Echinococcus multilocularis : developmental stage-specific expression of Antigen B 8-kDa-subunits

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Abstract: Antigen B (AgB) initially found in hydatid cyst fluid of Echinococcus granulosus is a polymeric lipoprotein of 160 kDa, and is an aggregate of several different but homologous small proteins with approximately 8 kDa. Four genes encoding these 8-kDa subunits have been identified from E. granulosus metacestode. In this study we isolated five genes encoding 8-kDa subunits of AgB from Echinococcus multilocularis. Sequence comparison of isolated cDNA clones demonstrated that one of these five clones was completely identical to EmAgB8/1which had been cloned previously by our group, and three of them were 94.5, 90.8 and 91.9% homologous to E. granulosus antigen B 8-kDa subunit genes, EgAgB8/2, EgAgB8/3 and EgAgB8/4, respectively. The remaining clone showed 51 to 58% homology with the

nucleotide sequences of AgB genes. Gene-specific RT-PCR and Western blot analyses revealed that these genes were expressed in a developmentally regulated manner in E. multilocularis vesicles, protoscoleces and immature adult worms. Possible functions of different expression manners are also discussed.

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Dear Sir,

On behalf of all authors, I am submitting the manuscript entitled "*Echinococcus multilocularis*: Developmental stage-specific expression of Antigen B 8-kDa subunits" by Mamuti W. and others. The manuscript consists of 18 pages of text (including 1 page of legends for figures), two Tables (3 pages) and three figures (3 pages). This paper describes isolation, characterization and developmental stage-specific expression of a group of genes encoding *Echinococcus multilocularis* Antigen B 8-kDa subunits.

In this study, (1) five cDNA and their genomic DNA clones (four novel clones and one reported previous by our group) encoding Antigen B 8-kDa subunits from *E. multilocularis* were isolated and characterized, (2) transcription and translation patterns of these genes in vesicles, protoscoleces and immature adult worms were analyzed, and the possible functions of different expression manners were also discussed. This is the first report demonstrating of developmental stage-specific expression of echinococcal Antigen B 8-kDa subunits.

We would be very grateful if the manuscript is considered for publication in the Experimental Parasitology.

Yours sincerely

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Echinococcus multilocularis: Developmental stage-specific expression of Antigen B 8-kDa subunits[☆]

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Abstract

Antigen B (AgB) initially found in hydatid cyst fluid of *Echinococcus granulosus* is a polymeric lipoprotein of 160 kDa, and is an aggregate of several different but homologous small proteins with approximately 8 kDa. Four genes encoding these 8-kDa subunits have been identified from *E. granulosus* metacestode. In this study we isolated five genes encoding 8-kDa subunits of AgB from *Echinococcus multilocularis*. Sequence comparison of isolated cDNA clones demonstrated that one of these five clones was completely identical to *EmAgB8/1* which had been cloned previously by our group, and three of them were 94.5, 90.8 and 91.9% homologous to *E. granulosus* antigen B 8-kDa subunit genes, *EgAgB8/2*, *EgAgB8/3* and *EgAgB8/4*, respectively. The remaining clone showed 51 to 58% homology with the nucleotide sequences of AgB genes. Gene-specific RT-PCR and Western blot analyses revealed that these genes were expressed in a developmentally regulated manner in *E. multilocularis* vesicles, protoscoleces and immature adult worms. Possible functions of different expression manners are also discussed.

1. Introduction

Alveolar echinococcosis (AE) and cystic echinococcosis (CE) are caused by accidental ingestion of eggs of *Echinococcus multilocularis* and *Echinococcus granulosus*, respectively. The metacestode of *E. granulosus* usually develops in patients into fluid-filled unilocular cyst. In contrast, the metacestode of *E. multilocularis* exhibits a multivesicular, tumor-like infiltrating structure with poorly defined barrier between parasite and host tissue, and usually contains semisolid matrix rather than fluid (Siles-Lucas et al., 2001).

