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Molecular cloning and characterization of major vault protein of *Echinococcus multilocularis*

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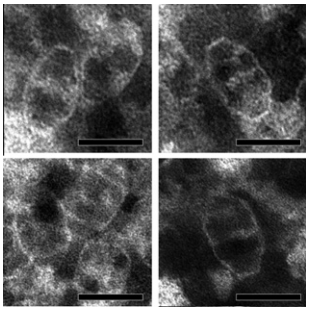
HIGHLIGHTS

- The major vault protein of *Echinococcus multilocularis* (EmMVP) was newly isolated.
- The deduced amino acid sequence of EmMVP had the motifs which were specific to MVPs.
- Immunoblot analysis revealed that *E. multilocularis* crude antigens included EmMVP.
- The expression of EmMVP in an insect cell line formed the vault-like particles.

GRAPHICAL ABSTRACT



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ABSTRACT

The cDNA clone coding a major vault protein (MVP)-like protein was derived from *Echinococcus multilocularis* cysts. MVP is a main component of vault particles, which are the largest cytoplasmic ribonucleoprotein particles in eukaryotic cells. We sequenced and characterized *E. multilocularis* MVP (EmMVP). The nucleotide sequence of the *emmvp* cDNA clone was 2607 bp in the full length open reading frame and its deduced amino acid sequence had several signature motifs which were specific to MVP families. Immunoblot analysis with mouse anti-EmMVP antiserum revealed that crude antigens of *E. multilocularis* included EmMVP protein. Furthermore, our results showed that the expression of EmMVP protein in an Sf9 insect cell line using a baculovirus vector directed the formation of particles that shared similar biochemical characteristics with other vault proteins and the distinct vault-like morphology when negatively stained and examined by electron microscopy.

1. Introduction

Alveolar echinococcosis (AE), which is caused by the larvae of *Echinococcus multilocularis* is a life-threatening zoonotic parasitosis in humans. *E. multilocularis* is widely distributed throughout the Northern hemisphere, and the endemic areas have been reported in central Europe, North America, China and Japan (Eckert and Deplazes, 2004). Human infection occurs by ingestion of eggs excreted in the faeces of infected foxes or dogs. The metacestode of *E. multilocularis* develops mainly in the liver and lungs and forms an infiltratively growing tumour. In most cases, no organ dysfunction is apparent until 10 or more years after initial infection. Once subjective symptoms appear, patient recovery from AE becomes difficult even after surgery, which is the only radical treatment for AE. Therefore, early diagnosis and treatment are important to reduce morbidity and mortality from AE (Eckert and Deplazes, 2004; Zhang and McManus, 2006).

Since 1990, many researchers have been attempting to find specific antigens for *E. multilocularis* and *E. granulosus* using molecular biological techniques to improve the quality and prevent false results in AE immunodetection (Gottstein, 1992; Zhang and McManus, 2006; Müller et al., 2007). Previously, we constructed an expression cDNA library of mRNA from the protoscolex and cysts

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of *E. multilocularis* isolated in Hokkaido, Japan (Sawada et al., 2002). During screening for new candidate antigens to be used in the immunodetection of AE, we detected a few clones encoding unknown proteins in *E. multilocularis* (Yamano et al., 2002, 2005; Katoh et al., 2008; Kouguchi et al., 2005; Sawada et al., 2002).

Recently, we cloned a new, unknown cDNA of protein homologous to the major vault protein (MVP) from an expression cDNA library constructed with mRNA from *E. multilocularis* cysts isolated in Hokkaido, Japan (Sawada et al., 2002). MVP is the main constituent of vault particles, which have a wide distribution and highly conserved morphology in eukaryotes (Suprenant, 2002; van Zon et al., 2003). Vault particles are identified as 13-MDa ribonucleoprotein particles with smooth, hollow barrel-like structures (Kong et al., 1999; Suprenant, 2002; van Zon et al., 2003). Vaults have been implicated in the regulation of several cellular processes including transport mechanisms, signal transmissions and innate immune responses (Yu et al., 2002; Kolli et al., 2004; Kolzov et al., 2006; Kowalski et al., 2007). In this study, we determined the complete nucleotide sequence of *E. multilocularis* MVP cDNA (*emmvp*) and compared the deduced amino acid sequence with those of other species. We also investigated the biological properties and antigenicity of recombinant *E. multilocularis* MVP protein (EmMVP) expressed in *Escherichia coli* and Sf9 insect cell line.

2. Materials and methods

2.1. Gene sequence analysis

Template DNA encoding putative MVP was cloned from an expression cDNA library constructed with mRNA from *E. multilocularis* cysts isolated in Hokkaido, Japan (Sawada et al., 2002). The plasmid was used as a template for DNA sequencing. Cycle sequencing was performed with a DNA sequence kit (Applied Biosystems, Foster City, CA), and the sequence was determined using a fluorescence autosequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems). The percentage homology between EmMVP protein and MVP protein of other species were calculated using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and GeneDB BLAST (http://www.genedb.org/Homepage; Logan-Klumpler et al., 2012). Deduced amino acid sequences were aligned using the online software CLUSTALW (http://www.genome.jp/tools/clustalw/). Secondary structure prediction and motif searches were performed using online software SMART (http://smart.embl-heidelberg.de/; Schultz et al., 1998) and Pfam (http://pfam.sanger.ac.uk/; Punta et al., 2012).

