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Death associated protein 1 (DAP 1) positively regulates virus replication and apoptosis of hemocytes in shrimp *Marsupenaeus japonicus*

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ABSTRACT

Death-associated protein 1 (DAP1) is a small proline-rich cytoplasmic protein that functions both in the apoptosis and autophage process of mammalian and in the clinical cancer of human. However, little knowledge is known about the homologue gene of DAP1 and its roles in the physiological process of invertebrates. In this paper, we report a novel function of DAP1 in the antivirus immunity of shrimp. A homologue gene of DAP1 was cloned from *Marsupenaeus japonicus* and named as *Mjdap*-1. The full-length of *Mjdap*-1 was 1761 bp with a 309 bp open reading frame that encoded 102 amino acids. Reverse transcription-PCR results showed that *Mjdap*-1 was expressed in all tested tissues, including hemocytes, gills, intestines, stomach, heart, hepatopancreas, testes, and ovaries. In shrimp, *Mjdap*-1 transcripts were up-regulated by white spot syndrome virus (WSSV) infection; *Mjdap*-1 knockdown decreased the virus copy *in vivo* and the mortality of *M. japonicus* to WSSV challenge. Conversely, injecting the purified recombinant *Mj*DAP1 protein promoted the amplification of virus in shrimp. Flow cytometric assay showed, the virus infection-induced apoptosis of hemocytes was enhanced by *Mj*DAP1 protein injection and inhibited in *Mj*DAP1 knockdown shrimp. Furthermore, the expression of apoptosis-inducing factor (AIF) was regulated by *Mjdap*-1, but the caspase transcripts were not affected. Our results suggested that *Mj*DAP1 facilitated the amplification of virus in shrimp, which may be attributed to the promotion of hemocyte apoptosis in an AIF-dependent manner. These results provided a new insight into the function of this protein that may be used for virus disease control.

1. Introduction

Virus infection is a complicated process that triggers various changes on the physiological function of its host [1,2]. Apoptosis is a programmed cell death, which is induced during virus infection and regulated by the interaction of virus and host [3,4]. Generally, apoptosis is regarded as a kind of antiviral immune response of the host at the early stage of viral infection, yet beneficial for viral amplification at the later stage [5,6].

In mammals, the molecular mechanisms of apoptosis are highly complex, and two main linked apoptotic pathways are distinguished according to the initial step of apoptosis: the death receptor (extrinsic) and mitochondrial (intrinsic) pathways. The classical extrinsic pathway is activated by the binding of death ligand to the death receptor, which is as member of the tumor necrosis factor receptor (TNFR) gene superfamily. Another extrinsic pathway (perforin/granzyme pathway) is specific for cytotoxic T lymphocytes that kill target cells, in which the transmembrane pore-forming molecule perforin and serine proteases granzyme A and granzyme B are needed. The intrinsic signaling pathway is an intracellular mitochondrial-initiated event triggered by non-receptor-mediated stimuli, such as deprival of cell survival factor, oxidative stress, and DNA damage [7]. However, such pathways are different and still in their infancy in invertebrate. For example, some molecules involved in the extrinsic apoptosis pathway in mammals do not exist in *Caenorhabditis elegans*, and their structures differ from those of *Drosophila* [8,9].

White spot syndrome virus (WSSV) is a large, enveloped *ds*DNA virus and one of the most destructive pathogens of shrimps and other crustaceans [10]. In shrimp, the signs of apoptosis induced by

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WSSV infection were reported [11]. To date, only several shrimp and viral proteins, such as the viral protein AAP-1 [12] (ORF390) and the caspase in shrimp [13,14], are identified as involved in apoptosis.

Death-associated protein 1 (DAP1) is a member of the DAP family, which comprises DAP1, DAP2 (DAP kinase), DAP3 (a nucleotide-binding protein), DAP4, and DAP5. DAPs are first isolated as a gene family involved in IFN-γ-induced HeLa cell apoptosis through a technical knockout strategy [15,16]. Given the lack of any identifiable functional motif, DAP1 is initially ascribed as nonfunctional and subsequently proven as a novel substrate of the Ser/Thr kinase mammalian target of rapamycin, which negatively regulates autophagy and serves as an anti-oncogenic molecule in some kinds of cancer [17,18]. In our previous work, a homologue gene of DAP1 (*Mjdap*-1) was identified as an up-regulating gene in virus-infected shrimp by differential expression profiling sequencing. We cloned the full-length *Mjdap*-1 gene, studied the expression pattern upon virus challenge, and investigated the mortality of shrimp and virus copy numbers after knockdown and overexpression of *Mjdap*-1. The result showed that *Mjdap*-1 is beneficial for amplification of virus. Further *in vivo* assay that integrated RNA interference showed that *Mjdap*-1 facilitated the apoptosis of shrimp hemocytes and affected the expression of apoptosis-inducing factor (AIF) gene during virus infection. To our knowledge, this study is the first report about the immune-related function of DAP 1 protein.