Antigen B (AgB), initially identified from *E. granulosus* hydatid cyst fluid, is a thermostable polymeric lipoprotein of 160 kDa (Oriol et al., 1971). On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions, the *E. granulosus* AgB (EgAgB) disassociates to generate several subunits with molecular sizes of 8, 16, 24 and 32 kDa, and the higher molecular weight subunits are composed of the 8-kDa subunit component (Lightowlers MW. et al., 1989; González et al., 1996). To date, four different but closely related cDNA clones encoding the 8-kDa subunit component of EgAgB have been identified and named as *EgAgB8/1* (Frosch et al., 1994), *EgAgB8/2* (Fernández et al., 1996), *EgAgB8/3* (Chemale et al., 2001) and *EgAgB8/4* (Arend et al., 2004). It is now evident that the EgAgB in hydatid cyst fluid is encoded by *EgAgB8/1* and *EgAgB8/2* genes (Fernández et al., 1996; González et al., 1996**).**

EgAgB may play an important role in parasite biology, since it presents in large amounts in hydatid cyst fluid (Musiani et al., 1978). The EgAgB has been characterized as a protease inhibitor with ability to inhibit recruitment of neutrophils and exploit activation of helper T

cell by eliciting a non-protective Th2 cell response (Shepherd et al., 1991; Reganò et al., 2001).

The EgAgB is also known as a major antigenic component of hydatid cyst fluid, and about 90% of CE and 40% of AE patients' sera exhibit positive reactions against this antigen (Maddison et al., 1989; Lightowlers. et al., 1989; Ito et al., 1999; Mamuti et al., 2002). This suggests that the AgB is also expressed in *E. multilocularis* metacestode. A partial cDNA encoding a homologue of EgAgB8/1 was isolated from *E. multilocularis* metacestode (Frosch et al., 1994). In our previous study, a full-length cDNA sequence of this gene (named as *EmAgB8/1*) was cloned and the antigenicity of recombinant EmAgB8/1 (rEmAgB8/1) was evaluated (Mamuti et al., 2004). The rEmAgB8/1 revealed almost same sensitivity and specificity with that those of rEgAgB8/1 in detecting IgG antibodies in serum samples from CE and AE patients. However, the existence of other related-genes encoding the 8-kDa subunits of *E. multilocularis* AgB (EmAgB) and expression patterns of AgB 8-kDa subunits in both of *E. multilocularis* and *E. granulosus* developmental stages still remain unclear.

In the present study, (1) four novel cDNA and genomic DNA clones of EmAgB 8-kDa subunits were isolated and characterized, and (2) the transcription and translation patterns of EmAgB 8-kDa subunit genes were analyzed in different developmental stages of the parasite.

2. Materials and methods

2.1. Parasite materials

Metacestodes were obtained from Chinese hamsters (*Cricetulus griseus*) experimentally infected with *E. multilocularis*, which was originally isolated from a naturally infected vole (*Clethrionomys rufocanus*) in Hokkaido, Japan. For preparation of immature adult worms, a dog was orally infected with about 10 g of fully developed metacestodes with official permission from animal ethical committee at Asahikawa Medical College. Immature adult

worms were collected from the dog's small intestine at the 17th day after infection as described previously (Zhang et al., 2003) and kept in liquid nitrogen in aliquots for further analysis. Vesicles and protoscoleces were obtained as follows: non-obese diabetic/Shi-*sever combined immunodeficiency* (NOD/Shi-*scid*) mice were infected intraperitoneally with metacestode homoginates (Nakaya et al., 2006). The parasite tissue recovered from NOD/Shi-*scid* mice was homogenized aseptically and suspended in a 10 times volume of sterile phosphate buffered saline (PBS pH 7.4). The suspension was filtered though a mesh with 300 μm of pore size to separate the vesicles and/or protoscoleces from large pieces of metacestode. Subsequently the flow-through was passed through a 150 μm pore-sized mesh to remove the most of fertile vesicles. Finally, the individual unfertile vesicles and protoscoleces were picked up separately with pipette under the microscope and kept in liquid nitrogen in aliquots for further analysis.

2.2. Total RNA isolation and cDNA synthesis

RNA samples from vesicles, protoscoleces and immature adult worms were prepared using a SV Total RNA Isolation Kit (Promega, Madison, WI, USA) according to manufacture's instructions. Approximately 20-25 mg of vesicles, protoscoleces or immature adult worms were used per isolation. First strands of cDNAs were synthesized using 10 μg of total RNA with 0.5 μM of Oligo $(dT)_{22}$ primer (Table 1), 70 units of Avian Myeloblastosis Virus Reverse Transcriptase XL (TaKaRa, Bio Inc., Shiga, Japan) and 0.25 mM of dNTPs (TaKaRa) in a 100 μl of total volume.