2.2. Expression and purification of recombinant EmMVPs from E. coli

EmMVP was divided into four regions for expression in *E. coli*. Each fragment of the *emmvp* cDNA was amplified by polymerase chain reaction (PCR) using the following primers: 5-TAG GGT ACC GAT GGC CTC TTC A-3 and 5-TGC CTG CAG TAC CAG CCA TAT CT-3 (RMVP-1; amino acids at positions 1–201); 5-ACG GGT ACC TGG GGA GAG AT-3 and 5-TTC TGC CAC AGC TGC AGG-3 (RMVP-2; amino acids at positions 196–420); 5-AGC GGT ACC GAC ATA TAT GCT-3 and 5-AAC TGT CGA CAC CGA CTG AAT ATC-3 (RMVP-3; amino acids at position 404–640); 5-CCT GGT ACC TAC AAG TGT GGA TA-3 and 5-TCT GTC GAC GTC CGT ATG GCC C-3 (RMVP-4; amino acids at positions 633–869). PCR products were gel-purified, digested with the appropriate restriction enzymes and cloned in-frame into pThioHis. Cloning and expression of recombinant DNA were performed in *E. coli* strain JM109. Recombinant clones were confirmed by sequencing.

Cultures were grown in Lennox broth at 37 °C with aeration to a cell density equivalent to an absorbance of 0.5–0.7 at 550 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM and induction was carried out at 32 °C for 5 h. The bacterial cells were harvested from 1 l of culture by centrifugation. The pellet was resuspended in 28 ml bacterial protein extraction reagent (B-PER; Pierce Biotechnology, Rockford, IL) with a protease inhibitor cocktail and placed on ice for 1 h. The insoluble materials were removed by centrifugation at 10,000*g* for 20 min. The clarified supernatant was mixed with 2 ml ProBond resin (Invitrogen), incubated at 4 °C for 2 h on a rotator and finally poured into a column. The resin was washed with 10 volumes of washing buffer [20 mM sodium phosphate buffer (pH 6.0) containing 500 mM NaCl] and the adsorbed proteins were eluted with 10 volumes of elution buffer [20 mM sodium phosphate buffer (pH 6.0) containing 500 mM NaCl and 500 mM imidazole]. The affinity fraction of RMVP-4 protein was dialyzed against 20 mM Tris–HCl (pH 7.8) at 4 °C overnight and further purified using ion exchange columns fitted on an AKTA Explorer (GE Healthcare UK Ltd, Buckinghamshire, England). The sample was applied to Mono Q HR 5/5 and eluted with a linear NaCl gradient ranging from 0 to 500 mM.

2.3. Preparation of antiserum

Female 7-week-old Balb/c mice (Japan SLC, Shizuoka, Japan) were immunized subcutaneously with 25 μg purified RMVP-4 protein and Freund's complete adjuvant for the first administration. Mice were kept at the animal facility of our institute and supplied water and pellet diets *ad libitum*. At 2 weeks post-immunization, a booster was inoculated intraperitoneally with Freund's incomplete adjuvant. After a week, the mice were sacrificed by exsanguination *via* cardiac puncture under anaesthesia, and blood was collected. Antiserum was obtained by centrifugation of the blood samples at 10,000*g* for 20 min. The animal experimental protocol was reviewed and approved by the ethics committee of Hokkaido Institute of Public Health.

2.4. SDS–PAGE and Western blotting

Recombinant EmMVP protein or crude antigens were dissolved in the same amount of sample buffer [62.5 mM Tris–HCl buffer (pH 6.8), 2% sodium dodecyl sulphate (SDS), 5% 2-mercaptethanol, 10% glycerol and 0.005% bromophenol blue]. After heating for 3 min at 100 °C, the antigens were separated by 10% SDS polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie Brilliant Blue R-250 (Wako Pure Chemical Industrial, Osaka, Japan). A molecular standard was used in each gel to calculate the molecular weight of each protein.

For Western blotting, proteins on the SDS–PAGE gels were transferred onto nitrocellulose membranes. The membranes were incubated with phosphate-buffered saline containing 0.05% Tween 20 (PBST; pH 7.4) and 5% milk diluent/blocking solution concentrate (KPL Inc., Gaithersburg, MD) and allowed to react for 1 h with the appropriately diluted anti-ThioHis mouse monoclonal antibody (1:5,000; Invitrogen), anti-MVP-4 mouse serum (1:500), AE positive control human serum (1:100; the component of ECHINOCOCCUS Western Blot IgG; Ldbio Diagnosis, Lyon, France) or normal mouse serum (1:100). The washed membranes were allowed to react with alkaline phosphatase (AP)-conjugated antibody to mouse or human IgG (1:2,500; Sigma, South San Francisco, CA) for 1 h, followed by washing. Proteins were visualized using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) system (PerkinElmer, Waltham, MA).

2.5. Immunoblot test

Purified RMVP-4 protein on the SDS–PAGE gels were transferred onto nitrocellulose membranes, which were cut into strips,