2. Materials and methods

2.1. Animals, virus, immune challenge, and sample preparation

Healthy shrimp *Marsupenaeus japonicus*, weighing 10–12 g, were purchased from the seafood market in Jinan city, Shandong Province, China. Shrimp were cultured in aquarium with air-pumped circulating seawater (salinity 24–26 ppt) at 25 °C for 24 h before usage. For immune challenge, 4×10^6 copies of WSSV were injected into the abdominal segment of each shrimp, and the same volume of phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4; pH 7.4) was used in control groups. In each group, the hemolymph of the shrimp was removed from the ventral sinus at indicated hour post injection (hpi) with a 1/10 volume of anticoagulant buffer (10% sodium citrate, pH 7; 200 mM phenylthiourea) in the injection syringe. The hemocytes were collected when the hemolymph was centrifuged at 700 g for 5 min (4 °C) immediately. Subsequently, various tissues (gills, intestines, stomach, hepatopancreas, heart, ovaries, and spermaries) were dissected and homogenized with TRIzon (Cwbio, Beijing, China) for RNA extraction. The tissues of three shrimp were mixed and used in each sample to eliminate individual differences. cDNA was synthesized using a FastQuant First Strand cDNA Synthesis kit (Tiangen, Beijing, China) according to the instruction of the manufacturer. The genomic DNA of shrimp tissues was extracted following the instruction of the Genomic DNA Extraction Kit (Toyobo, Japan).

2.2. Gene cloning and sequence analysis

The full-length cDNA of *Mjdap*-1 was obtained by PCR amplification with two specific primers *Mj*DAP1ExpF/ExpR (Table 1) designed according to transcriptome sequencing and cDNA library sequencing. The PCR criteria were as follows: 1 cycle at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 56 °C for 35 s, and 72 °C for 50 s; and 1 cycle at 72 °C for 5 min. The gene sequence was analyzed by online program ExPASy (http://www.au.expasy.org/) and BLAST (http://blast.ncbi.nlm.nih.gov/). Multiple protein alignments were performed using the GenDoc software. Phylogenetic analysis was conducted using MEGA 4.0 through the neighbor-joining method with a total of 1000 boot straps.

Table 1
Primer pairs for gene cloning, RNA interference and qRT-PCR.

Primers	Sequence (5′-3′)
*Mj*DAP1RTF	gcagggacagtgaaaagg
*Mj*DAP1RTR	tgtggcattacggggagt
*Mj*DAP1ExpF	catgcatatgtcatcctccgacgaggt
*Mj*DAP1ExpR	catgctcgagcttccttggttgttgaataat
*Mj*AIFRTF	atgttggctatgaggctatt
*Mj*AIFRTR	tgttgtctcgcaggtaaaac
*Mj*caspaseRTF	tccactcccaatggctgctat
*Mj*caspaseRTR	aagaactctgctttttcccgt
VP28RTF	agctccaaccctccttca
VP28RTR	ttactcggtctcagtgccaga
*Mj*ActinRTF	cagccttccttcctgggtatgg
*Mj*ActinRTR	gagggagcgagggcagtgatt
*Mj*DAP1RNAiF	gcgtaatacgactcactatagggcgtattagggaacttggaga
*Mj*DAP1RNAiR	gcgtaatacgactcactatagggtaactatgacagcccac
GFPRNAiF	gcgtaatacgactcactatagggtggtcccaattctcgtggaac
GFPRNAiR	gcgtaatacgactcactatagggcttgaagttgaccttgatgcc

2.3. Expression pattern analysis

Quantitative real-time PCR (qRT-PCR) and reverse transcription PCR (RT-PCR) were used to detect the mRNA transcription level changes of genes (*Mjdap*-1, *MjAIF*, and *VP28*). The total RNA of each sample was extracted with TRIzon, and cDNAs were synthesized using the Fast Quant First Strand cDNA Synthesis kit (Tiangen, Beijing, China) according to the manufacturer's instruction. The qRT-PCR was performed following the manufacturer's instructions of SYBR Green Supermix (Cwbio, Beijing, China) and using a real-time thermal cycler CFX96 RT System (Bio-Rad Laboratories, Inc., California, USA). The gene specific primers were named as RTF and RTR primers after the gene name, and sequences are shown in Table 1 β-actin was used as internal standardization and amplified with primers actin RTF/actin RTR (Table 1). qRT-PCR was performed using the following protocol: 94 °C for 10 min; 40 cycles of 94 °C for 15 s, 60 °C for 1 min, and 72 °C for 2 s; and a melt period with 0.5 °C gradient from 65 °C to 95 °C. The tested gene expression levels were calculated through $2^{-\Delta\Delta CT}$ methods. Data were analyzed by student's *t*-test using the software GraphPad Prism (GraphPad, San Diego, CA). Significant differences were considered at $P < 0.05$. RT-PCR was performed with the same primers used in qRT-PCR. The PCR procedure was as follows: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and 72 °C 10 min.

Western blot was used to detect the VP28 protein of WSSV in shrimp tissues. The gill and intestine tissues were homogenized in lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, and 3 mM EDTA). The supernatants were collected through centrifugation at 10,000 rpm and 4 °C for 10 min and submitted for concentration detection. Subsequently, the equivalent protein (approximately 10 μg) samples were separated by 12.5% SDS-PAGE and transferred into the nitrocellulose membrane. Polyclonal antibodies against *β*-actin (1:1000) or VP28 (1:500) or His-Tag (1:5,000, BioVision, China) were used as the primary antibodies, and horseradish peroxidase-conjugated goat antirabbit IgG (1:10,000) was used as the secondary antibody for Western blot analysis.

2.4. Recombinant protein expression and purification

The open reading frame (ORF) of *Mjdap*-1 was amplified with primer pairs *Mj*DAP1ExpF and *Mj*DAP1ExpR (Table 1) that were