2.3. Isolation of cDNAs encoding EmAgB 8-kDa subunits

The single-strand cDNAs prepared from vesicles, protoscoleces and immature adult worms were used as templates for amplification of AgB-related genes in 3'-RACE PCR. A forward

primer common for *Echinococcus* AgB, EchiAgB/F (Table 1) derived from a highly conserved region of nucleotide sequences of known AgB (GenBank accession nos. **Z26336**, **U15001**, **AF252859**, **AY357108**, and **AB100403**) and an adaptor primer (Table 1) were used. PCR reaction was carried out with *Ex Taq* DNA polymerase (TaKaRa) in a final volume of 50 μl, containing 0.4 μM of each primer, 200 μM of dNTPs and 2.5 units of *Ex Taq* DNA polymerase. Amplification was performed with 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 2 min. The amplicons were excised from agarose gels using NucleoSpin ExTract kit (MACHEREY-NAGEL, Düren, Germany) according to instruction manual and cloned into pT7Blue T-Vector (Novagen, Madison, WI, USA). Recombinant plasmids from 29 to 56 colonies per transformation (total 130 colonies) were purified using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

2.4. Amplification of 5′ ends of the cDNA clones

In order to obtain full-length cDNAs, 5′-RACE was performed using a GeneRacer Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The 5′ ends of the cDNA clones were amplified by PCR using a GeneRacer 5′ Primer and gene-specific primers (Nos. 1 to 6 in Table 1). The PCR products were excised from agarose gels and sub-cloned into pT7Blue T-Vector (Novagen) for sequencing.

2.5. Amplification of genomic DNA fragments of EmAgB 8-kDa subunit genes

Genomic DNA was extracted from the *E. multilocularis* protoscoleces using a DNeasy Tissue kit (Qiagen) and used as template DNA. PCR amplification was carried out with gene-specific primers (Nos. 14 to 17 and Nos. 2 to 6 in Table 1). The PCR condition was set up as

in 3′-RACE PCR in section 2.3 of Material methods. The PCR products were excised from agarose gels and sub-cloned into pT7Blue T-Vector (Novagen) for sequencing.

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2.6. DNA sequencing and sequence data analysis

Nucleotide sequencing was performed on an ABI PRSIM 377 Sequencer (Applied Biosystems, Foster City, CA., USA) with DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Piscataway, NJ, USA). The alignment of sequences was carried out using CLUSTAL W program (http://www.ddbj.nig.ac.jp/search/clustalw-e.html).

2.7. RT-PCR analysis of stage-specific gene transcription

The single-strand cDNAs from vesicles, protoscoleces or immature adult worms were used as template DNA. PCR amplification was carried out in a 50-µl total volume of reaction mixture using intron-spanning primers and gene-specific primers (Nos. 7 to 11 and Nos. 2 to 6 in Table 1). The PCR condition was set up same as described in section 2.3 of Material methods. The PCR cycles were designed as 20 to 35 cycles with five cycle intervals for each sample. *E. multilocularis* cyclophilin gene (*EmCyclo*) was amplified as an external control (Colebrook et al., 2002) with primers (Nos. 12 and 13) shown in table 1. The PCR products were resolved on 1.5% agarose gel electrophoresis. Band densities of amplified fragments were compared among samples following visualizing on a UV transilluminator after staining with ethidium bromide.

2.8. Expression and purification of recombinant AgB proteins

The coding regions for anticipated mature forms of EmAgB 8-kDa subunits were amplified by PCR with primer sets of EmAgB1/F-EmAgB1/R, EmAgB2/F-EmAgB2/R, EmAgB3/F-

EmAgB3/R, EmAgB4/F-EmAgB4/R and EmAgB5/F-EmAgB5/R (Nos. 18 to 27, in Table 1). In order to create cohesive ends for directional cloning into the expression vector, restriction enzymes *Bam*HI and *Hin*dIII recognition sequences were added to the 5′ end of these primers (underlined in Table 1). PCR amplification was performed under the same conditions as mentioned in section 2.3 of Materials and methods. The PCR products were purified from agarose gel, double digested with *Bam*HI and *Hin*dIII, and cloned into pET32a(+) (Novagen). The recombinant plasmids were transfected into *Escherichia coli* (*E. coli*) BL21 (DE3) pLysS (Novagen). Protein expression was induced with 1 mM of isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C. The recombinant proteins fused with thioredoxin (Trx) were purified with His Trap column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the Trx was removed by cleaving with recombinant enterokinase (Novagen) according to manufacturers' instructions. The purified recombinant proteins were analyzed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with 0.2% Coomassie brilliant blue R-250. Protein concentration was estimated by using a BCA Protein assay kit (Pierce, Rockford, Ill. USA).

