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Molecular characterization of the autophagy-related gene Beclin-1 from the olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

Autophagy is an important cellular response to starvation and stress, and plays critical roles in embryogenesis, development, cell death, cancer, and immunity. Beclin-1 is one of the central regulators of autophagy in mammals. In the present study, we isolated a PoBeclin-1 cDNA from the olive flounder (*Paralichthys olivaceus*) by screening a flounder gill cDNA library and rapid amplification of cDNA ends (RACE). The PoBeclin-1 cDNA we isolated encodes a 447-amino acid polypeptide containing a conserved Bcl-2-binding domain. The deduced amino acid sequence of PoBeclin-1 showed high degrees of sequence identity (80.5–95.3%) with Beclin-1 from human, frog, mouse, zebrafish, and pufferfish. PoBeclin-1 transcripts were detected from 1 day post-hatching and were found to be ubiquitously expressed in the healthy flounder. Expression of PoBeclin-1 mRNA was increased in the kidney and spleen of flounders challenged with viral hemorrhagic septicemia virus (VHSV). When infected with VHSV, PoBeclin-1-overexpressing HINAE cells had low level (about 26%) of VHSV G transcripts compared to control cells. Taken together, these results suggest that PoBeclin-1 may play a role in the innate immune response to viral infection in the flounder.

1. Introduction

Autophagy, a conserved mechanism of quality control in cells, plays roles in the elimination of defective proteins and organelles, the prevention of abnormal protein aggregate accumulation, and the removal of intracellular pathogens [1–4]. About 32 autophagy-related (ATG) genes encode components of the autophagy machinery in the yeast *Saccharomyces cerevisiae*, and many of them are also found in mammals and other higher eukaryotes [5]. Mutations in some of these ATG genes affect survival during nutrient starvation, embryogenesis, and development [6–8]. Additionally, autophagy has been implicated in protection against certain types of cancer, disease, oxidative stress, and infection [9–11].

Autophagy plays a key role in innate and adaptive immunity to viruses [12,13]. It is critical for recognizing signatures of viral infection, and represents an important effector mechanism to restrict viral replication. In Atg5-deficient mice, plasmacytoid dendritic cells (pDCs), a subset of DCs known for their ability to secrete large amounts of IFN-α in response to viral infection, failed to mount an IFN-α response following systemic infection with VSV or herpes simplex virus (HSV)-1 [14]. In plants, autophagy exerts antiviral effects against tobacco mosaic virus (TMV) by restricting cell death to the infection site, and limiting the replication and cell-to-cell movement of TMV [15]. On the other hand, autophagosomes have been exploited by certain viruses as a niche for viral replication [16]. Atg6/Beclin-1, Atg4B, Atg5, and Atg12 are proviral factors required to initiate hepatitis C virus replication [17].

Beclin-1, the mammalian ortholog of the yeast protein Atg6/Vps30 (vacuolar protein sorting 30), was first identified as a Bcl-2-interacting protein through yeast two-hybrid screening [18]. It is a component of the class III PI3K complex, which helps to localize autophagy proteins to the preautophagosomal membrane [19]. Beclin-1 plays a central role in coordinating the cytoprotective function of autophagy and in protecting against apoptosis. Beclin-1-mediated autophagy plays a role in antiviral host defense [20,21]. Overexpression of mammalian Beclin-1 protects mice against lethal

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Sindbis virus encephalitis through its Bcl-2-binding domain and is involved in the clearance of HSV-1 from infected host cells. TMV replication was found to be increased in Beclin-1-silenced plants [15]. Beclin-1 is a mammalian tumor suppressor. Monoallelic deletion of Beclin-1 was, in one study, observed in up to 75% of ovarian cancers, 50% of breast cancers, and 40% of prostate cancers [22]. The BEC-1 gene of *Caenorhabditis elegans*, which shares 31% homology with human Beclin-1, is required for life span extension in this organism [23,24].

Recently, Beclin-1 homologs were identified in the pufferfish (*Tetraodon nigroviridis*; GenBank accession no. CAJ19379), fugu rubripes (*Takifugu rubripes*; NP_001032963), European seabass (*Dicentrarchus labrax*; CBN81459), three-spined stickleback (*Gasterosteus aculeatus*; CAJ19735), Atlantic salmon (*Salmo salar*; NP_001133290), medaka (*Oryzias latipes*; NM_001 098248), rainbow trout *Oncorhynchus mykiss* (NM_001117901), and zebrafish (*Danio rerio*; NP_957166). However, fish Beclin-1 genes have not been characterized. In this study, we describe the identification and characterization of the olive flounder (*Paralichthys olivaceus*) Beclin-1 (PoBeclin-1). We report that it is a homolog of the yeast and mammalian autophagy gene ATG6/VPS30/Beclin-1.

