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Short communication

Beclin-1 is involved in tongue sole *Cynoglossus semilaevis* immune defense against bacterial infection

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ABSTRACT

In mammals, beclin-1 is a key player that regulates autophagic activity. In fish, the immune function of beclin-1 is essentially unknown. In this study, we analyzed the involvement of tongue sole (*Cynoglossus semilaevis*) beclin-1 (named CsBECN1) in antibacterial immunity. CsBECN1 is composed of 451 amino acid residues and shares 84.5–95.1% overall sequence identities with other teleost beclin-1. CsBECN1 possesses a typical Bcl-2 homology domain 3 and an Atg6 domain. Expression of CsBECN1 occurred in multiple tissues and was upregulated during bacterial infection. Knockdown of CsBECN1 significantly enhanced bacterial dissemination in the tissues of tongue sole, whereas overexpression of CsBECN1 significantly reduced bacterial dissemination. Taken together, these results indicate that CsBECN1 is required for the antibacterial immunity of tongue sole.

1. Introduction

Autophagy is an evolutionarily conserved cellular response to stress and plays crucial roles in the recycling of defective proteins and organelles [1–4]. Autophagy is involved in both innate and adaptive immunity and participates in immunological processes including direct pathogen elimination, pathogen-associated molecular pattern recognition, inflammasome regulation, and cytosolic antigen processing for MHC-II presentation [5–8]. The autophagic machinery is highly complex, and in yeast about 32 different “autophagy-related genes” (ATG) have been characterized, most of which are evolutionarily conserved among eukaryotes from yeast to human [9].

Beclin-1 is the mammalian homologue of yeast Atg6/Vps30 (vacuolar protein sorting 30) and a key component of PI3K complex that initiates the formation of autophagosomes by helping to localize other autophagy proteins, such as Atg14 and UV-irradiationresistance-associated gene (UVRAG), to the pre-autophagosomal membrane [10,11]. Beclin-1 was first characterized in mouse as a novel Bcl2-interacting protein that plays a central role in regulating the balance between the cytoprotective function of autophagy and protecting against apoptosis [12,13]. Beclin-1 may serve as a platform upon which cellular signals converge and function to regulate the crosstalk of multiple processes [14]. Gene knockout/knockdown studies indicate a conserved requirement for Beclin-1 in autophagy in plants, slime molds, nematodes, fruit flies, mice, and human cells [15]. Decreases in beclin 1 expression and/or functional activity have been linked to increased susceptibility to cancer, Alzheimer's disease, Huntington's disease, and alterations in microbial pathogenesis [16,17]. Overexpression of mammalian beclin-1 protects mice against lethal Sindbis virus encephalitis and promotes clearance of HSV-1 from infected host cells [12,18]. In addition, beclin-1 is essential for early embryonic development and is a haploinsufficient tumor suppressor gene [19,20].

In teleost, beclin-1 orthologues have been identified in pufferfish (*Tetraodon nigroviridis*), Atlantic salmon (*Salmo salar*), zebrafish (*Danio rerio*), olive flounder (*Paralichthys olivaceus*) [21], common carp (Cyprinus carpio) [22], and rare minnow (*Gobiocypris rarus*) [23]. However, most of the studies of fish becin-1 focused on monitoring of mRNA expression. The immune function of fish beclin-1 is essentially unknown.

Half-smooth tongue sole (*Cynoglossus semilaevis*) is a flatfish and an important farmed species in China. In this study, we cloned the beclin-1 gene of tongue sole (named CsBECN1) and investigated its role in immune defense against bacterial infection.