2.9. Production of monospecific polyclonal antibodies against EmAgB 8-kDa subunits

Anti-sera against five different rEmAgB were obtained by immunizing Japanese white rabbits subcutaneously with approximately 200 µg of each purified recombinant protein emulsified 1:1 in Freund's complete adjuvant. The rabbits received booster injections thrice with same antigens emulsified 1:1 in Freund's incomplete adjuvant with 14 days intervals and bled 12 days after the third boost. In order to obtain monospecific polyclonal antibodies, the cross-reactive IgGs in serum samples raised against each recombinant protein were absorbed as follows; 200 µl of each immunized rabbit sera were incubated with 800 µl of 1% *E. coli* lysate (in 0.02 M Tris-HCl containing 1% casein and 0.15 M NaCl, pH 7.6) at 37°C for 1 h

and centrifuged at 20,000g for 30 min at 4°C. Afterwards the supernatants were incubated with 5 µg of each homologous recombinant antigen for 1 h at 37°C followed by overnight at 4°C and then centrifuged at 20,000g for 30 min to leave behind the cross-reactive antibody-antigen complexes. Specificities of absorbed polyclonal antibodies were tested by Western blot using the recombinant antigens.

2.10. Extraction of parasite somatic antigens

To prepare parasite somatic proteins extracts, 1 ml of PBS was added to 200 µl each of frozen vesicles, protoscoleces or immature adult worms, and disrupted first by homogenizing on ice with a glass homogenizer and finally by repeated freeze-thaw lysis. Then the suspensions were subjected to centrifugation at 20,000g for 30 min at 4°C. The resulting supernatants were used as parasite somatic antigens.

2.11. Western blot analysis

To analyze EmAgB 8-kDa subunits in different developmental stages of the parasite, SDS-PAGE and Western blots were performed as described previously (Ito et al., 1993) with slight modification. Briefly, protein extracts from the vesicles, protoscoleces and immature adult worms were resolved by SDS-PAGE under reduced condition on 15% polyacrylamide gels and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Tokyo, Japan). The membranes were probed with absorbed monospecific polyclonal antibodies prepared against each recombinant EmAgB 8-kDa subunit, as described in section 2.9 of Material methods. A biotin-conjugated goat anti-rabbit IgG antibody (Sigma, Saint Louis, MO. USA) was used as secondary antibody at 1:1000 dilution. Then the membranes were incubated with avidin-biotin complex VECTASTAIN ABC kit (Vector

Laboratories, Burlingame, USA) according to manufacturer's instructions. As a color substrate, 0.05% 4-chloro-1-naphthol (Nacalai Tesque, Koyoto, Japan) was used.

3. Results

3.1. Isolation and characterization of EmAgB 8-kDa subunit genes

In order to obtain AgB-related genes, 3′-RACE and 5′-RACE were performed using single-strand cDNAs prepared from vesicles, protoscoleces and immature adult worms. Finally, five clones were isolated. One of 5 cDNA clones was completely identical to the previously isolated *EmAgB8/1* (GenBank accession no. **AB100403**) (Mamuti et al., 2004). One of remaining 4 clones showed 94.5% homology with the *E. granulosus AgB8/2* (*EgAgB8/2*; GenBank accession no. **U15001**). Two of them showed 90.8% and 91.9% homologies with *EgAgB8/3* (GenBank accession no. **AF362442**) and *EgAgB8/4* (GenBank accession no. **AY357110**), respectively. Therefore, these clones were named accordingly as *EmAgB8/1*, *EmAgB8/2*, *EmAgB8/3* and *EmAgB8/4*, respectively. However, the last one showed 51% to 58% homology with the echinococcal *AgB* genes and no similar genes were found other than the *AgB*. Thus this clone was named as *EmAgB8/5*. Genomic DNA sequence analysis demonstrated that all of these genes consisted of two exons and one intron (data not shown). Exon 1 encoded N-terminal signal peptide and exon 2 encoded a mature protein, respectively. Sequence comparison among five EmAgB 8-kDa subunits revealed 43-73% and 35-64% overall identity to each other at the nucleotide and amino acid sequences, respectively (Table 2). Between the EmAgB8/1 and EmAgB8/3 as well as between the EmAgB8/2 and EmAgB8/4 there appeared to be a greater identity to each other rather than to the other clones (67% and 73% at nucleotide sequences, 53% and 64% at amino acid sequences, Table 2). Sequence alignment revealed that amino acid residues conserved among the mature proteins

