Fish and Shellfish Immunology 81 (2018) 343–353

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular cloning and characterization of Aos1 and Uba2 from the orange-spotted grouper (*Epinephelus coioides*)

Jingguang Wei[a,**], Chen Li[a], Xin Zhang[a], Sheng Zhou[a], Shaoqing Zang[b,c], Shina Wei[a], Qiwei Qin[a,d,*]

[a] College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, PR China
[b] CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, 510301, PR China
[c] University of Chinese Academy of Sciences, Beijing, 100049, PR China
[d] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266000, PR China

ARTICLE INFO

Keywords:
Epinephelus coioides
Aos1
Uba2
RGNNV
Cellular localization

ABSTRACT

Small ubiquitin-related modifiers (SUMOs) are post-translationally conjugated to other proteins and are essential regulators of a wide range of cellular processes. Covalent attachment of SUMO requires an enzymatic cascade consisting of a single E1-activating enzyme (Aos1 and Uba2 heterodimer), a single E2-conjugating enzyme (Ubc9), and one of several E3 ligases that facilitate transfer of SUMO from Ubc9 to the substrate. In the present study, the Aos1 and Uba2 homologues (EcAos1 and EcUba2) from the orange-spotted grouper (*Epinephelus coioides*) were cloned and their possible roles in fish immunity were analyzed. The open reading frame (ORF) of EcAos1 contains 1050 base pairs (bp) encoding a 350 amino acid protein with a predicted molecular mass of 38.97 kDa EcAos1 has a nuclear localization signal (NLS) at residues 193–203. The ORF of EcUba2 contains 1950 bp encoding a 650 amino acid protein with a predicted molecular mass of 71.3 kDa EcUba2 has a NLS at residues 608–630. Quantitative real-time polymerase chain reaction analysis indicated that both EcAos1 and EcUba2 were distributed in all examined tissues. The expression levels of EcAos1 and EcUba2 in the spleen and head kidney of *E. coioides* were differentially up-regulated when challenged with polyinosine-polycytidylic acid. Green fluorescence of both pEGFP-C1-EcAos1 and pEGFP-C1-EcUba2 was distributed in the nucleus of GS cells. When the NLSs of EcAos1 and EcUba2 were deleted, the cellular localizations all changed. Over-expression of EcAos1 and EcUba2 inhibited red-spotted grouper nervous necrosis virus infection and replication. These results are important for better understanding of the SUMO pathway in fish and provide insights into the regulatory mechanism of viral infection in *E. coioides* under farmed conditions.

1. Introduction

SUMOylation, or reversible attachment of small ubiquitin-related modifiers (SUMOs), serves to regulate hundreds of proteins [1]. Covalent attachment of SUMO requires an enzymatic cascade consisting of a single E1-activating enzyme (Aos1 and Uba2 heterodimer), a single E2-conjugating enzyme (Ubc9), and one of several E3 ligases that facilitate transfer of SUMO from Ubc9 to the substrate. The majority of known SUMO targets are nuclear proteins, and, consistent with this, most of the known SUMO enzymes are enriched in this compartment [2].

The SUMO E1-activating enzyme (SAE) is a heterodimer consisting of the two subunits: Aos1 (SAE1) and Uba2 (SAE2) [3,4]. Both Aos1 and Uba2 contain nuclear localization sequences (NLSs) [2,5]. Aos1 resembles the N terminus of the ubiquitin E1 enzyme with a classical NLS in its mid region defined by the short sequence motif of K (K/R)*X* (K/R), where K is lysine, R is arginine, and *X* can be any residue. Uba2 is homologous to the C terminus of ubiquitin E1, but it has an 80 amino acid extension that contains a bipartite NLS consisting of two clusters of basic amino acids separated by a 9–10 amino acid segment [2,5,6]. The C-terminal extension is unstructured, and its deletion causes Uba2 to localize to the cytoplasm but otherwise has no effect on *in vitro* enzymatic activity [5,7]. Each component of the SAE can localize to the nucleus separately using its own NLS. Although the Aos1 NLS is blocked in the heterodimer, the Uba2 NLS can still transport the SAE1/2 heterodimer to the nucleus [2]. In many species, SUMO proteins are highly conserved and are important to many eukaryotic cell processes [3,8,9].