2. Materials and methods

2.1. Fish

Clinically healthy tongue sole (average 25.3 g) were purchased from a commercial fish farm in Shandong Province, China and maintained in the laboratory for two week before experiment. During laboratory maintenance, fish were kept at 20 °C in 50-L tanks (< 20 fish/tank)

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containing aerated seawater. The seawater was sand-filtered and activated carbon-absorbed, with pH of 8.1, oxygen > 6 mg/L, and ammonia < 0.1 mg/L. Fish were fed daily with commercial dry pellets (Shandong Sheng-suo Fish Feed Research Center, Shandong, China). Before experiment, fish were randomly sampled and verified to be absence of common pathogens in liver, head kidney, and spleen as reported previously [24]. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA) as reported previously [24]. Live fish study was approved by the Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences.

2.2. Sequence analysis

The cDNA and amino acid sequences of CsBECN1 (GenBank accession no. XP_008312022.1) were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. Multiple sequence alignment was created with DNAMAN.

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

qRT-PCR was performed as reported previously [25]. Briefly, tissues (spleen, head kidney, intestine, gill, heart, liver, brain, blood, and muscle) were taken aseptically from five tongue sole and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, USA). The RNA was treated with RNase-free DNase I (TaKaRa, Dalian, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase Kit (Invitrogen Corporation, Carlsbad, USA). The cDNA synthesis reaction was as follows: 1 μg total RNA was mixed with 1 μl Oligo (dT) $_{18}$ primer and 10 μl H_2O; the mixture was incubated at 65 °C for 5 min and chilled on ice. Four microliters of 5 × reaction buffer, 1 μl RiboLock RNase Inhibitor (20U/μl), 2 μl 10 mM dNTP Mix, and 1 μl RevertAid M-MuLV RT (200U/μl) were added to the above RNA mixture, followed by incubation at 42 °C for 60 min, and the reaction was terminated at 70 °C for 5 min qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China). The primer sequences for CsBECN1 were F1 (5′–CCCCTCAAA CTGGACACATC–3′) and R1 (5′–TTCAAAGGTCTCCTCTTGTGCC–3′), which produced 138 bp DNA fragment. The expression level of CsBECN1 was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin (ACTB) as an internal reference [26,27]. The PCR efficiency was 96.8%, and the Correlation coefficient (R2) was 0.998. The experiment was performed in replicates.

For qRT-PCR analysis of CsBECN1 expression in response to bacterial infection, *Edwardsiella tarda* and *Vibrio harveyi* were cultured in LB medium to an OD_{600} of 0.8, and the cells were washed with PBS and resuspended in PBS to 2×10^7 CFU/ml and 5×10^7 CFU/ml, respectively. Tongue sole were divided randomly into three groups (20/group) and injected intraperitoneally (i.p.) with 50 μl *E. tarda* (i.e. 1×10^6 CFU/fish), *V. harveyi* (i.e. 2.5×10^6 CFU/fish) or PBS. Tongue sole began to die on the third day after *E. tarda* or *V. harveyi* injection. At 6 h, 12 h, 24 h, and 48 h post-infection, five fish were taken for tissue collection, and CsBECN1 expression was determined by qRT-PCR as above. The internal references for head kidney and spleen were ACTB and ribosomal protein L18 (RPL18) respectively [27]. The experiment was performed in replicates.

2.4. CsBECN1 knockdown

CsBECN1 knockdown was performed by small RNA (siRNA) interference as reported previously [28]. To select effective siRNA for CsBECN1, the sequences of three siRNA targeting CsBECN1 were designed according to https://www.genscript.com/ssl-bin/app/rnai and inserted into the siRNA expression vector pRNAT-CMV3.1 (GenScript, Piscataway, USA) between BamHI/AlfII sites, resulting in plasmids pCsBECN1si-1, pCsBECN1si-2, pCsBECN1si-3. The plasmid pCsBECN1siC, which expresses a scramble siRNA, was constructed similarly. Endotoxin-free plasmid DNA was prepared using Endo-Free plasmid Kit (Omega Bio-Tek, Doraville, USA) and diluted in PBS to 400 μg/ml. Tongue sole were divided randomly into five groups (5 fish/group) and injected intramuscularly (i.m.) with 100 μl pCsBECN1si-1, pCsBECN1si-2, pCsBECN1si-3, or pCsBECN1siC; the control group of fish was injected with PBS. At 3d post-plasmid injection, expression of CsBECN1 in head kidney and spleen was determined by qRT-PCR as described above. The plasmid showing the strongest inhibitory effect on CsBECN1 expression was re-named pCsBECN1si. The experiment was performed in replicates. The siRNA sequences expressed by pCsBECN1si and pCsBECN1siC are 5′-GAGCGTCGACAACCAGATG-3′ and 5′-ACCG AATACGCGATGCGGA-3′ respectively.