of more than four EmAgB 8-kDa subunits appeared at 24 positions (gray boxes in Fig. 1). Especially, a highly conserved region was observed between position 45 to 52 amino acid sequences corresponding to EmAgB8/1. The clones *EmAgB8/1* to *EmAgB8/4* were isolated from vesicles and protoscoleces, but the *EmAgB8/5* was not isolated in these developmental stages. In contrast, the *EmAgB8/5* was isolated from immature adult worms, while the *EmAgB8/1* and *EmAgB8/2* were not isolated from this stage. These results raised the possibility that expression patterns of AgB 8-kDa subunit genes were regulated stage specifically in *E. multilocularis* life-cycle.

3.2. Stage-specific transcription of EmAgB 8-kDa subunit genes

In order to confirm whether the five genes encoding EmAgB 8-kDa subunits are transcribed stage-specifically in parasite development, gene-specific RT-PCR was performed. As a result, different transcription patterns of these genes were observed in different developmental stages of the parasite. In vesicles: *EmAgB8/1* was detected just at 20 cycles of amplification, while the bands representing for *EmAgB8/2*, *EmAgB8/3* and *EmAgB8/4* were detected at 25 cycles; however, no product of EmAgB8/5 was detected in this stage even after 35 cycles (Fig. 2A). In protoscoleces: the products of *EmAgB8/1* and *EmAgB8/3* were detected at 25 cycles, but products corresponding to *EmAgB8/2* or *EmAgB8/4* were detected at 30 cycles, while a very faint band of *EmAgB8/5* was detectable after 35 cycles of amplification (Fig. 2B). In immature adult worms: a clear band of *EmAgB8/3* was detected at 20 cycles of amplification, while the product of *EmAgB8/1* and *EmAgB8/4* became detectable at 35 cycles; a clear band of *EmAgB8/5* was detectable at 30 cycles and no product of *EmAgB8/2* was detectable in this developmental stage even after 35 cycles (Fig. 2C).

3.3. Western blot analysis of EmAgB 8-kDa subunit proteins in different developmental stages

The expression patterns of native proteins of EmAgB 8-kDa subunits in vesicles, protoscoleces and immature adult worms were analyzed by Western blots using monospecific polyclonal antibodies against respective recombinant EmAgB 8-kDa subunit. As results, the monospecific polyclonal antibodies against recombinant EmAgB8/1, EmAgB8/2, EmAgB8/3 and EmAgB8/4 detected a clear band at about 8-kDa in protein extracts from vesicles and protoscoleces (lanes 1-4 in Fig. 3A and B). However, the corresponding bands to EmAgB8/1, EmAgB8/2 and EmAgB8/4 were not detected in immature adult worms (lanes 1, 2 and 4 in Fig. 3C). Two reaction bands were detected in protein extract from immature adult worms by polyclonal antibody against recombinant EmAgB8/3: one band at about 8 kDa and another relatively faint band at about 16 kDa (lane 3 in Fig 3C). EmAgB8/5 was not detected in all developmental stages examined (lane 5 in Fig. 3A-C).

4. Discussion

AgB has been recognized as a predominant secreted component of the larval stage of *E. granulosus* (Oriol et al., 1971; Musiani et al., 1978) and is a highly antigenic molecule in human echinococcal infections (Maddison et al., 1989; Lightowlers. et al., 1989; Ito et al., 1999; Mamuti et al., 2002). Previous studies were mainly focused on serological and molecular biological characterization of this antigen from *E. granulosus*. However, there is little information available about AgB from the closely related parasite, *E. multilocularis* and no data is available on expression patterns of AgB 8-kDa subunits in different developmental stages of both parasites. In this study, we report isolation, characterization and developmental differential expression of five genes encoding EmAgB 8-kDa subunits. One of these 5 clones was completely identical to *EmAgB8/1*; three of them were more than 90% homologous with corresponding genes of EgAgB 8-kDa subunits. The remaining clone did not show a high degree of identity with other echinococcal *AgB* genes. However, the amino acid sequence

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alignment showed that its predicted protein had conserved amino acid residues in many positions with all other EmAgB 8-kDa subunits (Fig. 1). In addition, the gene structure of this clone was also consisted of two exons and one intron inserted at the junction between the sequences encoding the signal peptide and the mature protein. Thus, this clone was considered to be belonging to the AgB gene family, and named as EmAgB8/5. Taken together these data, it can be concluded that the EmAgB is encoded by a multigene family which comprises five members at least.

