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Identification and characterization of protein 14-3-3 in carcinogenic liver fluke *Opisthorchis viverrini*

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ABSTRACT

Protein 14-3-3s are abundant phospho-serine/threonine binding proteins, which are highly conserved among eukaryotes. Members of this protein family mediate metabolism and signal transduction networks through binding to hundreds of other protein partners. Protein 14-3-3s have been studied in other species of parasitic helminthes, but little is known about this protein in the carcinogenic liver fluke *Opisthorchis viverrini*. In this study, we identified and characterized protein 14-3-3s of *O. viverrini*. Seven protein 14-3-3 encoded sequences were retrieved from the *O. viverrini* genome database. Multiple alignment and phylogenetic analysis were performed. Two isoforms (protein 14-3-3 zeta and protein 14-3-3 epsilon) that have been previously found in the excretory-secretory (ES) products of *O. viverrini* were produced as recombinant protein in *E. coli* and the proteins were then used to immunize mice to obtain specific antibodies. Western blot analysis showed that both proteins were detected in all obtainable developmental stages of *O. viverrini* and the ES products. Immunolocalization revealed that both isoforms were expressed throughout tissues and organs except the gut epithelium. The highest expression was observed in testes especially in developing spermatocytes, suggesting their role in spermatogenesis. Prominent expression was also detected on tegumental surface of the parasite and on epical surface of bile duct epithelium indicates their additional role in host-parasite interaction. These findings indicate that protein 14-3-3s play important role in the life cycle of the parasite and might be involved in the pathogenesis of *O. viverrini* infection.

1. Introduction

The human liver fluke *Opisthorchis viverrini* infection remains a major public health concern in Thailand, Laos PDR and Cambodia, where the life style contributes largely to sustained infections. People acquire the liver fluke infection by consuming raw or improper cooked fresh water fishes (in the family Cyprinidae such as *Henicorhynchus siamensis*, *Hampala macrolepidota*, etc.) in endemic areas. Chronic infection with *O. viverrini* results in hepatobiliary diseases including a lethal bile duct cancer called cholangiocarcinoma (CCA) [1]. Carcinogenesis of cholangiocarcinoma caused by the liver fluke infection has been postulated to be a multi-factorial process, including mechanical damages, immunopathology and toxic effect of parasite ES products [2]. Among other factors, parasite molecules potentially play important role in this process.

More than 16,000 protein-encoding genes were predicted from the genome of *O. viverrini* [3]. Transcriptome analysis revealed a significant difference in gene expression profile between the juvenile and adult worms [4]. However, most of these genes have unknown function. Tegumental proteins and ES products that are expressed in adult worm during the chronic infection stage likely involve in parasite survival and host-parasite interaction. There were 437 proteins computationally defined as *O. viverrini* secretome [3]. Forty-three unique proteins of surface and secreted proteins have been previously identified [5]. Moreover, 108 proteins were identified from the extracellular vesicles (EVs) secreted by *O. viverrini* [6]. Protein 14-3-3s were among several proteins that were detected in both ES products and extracellular vesicles (EVs).

Protein 14-3-3s are abundant phospho-serine/threonine binding proteins, which are highly conserved among eukaryotes- from plants

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to mammals [7,8]. These proteins mediate metabolism and signal transduction networks through binding to hundreds of other protein partners. Protein 14-3-3 prevents apoptosis by binding to BH3-containing proteins, FOXO transcription factors and apoptotic kinases. Protein 14-3-3 is binding partner of Snail transcriptional repressor that functions as a master regulator of EMT events and directly regulates genes affecting cell adhesion, motility, and polarity [9,10]. The protein 14-3-3 binding affects the conformation and function of target proteins, which provide a multitude of regulatory functions, thus playing key roles in many cellular processes [11]. Therefore, deregulations of these proteins are involved in pathogenesis and progression of many diseases.

Here we report the identification and characterization of protein 14-3-3 in the liver fluke *O. viverrini*. Sequence and phylogenetic tree analysis were performed to categorize the identified proteins into specific group. Recombinant protein of two isoforms (epsilon and zeta), which have been previously detected in the ES product of *O. viverrini*, was produced and purified from *Escherichia coli*. Western blot analysis and immunohistochemistry were conducted to study the gene expression profile and protein localization. The final aim of this study is to understand the function of protein 14-3-3 in parasite biology and its role in host-parasite interaction, which in turn will help to elucidate the pathogenesis of *O. viverrini* infection.