2. Materials and methods

2.1. Cloning of PoBeclin-1 from the olive flounder (P. olivaceus)

A partial PoBeclin-1 cDNA sequence was isolated by screening an olive flounder gill cDNA library with the EST clone gill-1-E05 identified as a flounder Beclin-1 homolog from the EST analysis of *P. olivaceus* cDNA library (unpublished data). It showed homology to other Beclin-1 sequences. 5′-Rapid amplification of cDNA ends (RACE) was performed using the SMART RACE cDNA Amplification Kit (Clontech) to obtain a full-length PoBeclin-1 cDNA. Based on a partial sequence, we designed internal primers (P3, 5′-GCA ATT AAT GAA CTG CTC TCC TTC C-3′; P4, 5′-AGC TGT CGT CCA GCT CCG AGA G-3′) and used them in combination with the universal primer supplied with the kit to amplify the 5′-end of the PoBeclin-1 transcript. DNA sequencing was performed with universal T7 and T3 primers (Promega) and internal primers using an ABI3730xl autosequencer (Applied Biosystems). The full-length sequence of the PoBeclin-1 cDNA was obtained by assembling the DNA sequences of the partial sequences of clones and 5′-RACE PCR products. The cDNA sequence was annotated in GenBank using BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2. Multiple sequence alignment and phylogenetic analysis

A multiple sequence alignment was performed using CLUSTALW (www.ebi.ac.uk/clustalw/). MEGA (ver. 4.1) was used to assess similarities among the aligned sequences. A phylogenetic tree based on the deduced amino acid sequences was constructed using a neighbor-joining algorithm, and the reliability of the branching was tested using bootstrap resampling with 1,000 pseudo-replicates.

2.3. Fish rearing condition

Fish were maintained at the Genetics and Breeding Research Center of the National Fisheries Research and Development Institute (NFRDI) in Geoje, Republic of Korea. Artificially fertilized flounder eggs were stocked in a tank with a flow through system of filtered seawater. A total of 98% of the eggs hatched 3 days later. Feeding program was modified from Sakakura (2006) [25]. Enriched L-type rotifers (*Brachionus plicatilis* complex) were fed from day 3 to day 14; enriched *Artemia franciscana nauplii* were supplied from day 13 to day 28; commercial fish diets (Maruwa Co., Ltd.; crude protein: 48–54%, crude fat: 9–12%) were offered from day 21. Feeding was given six times per day for ensuring sufficient food supply. Fish (approximately 12–15 cm in body length) were fed a commercial fish diet (crude protein, 52%; crude fat, 11%) three times per day. The temperature of the rearing tanks was maintained at 18 °C.

2.4. Quantitative real-time RT-PCR

Whole mount of the larva during early development or tissues were immediately frozen in liquid nitrogen, and stored at −80 °C before use. Total RNA was prepared from tissues using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA concentrations were quantified, and 1 μg of total RNA reverse transcribed. The first-strand cDNA was synthesized using an Advantage RT-for-PCR kit (BD Sciences). Quantitative real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche) and the following forward and reverse primers: PoBeclin-1-RT-F (5′-AGC CGG AGA GGA GAT TCT GA-3′) and PoBeclin-1-RT-R (5′-GCC AAA GGT AGG AAG GTT GG-3′); 18S rRNA-F (5′-ATG GCC GTT CTT AGT TGG TG-3′), 18S rRNA-R (5′-CAC ACG CTG ATC CAG TCA GT-3′); VHSV G–F (5′-AGA TGA GGG GAG CCA CAG AC-3′), VHSV G–R (5′-GGG ATG ATC AAT TTG TCC CC-3′). Following an initial Taq activation step (95 °C for 10 min), 40 cycles of LightCycler PCR were performed using the following cycling conditions: 95 °C for 10 s, 57 °C for 5 s, 72 °C for 30 s, and fluorescence reading.

2.5. Localization of PoBeclin-1 mRNA by in situ hybridization (ISH)

Tissues were prepared as described previously [26]. After deparaffinization and rehydration, sections (4–5 μm) were washed for 5 min in 100% ethanol at room temperature and pretreated with proteinase K (10 μg/mL) for 30 min at 37 °C. Prehybridization and hybridization were performed in DIG Easy Hyb solution (Roche). Digoxigenin (DIG)-labeled probes were prepared using a DIG RNA Labeling Kit (Roche) according to the manufacturer's instructions. PoBeclin-1, amplified with specific primers (PoBeclin-1-RT-F and PoBeclin-1-RT-R), was used to synthesize DIG-labeled cRNA probes. A hybridization mix was prepared by adding one volume of hybridization buffer to one volume of the antisense and sense probes. Slides were washed with DIG Wash and Block Buffer (Roche), and the signal was detected using a DIG Luminescent Detection Kit (Roche) according to the manufacturer's instructions. Sections were counterstained with Bismarck brown Y before observation.

2.6. Experimental challenge

Experimental challenge was conducted on 100 fish (approximately 12–15 cm in body length) with a dose of 1×10^6 $TCID_{50}$ of VHSV being administered by immersion at 16 °C [27]. After infection for 1.5 h, the virus was removed by replacing the water in the tank. Tissues were removed from three fish each at 3, 9, 12, 24, and 48 h post-infection. Cumulative mortality was monitored daily over the course of 3 weeks (data not shown).

2.7. Construction of the expression plasmid

Amplification of the open reading frame (ORF) of PoBeclin-1 was carried out using Ex Taq DNA polymerase (TaKaRa) and primers specific for the 5′-end (starting at the ATG initiator codon) and 3′-end of the PoBeclin-1 cDNA. The primers used were designed such that the amplified DNA would contain *Eco*RI and *Xho*I restriction endonuclease sites at the 5′- and 3′-ends, respectively. The