* Corresponding author. College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, PR China.
** Corresponding author.
E-mail addresses: weijg@scau.edu.cn (J. Wei), qinqw@scau.edu.cn (Q. Qin).

https://doi.org/10.1016/j.fsi.2018.07.043
Received 21 April 2018; Received in revised form 18 July 2018; Accepted 20 July 2018
Available online 21 July 2018

In fish, many sequences of Aos1 and Uba2 have been reported in National Center for Biotechnology Information (NCBI). However, to the best of our knowledge the function of grouper Aos1 and Uba2 in response to viral infection has not been studied.

The orange-spotted grouper (*Epinephelus coioides*) is one of the main commercial marine aquaculture fishes in South China and Southeast Asia. However, the growing scale of grouper aquaculture and continuous deterioration of the culture environment have led to heavy economic losses to the grouper market due to various infectious diseases, particularly nervous necrosis viruses (NNVs) and iridoviruses, which can result in as much as 80% mortality [10,11]. NNVs are non-enveloped RNA viruses and the causative agents of viral encephalopathy and retinopathy in fish. NNVs are divided into four genogroups based on the RNA2 sequence: barfin flounder NNV, red-spotted grouper NNV (RGNNV), striped jack NNV, and tiger puffer NNV [12]. To understand the virus–host interaction, two cDNA libraries were constructed in our lab from spleens of grouper infected with viruses, and some genes were identified and characterized as having antiviral function against RGNNV [11,13–16].

In the present study, Aos1 and Uba2 genes of grouper were cloned, and their expression at the transcript level in RGNNV-infected grouper and in control fish were analyzed. The intracellular localization of EcAos1 and EcUba2 and their effects on virus replication were also studied. Results of this study provide a better understanding of the innate immune mechanisms in the virus response of fishes.

2. Materials and methods

2.1. Cells and virus

Grouper spleen (GS) cells were grown in Leibovitz's L15 culture medium with 8% fetal bovine serum (Gibco, USA) at 28 °C [17]. Propagation of RGNNV was performed as described in the literature [18]. The viral titers of RGNNV were 10^5 $TCID_{50}$/ml.

2.2. Fish and immunization experiments

The orange-spotted groupers (weight 20–30 g) were purchased from Shenzhen Marine Fish Farm, Guangdong Province, China. Fishes were maintained in a laboratory recirculating seawater system at 24–28 °C and fed twice daily for 2 weeks. Tissue samples, including liver, spleen, kidney, brain, intestine, heart, skin, muscle, stomach, gill, and head kidney, were collected from five fish and immediately frozen in liquid nitrogen followed by storage at −80 °C prior to analysis.

For the pathogen challenge test, fishes were intraperitoneally injected (i.p.) with 100 μl of polyinosine-polycytidylic acid (poly [I:C]) (1 μg/ml) purchased from Sigma-Aldrich. A group of untreated fishes was used as the control. At 3, 6, 12, 24, 48, and 72 post-inoculation, five fishes from each group were sacrificed, and spleen and head kidney were removed for RNA extraction and cDNA synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR) was used for the expression analysis.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted using the SV Total RNA Isolation System (Promega, USA) according to the manufacturer's protocol. The quality of total RNA was assessed by electrophoresis on 1% agarose gels. Total RNA was reverse-transcribed to synthesize the first-strand cDNA using the ReverTra Ace kit (TOYOBO, Japan) according to the manufacturer's instructions.

2.4. Cloning and sequencing Aos1 and Uba2 from E. coioides

The primers F1 and R1 for the open reading frame (ORF) of grouper Aos1 were designed based on the nucleotide sequences of Aos1 from *E. coioides* (accession no. KT750034.1) (Table 1). The forward primer F2 for the ORF of grouper Uba2 was designed based on the nucleotide sequences of Uba2 from *E. coioides* (KT750035.1), whereas the reverse primer R2 of grouper Uba2 was designed based on the conserved nucleotides of two fish Uba2 sequences reported previously (*Kryptolebias marmoratus*, accession no. XP_017295430.1 and *Lates calcarifer*, accession no. XP_018559587.1) (Table 1). The ORF sequences of grouper Aos1 and Uba2 were amplified with F1/R1 and F2/R2 using grouper spleen cDNA as amplification templates. PCR was performed with an initial denaturation step of 5 min at 94 °C, and then 35 cycles were run as follows: 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1.5 min, and 72 °C elongation for 5 min.