2.5. Effect of CsBECN1 knockdown on bacterial infection

Tongue sole (as above) were divided randomly into three groups (N = 15) and administered with pCsBECN1si, pCsBECN1siC, or PBS as above. At 3 d post-plasmid administration, the fish were infected as above with 50 μl *E. tarda* (2×10^7 CFU/ml). At 12 h, 24 h, and 48 h post-infection, head kidney and spleen were taken from the fish (5 fish/time point) and examined for bacterial recovery by plate count as reported previously [25]. The experiment was performed in replicates.

2.6. CsBECN1 overexpression and its effect on bacterial infection

Overexpression of CsBECN1 in fish was performed as reported previously [29]. Briefly, the plasmid pCNBeclin-1, which expresses His-tagged CsBECN1, was created as follows. The coding sequence of CsBECN1 was amplified by PCR with the primer pair of CsBECN1-F1 (5′–GATATCGCCACCATGACAATGGCAATGGAAGGCTCAA–3′, underlined sequence, EcoRV site) and CsBECN1-R1 (5′–GATATCTCTGTTGT AGAACTGTGACGTGACC–3′, underlined sequence, EcoRV site); the 1370 bp PCR products were ligated with the T-A cloning vector T-Simple (Tiangen, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the CsBECN1-containing fragment, which was inserted into pCN3 [30] at the EcoRV site, resulting in plasmid pCNBeclin-1. Endotoxin-free plasmid DNA was prepared using Endo-Free plasmid Kit (Omega Bio-Tek, Doraville, USA) and diluted in PBS to 400 μg/ml. Tongue sole were divided randomly into three groups (5 fish/group) and injected i.m. with 100 μl pCNBeclin-1, pCN3, or PBS. Head kidney, spleen and muscle were taken from the fish at 3 d post-plasmid administration. For PCR analysis of plasmid presence, DNA was extracted from the tissues with the TIANamp DNA Kit (Tiangen, Beijing, China); PCR detection of pCNBeclin-1 and pCN3 was performed with the primer pairs CN-F1 (5′–CTTGCGTTTCTGATAGGC ACCTA–3′)/CsBECN1-R1 and CN-F1/CN-R1 (5′–TGCGGGCCTCTTCGC TATT–3′) respectively. To examine transcription of plasmid-encoded CsBECN1, total RNA was extracted from the tissues as described above and used for RT-PCR with the primer pair of CsBECN1-F1 and His-R (5′–GTGGTGGTGGTGGTGGTG–3′), in which the forward primer is specific to the target gene, while the reverse primer is specific to the His-tag of the backbone plasmid. As an internal control, RT-PCR was also performed with the primer pair of β-actin F (5′–GCACGGTATTGTGACCA ACTGG–3′)/β-actin R (5′–CAGGGGAGCCTCTGTGAGC–3′), which are specific to β-actin. To examine the CsBECN1 protein encoded by plasmid pCNBeclin-1, Western blot was performed as follows. The spleen was homogenized in PBS, and the supernatant was collected by centrifugation at 12,000 g, 4 °C for 20 min. The protein concentration was measured by BCA Protein Assay Kit (Pierce, USA). The samples with the same amount of protein were separated by SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (Millipore, USA), and the membrane was soaked in PBST (0.05% Tween 20 of PBS) with 5% skim milk. The membrane was incubated with anti-