2. Materials and methods

2.1. Parasite stages

O. viverrini eggs, metacercariae, adult worms and excretory-secretory products were prepared as previous described [12]. Briefly, *O. viverrini* metacercariae were obtained from naturally infected cyprinoid fish (*Henicorhynchus siamensis*, *Hampala macrolepidota*, etc.). The fish were grinded and digested by pepsin-HCl. After several washings with normal saline, the metacercariae were identified and collected under a dissecting microscope. Viable metacercariae were used to infect hamsters. After 2–3 months of infection, hamsters were euthanized and necropsied, and adult worms were recovered from the bile ducts. Adult *O. viverrini* were washed several times with phosphate buffered saline (PBS, pH 7.4) containing antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin G) and cultured in serum free RPMI-1640 medium contain 1% glucose, antibiotics and protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 2 μM E-64 and 10 μM leupeptin) at 37 °C with 5% CO_2. Culture medium was collected every 6 h and centrifuged at 1500*g* to separate the ES products and eggs into supernatant and pellet, respectively. ES products were then concentrated and diafiltrated into PBS using Amicon centrifugal filter devices with a cut-off size of 10 kDa (Millipore, USA). Egg, metacercaria and adult worm were resuspended in PBS and subjected to sonication. Soluble protein extracted was collected from supernatant after centrifuged at 14,000 rpm for 30 min. Protein concentrations of the extracts were determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA), after which parasite extracts and ES products were stored at −80 °C until use. The animals used in our investigations were undertaken with the approval of the Institutional Animal Use and Care committee of the Faculty of Science at Mahidol University.

2.2. Sequence retrieval, alignment, conserved motifs and phylogenetic analysis

Putative DNA sequences annotated as protein 14-3-3 were retrieved from genome databases of *O. viverrini* at NCBI nucleotide database using keyword "*Opisthorchis viverrini* protein 14-3-3." To verify the obtained nucleotide sequence, a BLASTn search against *O. viverrini* transcriptome in Sequence Read Archive database (accession no. SRX018025) was performed with the default settings. Retrieved short read sequences were assembled by an online sequence assembling program (CAP3) [13]. The assembled contig was then translated into putative amino acid sequences. Multiple alignments of the translated protein sequences were assembled by ClustalW using BioEdit program [14]. Phylogenetic relationship of protein 14-3-3s was inferred by maximum parsimony method using the MEGA 6 program [15].

2.3. Expression and purification of recombinant O. viverrini protein 14-3-3

The *O. viverrini* protein 14-3-3 ε1 and 14-3-3 ζ1 coding sequences were amplified from a full length cDNA library of adult worm constructed with the pTriplEx2 plasmid backbone (BD Bioscience) [16] using specific primers (14-3-3 ε1 – Forward, 5′GCGCGCCATATGACTGAACGTG AGACTCTCGTATAC3′; 14-3-3 ε1 – Reverse, 5′CGCGCGCTCGAGCGCC TTCTCCTCCGGCGAT3′; 14-3-3 ζ1 - Forward, 5′GCGCGCCATATGGCT CAGTCTTGGGTGAC3′; 14-3-3 ζ1 – Reverse, 5′CGCGCGCTCGAGGTTAT CGCCAGCGTCGGCCTCCTCGT3′). The polymerase chain reaction (PCR) thermal cycle was the following; initial denatured at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and final extension at 72 °C for 7 min. PCR products of 750 bp were then double digested and ligated into pET32a (+) via *Nde* I and *Xho* I sites. Recombinant plasmids were then transformed into BL21 (DE3) *E. coli* cells. Following after, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG). The expression of recombinant proteins was determined by SDS-PAGE (15% separating gel and 5% stacking gel) followed by staining with Coomassie Brilliant Blue. Recombinant proteins were then purified from *E. coli* lysates using HisPur Cobalt Resin affinity chromatography (Thermo Fisher Scientific Inc., USA). Purified proteins were dialysed into PBS. The yields of recombinant proteins were quantified using the Bradford assay (Bio-Rad, Hercules, CA, USA), and stored at −80 °C.

2.4. Preparation of anti-protein 14-3-3 antibody

BALB/c mice were immunized subcutaneously with purified recombinant protein of protein 14-3-3 ε1 or 14-3-3 ζ1 (25 μg per immunization, 5 mice per protein). The first immunization was carried out with recombinant protein formulated with Freund's complete adjuvant; the second and third immunizations were carried out with recombinant protein formulated with Freund's incomplete adjuvant [17]. Immunizations were conducted on days 1, 15 and 29. Antisera were obtained two weeks after the third immunization.

2.5. Western blot analysis

Ten micrograms of egg, metacercaria and adult worm extracts, ES products and 1 μg of recombinant protein were separated by SDS-PAGE (15% separating gel and 5% stacking gel) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 3% bovine serum albumin (BSA) in PBS tween-20 (0.05% tween-20 in PBS) (PBS-T) for 2 h. The mouse anti-protein 14-3-3 serum diluted 1:1000 in 1% BSA in PBS-T was then applied to the membrane and incubated for 2 h at room temperature. After washing 5 min for 3 times (3 × 5 min) with PBS-T the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody diluted 1:1000 in 1% BSA in PBS-T for 1 h. The membrane was then washed again 3 × 5 min with PBS-T followed by a final 10 min rinse with PBS. Reactive bands were developed using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., USA). Band intensity was measured by image J [18].

2.6. Immunohistochemistry

Two-three month *O. viverrini* infected hamster liver were fixed and cut into sections of 4 μm with a microtome as described elsewhere [19]. Briefly, sections were deparaffinized in xylene, hydrated in a series of ethanol and distilled water, respectively. The endogenous peroxidase was eliminated by treating sectioned tissues with absolute methanol