Gene-specific RT-PCR analyses revealed that the transcription levels of some genes were down-regulated or up-regulated depending on the parasite developmental stages: The transcription levels of *EmAgB8/1*, *EgAgB8/2* and *EmAgB8/4* appeared to decrease gradually following the parasite development from vesicles to immature adult worms; especially the mRNA corresponding to *EmAgB8/2* became undetectable in immature adult worms. In contrast, the *EmAgB8/5* increased apparently from the protoscoleces to immature adult stage and was not detectable in vesicles. However, the *EmAgB8/3* was transcribed constitutively in all three stages examined. In Western blot analyses, EmAgB8/1, EmAgB8/2 and EmAgB8/4 were detectable in vesicles and protoscoleces but not in immature adult worms, while the EmAgB8/3 was detectable but EmAgB8/5 was not detectable in all three stages of the parasite. The failure of detection of EmAgB8/1, EmAgB8/4 and EmAgB8/5 at protein level in spite of the presence of their transcriptions could reflect the sensitivity of RT-PCR and Western blot methods to identify particular mRNA or protein, respectively. Alternatively, these genes were transcribed but regulated at the process of translation (Dallagiovanna et al. 2001). The matured adult stages of the parasite were not included in this study due to the regulation for obtaining the sample. Therefore it is not ruled out that the EmAgB8/5 might be expressed in mature adult stage of this parasite, since its transcript was showing a trend of increase through the parasite development. In order to elucidate this hypothesis, an extensive study will be

carried out to analyses the expression patterns of EmAgB 8-kDa subunits in matured adult worms. The continuous expression of EmAgB8/3 may be expected to have essential metabolic functions throughout all life-cycle stages of this parasite, while the EmAgB8/1, EmAgB8/2 and EmAgB8/4 may be essential factors for survival of metacestodes in intermediate hosts.

AgB has high homology and shares apparent structural similarities with a group of helix-rich hydrophobic ligand binding proteins (HLBPs) from other cestode adult worms (Shaghir et al. 2001). Some of these proteins are shown to be involved in lipid detoxification, transport and metabolism with their fatty acid binding properties (Barrett et al. 1997; Shaghir et al. 2001). Recently, it has been demonstrated that EgAgB has distinct hydrophobic binding properties when compared to HLBPs from other cestodes (Chemale et al. 2005). For instance, unlike other cestode HLBPs, native EgAgB and its two recombinant subunits (rEgAgB8/1 and rEgAgB8/2) bind 16-(9-Anthroyloxy) palmitate but not bind the other fluorescent probes that they examined. Their results indicated that EgAgB lost the ability to rapidly exchange fatty acids and this protein may be not directing fatty acid metabolic pathways but involved in different roles for parasite survival in host microenvironment (Chemale et al. 2005). Although the hydrophobic ligand binding properties and other possible biological functions of EmAgB and its 8-kDa subunits have not been evaluated so far, the differential expression of EmAgB 8-kDa subunits in different developmental stages of the parasite might be relevant to its distinct roles in parasite survival mechanism. We have herein disclosed that the EmAgB 8-kDa subunits are differentially expressed in parasite different developmental stages. This is the first report demonstrating of developmental stage-specific expression of echinococcal AgB 8-kDa subunits, and may shed some light on further investigation of biological functions of this protein in host-parasite interactions.

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Legends to figures

FIG. 1. Multiple alignments of amino acid sequences deduced from five cDNA sequences encoding the *E. multilocularis* AgB 8-kDa subunits. Amino acid residues conserved among the more than four mature proteins are highlighted by gray boxes. Dashes are introduced to maintain optimal alignment. The putative signal sequences are shown in boldface.

