. Real-time PCR

Total RNAs from different tissues of healthy abalones and hemocytes incubated with PHA or hemocytes of abalones challenged with LPS were isolated using TRIzol reagent (Invitrogen, USA). The specific primers for ab-Rbx1 and 28s rDNA (Table 1) were designed by primer 3.0 software. Real-time PCR was performed using the SYBR Premix Ex Tag™ KIT (Takara, Japan). Briefly, the RNA was reverse-transcribed into cDNA with M-MLV reverse transcriptase and the PCR reaction was performed in a 25 μL volume using a 7500 real-time PCR system (ABI, USA). Experiments were performed in triplicate and the data were represented as the mean ± SEM ($n = 3$) for Student's *t*-test analysis. Differences were considered statistically significant when p values were less than 0.05.

2.6. Protein expression and purification

Based on the entire ab-Rbx1 coding region sequence, specific PCR primers (Table 1) were designed to amplify the mature protein. PCR products were digested with restriction enzymes (EcoRI, XhoI) and ligated to the PET-28 (a+) expression vector (Novagen, USA). After the recombinant plasmids were propagated in *E. coli* DH5a, they were transformed into *E. coli* BL21 (DE3) for protein expression. Then the recombinant fusion proteins were purified by affinity chromatography using the nickel-nitrilotriacetic acid agarose (Ni-NTA) resins (Qiagen, Germany) following the manufacturer's protocol. The purified recombinant protein was analyzed by 12% SDS-polyacrylamide gel electrophoresis at 25 mA for 2 h. The protein separation was visualized with Coomassie brilliant blue R-250 (Sigma, USA). Molecular weight protein standards (Fermentas, USA) were used to determine the target protein size. Protein concentration was measured by the Bradford method (Bradford, 1976).

2.7. Antibody preparation

Anti-ab-Rbx1 antibodies were prepared as previously reported (Zhu and Wu, 2008a). In brief, purified proteins were homoge-