Table 1
Primers used in the present study.

Primer	Sequence (5′-3′)
F1	ATGATCGATATGATCGAGAAGGAGG
R1	TTAGTTTGGTCCAAAGTAGTCAACC
F2	ATGGTCCAACTAGTGGGTTCCCTCC
R2	TTAATCCAGAGCGATGATGTCGTCA
F3	GCCAAAGCCGGGATGAAGCGAACAC
R3	GCGAATCTGCCTCAGGAGCTGACTG
F4	CTGTCATCGCTGGACTCATTGTGCT
R4	CTTCCTAAGGTTGGGATGCTTGTTC
pEGFP-C1-Aos1-S	GCTCAAGCTTCGATGATCGATATGATCGAGAAGGAGG
pEGFP-C1-Aos1-N-R	GCGGATCCCGTTAAATTTCCAGTTGGTTTCACA
pEGFP-C1-Aos1-C-S	GCTCAAGCTTCGATGTCCAGCGAGACAACCATGGTGA
pEGFP-C1-Aos1-R	GCGGATCCGTTTGGTCCAAAGTAGTCAACCATG
pEGFP-C1-Uba2-S	GCGAATTCTATGGTCCAACTAGTGGGTTCCCTCC
pEGFP-C1-Uba2-N-R	GCGGTACCCTGGTTGCCGCTGCTGCTGCAGAGC
pEGFP-C1-Uba2-R	GCGGTACCATCCAGAGCGATGATGTC
pcDNA3.1-Aos1-S	GCGGATCCATGATCGATATGATCGAGAAGGAGG
pcDNA3.1-Aos1-N-R	CGCTCGAGTTACGTTAAATTTCCAGTTGGTTTC
pcDNA3.1-Aos1-C-S	GCGGATCCATGTCCAGCGAGACAACCATGGTGA
pcDNA3.1-Aos1-R	CGCTCGAGTTAGTTTGGTCCAAAGTAGTCAACC
pcDNA3.1-Uba2-S	GCGGTACCATGGTCCAACTAGTGGGTTCCCTCC
pcDNA3.1-Uba2-N-R	GCGAATTCTTACTGGTTGCCGCTGCTGCTGCAG
pcDNA3.1-Uba2-R	GCGAATTCTTAATCCAGAGCGATGATGTCGTCA
CP-F	CAACTGACAACGATCACACCTTC
CP-R	CAATCGAACACTCCAGCGACA
RdRp-F	GTGTCCGGAGAGGTTAAGGATG
RdRp-R	CTTGAATTGATCAACGGTGAACA
18 S-F	ATTGACGGAAGGGCACCACCAG
18 S-R	TCGCTCCACCAACTAAGAACGG

2.5. Cloning, sequencing, and sequences analysis

The PCR products were analyzed on 1% agarose gels, extracted with an AxyPrep DNA gel extraction kit (AxyGEN), then ligated into pMD18-T vectors (TaKaRa) and transformed into competent *Escherichia coli* DH5α cells. Positive colonies were screened by PCR and at least three recombinant plasmids were sequenced [19].

Sequences were analyzed based on nucleotide and protein databases using the online BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). The coding sequences were predicted using the online ORF finder tool (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The protein molecular weights were deduced using the Bio-Soft website (http://www.bio-soft.net/sms/index.html). The signal peptides were predicted using Signalp3.0 (http://www.cbs.dtu.dk/services/SignalP/). The NLSs were predicted using cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi#opennewwindow). Multiple alignments of amino acid sequences were analyzed by ClustalX, and the neighbor-joining (NJ) method implemented in MEGA 4.0 was used for phylogenetic analysis. The robustness of bifurcations was estimated with bootstrap analysis, and bootstrap percentages were obtained with 1000 replicates.