FIG. 2. RT-PCR analyses of stage-specific gene transcription of EmAgB 8-kDa subunits in *E. multilocularis*. cDNAs from vesicles (panel A), protoscoleces (panel B) and immature adult worms (panel C) were used. PCR was undertaken using gene specific primer sets for *EmAgB8/1*(*AgB1*), *EmAgB8/2* (*AgB2*), *EmAgB8/3*(*AgB3*), *EmAgB8/4* (*AgB4*) and *EmAgB8/5* (*AgB5*). *EmCyclo* (*Cyclo*) was amplified as an external control. The lanes 1 to 4 are corresponding to the PCR cycle numbers 20, 25, 30 and 35 cycles, respectively. DNA sizes in base pairs (bp) are shown on the left.

FIG. 3. Western blot analysis of EmAgB 8-kDa subunits at different developmental stages of *E. multilocularis*. The soluble somatic extracts of *E. multilocularis* vesicles (panel A), protoscoleces (panel B) and immature adult worms (panel C) were probed with monospecific polyclonal IgG antibodies against EmAgB8/1 (lane 1), EmAgB8/2 (lane 2), EmAgB8/3 (lane 3), EmAgB8/4 (lane 4) and EmAgB8/5 (lane 5)   or with normal rabbit sera (lane 6) as a negative control, respectively. The molecular sizes in kilodaltons (kDa) are shown on the left.

Tables

Table 1

Oligonucleotide primers used in this study

Application	Primer name	Sequence (5′-3′)
cDNA Synthesis	Oligo (dT)$_{22}$-Adaptor Primer	CTGATCTAGAGGTACCGGATCC(dT)$_{22}$
3′-RACE PCR	EchiAgB/F	CTTGCTCTCGTGGCTTTCGTG
	Adaptor Primer	CTGATCTAGAGGTACCGGATCC
No. 1 to No. 6 5′-RACE PCR	1. Gene Racer 5′ Primer	CGACTGGAGCACGAGGACACTGA
	2. EmAgB8/1gsp/R	TTCAGCAACCAACTCTCTGAG
	3. EmAgB8/2gsp/R	CTTCTTCAGCACCTCACGAAT
	4. EmAgB8/3gsp/R	TGACATACTCCTTCAGTGCCA
	5. EmAgB8/4gsp/R	CGTGAACCTTCAAATGCTGCTTT
	6. EmAgB8/5gsp/R	GAGCTTTTGTCCTGGCCAAAA
No. 2 to No. 13 RT-PCR analysis	7. EmAgB8/1csp/F	GTTACTCAGGCTGATGATGGT
	8. EmAgB8/2csp/F	GTCGTTCAAGCTAAAGATGAG
	9. EmAgB8/3csp/F	GTCGTTCAAGCGAAAGCTGAA
	10. EmAgB8/4csp/F	GTTGCTCGTGCTGATGATGAT
	11. EmAgB8/5csp/F	ATCGCTTTGGCGGAAGACGAC
	12. EmCyclo/F2	CGTCTTTGCTCTGTTTGATG

No. 2 to No. 6 and No. 14 to No. 17 genomic DNA sequence analyses	13. EmCyclo/R2	ATTTGCTCCCGTATATGCTC
	14. EmAgB8/1,3g/F	CTGGCTCTCGTCTCATTCGTG
	15. EmAgB8/2g/F	CTTGCTCTTGTAGCTTTCGTGGC
	16. EmAgB8/4g/F	CTTGTTCTCGTGGCTTTTGTGGC
	17. EmAgB8/5g/F	CTGGCTCTCGTAGCCTTCGT
No. 18 to No. 27 Recombinant protein expression	18. EmAgB8/1/F	CGC<u>GGATCC</u>GATGATGGTCTCACCTCGACG
	19. EmAgB8/1/R	CGC<u>AAGCTT</u>CTATTTACCTTCAGCAACCAA
	20. EmAgB8/2/F	CGC<u>GGATCC</u>AAAGATGAGCCAAAAGCACAC
	21. EmAgB8/2/R	CGC<u>AAGCTT</u>TTACTTTGAATCATCATCTTT′
	22. EmAgB8/3/F	CGC<u>GGATCC</u>GATGATGATGAAGTGACCCAG
	23. EmAgB8/3/R	CGC<u>AAGCTT</u>CTACTCATCCTCTTTAATCAA
	24. EmAgB8/4/F	CGC<u>GGATCC</u>AAAGCTGAACCCGAGAGATGC
	25. EmAgB8/4/R	CGC<u>AAGCTT</u>TTACTTTAAATCATCCTCATC
	26. EmAgB8/5/F	CGC<u>GGATCC</u>GAAGACGACATCGATTCGAAA
	27. EmAgB8/5/R	CGC<u>AAGCTT</u>CTACTCCCCTTCATCCATCAA

Table 2

Nucleotide (above diagonal) and amino acid (below diagonal) identities among EmAgB 8-kDa

subunits

clones	*EmAgB8/1* (%)	*EmAgB8/2* (%)	*EmAgB8/3* (%)	*EmAgB8/4* (%)	*EmAgB8/5* (%)
EmAgB8/1	—	43	67	44	54
EmAgB8/2	40	—	46	73	48
EmAgB8/3	53	38	—	46	58
EmAgB8/4	41	64	40	—	51
EmAgB8/5	40	40	50	35	—

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EmAgB8/1   M-RFCLLLALALVSFVVVTQADDG-LTSTSRSMMKMLGEMKYFFER   44
EmAgB8/2   M-RNYVLLSLALVAFVAVVQAKDEPKAHLGQGIKKRWGELRDFFKN   45
EmAgB8/3   MMKFCMLLALALVSFVVVARADDDEVTQTKKGVMKAISEIKHFFQS   46
EmAgB8/4   M-RTCILLSLVLVAFVAVVQAKAEPERCKCL-IMRKLSEIRDFFRS   44
EmAgB8/5   M-KLYIILALALVAFVAIALAEDDIDSKSKKGVMKSVAELKEFFAS   45


EmAgB8/1   DPLGQKLVDLLKELEEVFQMLRKKLRTALKSHLRELVAEGK----   85
EmAgB8/2   DPLGQRLVALGNDLTAICQKLQLKIREVLKKYVKNLVEEKDDDSK   90
EmAgB8/3   DPLGKKLVEVMKEVGSVCQMVRKKARMALKEYVRKLIKEDE----   87
EmAgB8/4   DPLGQRLVALGRDLTAICQKLHLKVHEVLKKYVKDLLEEDEDDLK   89
EmAgB8/5   DPMGQKLAAICKELKDFFLLARTKARSALRDYVKRLMDEGE----   86
```

Fig. 1. Mamuti et al.
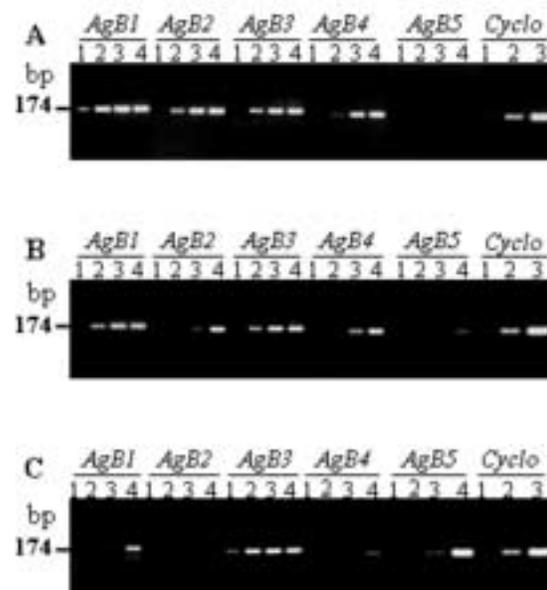
Fig. 2. Mamuti et al.
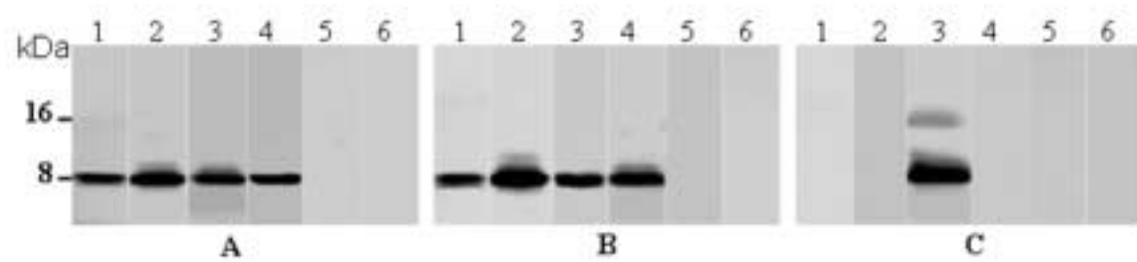
Fig. 3, Mamuti et al.

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