Sequencing, bioinformatic characterization and expression pattern of a putative amino acid transporter from the parasitic cestode *Echinococcus granulosus* ☆

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Abstract

We have sequenced and partially characterized an *Echinococcus granulosus* cDNA, termed *egat1*, from a protoscolex signal sequence trap (SST) cDNA library. The isolated 1627 bp long cDNA contains an ORF of 489 amino acids and shows an amino acid identity of 30% with neutral and excitatory amino acid transporters members of the Dicarboxylate/Amino Acid Na^+ and/or H^+ Cation Symporter family (DAACS) (TC 2.A.23). Additional bioinformatics analysis of EgAT1, confirmed the results obtained by similarity searches and showed the presence of 9 to 10 transmembrane domains, consensus sequences for N-glycosylation between the third and fourth transmembrane domain, a highly similar hydropathy profile with ASCT1 (a known member of DAACS family), high score with SDF (Sodium Dicarboxilate Family) and similar motifs with EDTRANSPORT, a fingerprint of excitatory amino acid transporters. The localization of the putative amino acid transporter was analyzed by *in situ* hybridization and immunofluorescence in protoscoleces and associated germinal layer. The *in situ* hybridization labelling indicates the distribution of *egat1* mRNA throughout the tegument. EgAT1 protein, which showed in Western blots a molecular mass of ~60 kD, is localized in the subtegumental region of the metacestode, particularly around suckers and rostellum of protoscoleces and layers from brood capsules. The sequence and expression analyses of EgAT1 pave the way for functional analysis of amino acids transporters of *E. granulosus* and its evaluation as new drug targets against cystic echinococcosis.

Keywords: Cystic echinococcosis; Helminth parasites; DAACS; EgAT1; ASCT

Abbreviations: ASCT, Alanine/serine/cysteine/threonine transporter; BCIP, 5-bromo-1-chloro-3-indolyl phosphate; BSA, bovine serum albumin; cDNA, DNA complementary to RNA; DAACS, Dicarboxylate/Amino Acid Na^+ and/or H^+ Cation Symporter family; ELISA, enzyme-linked immunosorbent assay; NBT, nitro blue tetrazolium; ORF, open reading frame; PBS, phosphate-buffered saline; PBST, PBS containing 0.1%; Tween-20; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SDF, sodium dicarboxilate family; SST, signal sequence trap; SSC, sodium saline citrate; Trx, *Escherichia coli* thioredoxin; TrxEgAT1, *Escherichia coli* thioredoxin fused to c-terminal portion of EgAT1 protein; UTR, untranslated region.

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1. Introduction

Echinococcus granulosus is a small parasitic cestode whose larval stage causes cystic echinococcosis, a disease that affects humans and ungulate animals in many countries around the world. *E. granulosus* has a complex life cycle involving a definitive and an intermediate host. The larval stage, or hydatid cyst, which develops in the intermediate host, is lined with an inner germinal layer that produces small immature worms named protoscoleces. Protoscoleces are capable of developing into adult worms in the gut of the definitive host.

Since cestodes lack a digestive tract, all nutrients must enter through the tegument of the worm or the larvae. As it was reviewed by Pappas and Read (1975), cestodes absorb amino acids, in part, by mediated processes. As a consequence of a parasitic way of life, the amino acid metabolism is very limited and the adult worm must fulfill most of its amino acid requirements by active tegumental absorption from the intestinal amino acid pool (Wack et al., 1983). It was shown that *E. granulosus* protoscoleces are able to accumulate L-amino acids and it was suggested the existence of various systems for the absorption of amino acids (Jeffs and Arme, 1987; Allen and Arme, 1991). Also, it was found that amino acids in the hydatid cyst fluid were severalfold more concentrated than in host plasma (Jeffs and Arme, 1988; Hurd, 1989; Çelik et al., 2001) suggesting that the cyst wall is not freely permeable to metabolites.

In a recent work (Rosenzvit et al., 2006), the signal sequence trap technique (Chen and Leder, 1999) was used to identify secreted and membrane bound proteins that could be involved in molecular mechanisms of host–parasite interactions. Several of the clones obtained shared between 30 and 40% amino acid identity with amino acid transporters belonging to the DAACS family (TC 2.A.23). According to the classification developed by Saier (2000), the members of the DAACS family catalyze Na^+ and/or H^+ transport together with a Krebs cycle dicarboxylate or an amino acid. Several homologous types of Na^+-dependent high affinity glutamate transporters and neutral amino acid carriers belonging to the DAACS family were described in Bacteria, Archaea and Eukarya. In mammals, members of the DAACS family have important and diverse roles as termination of the glutamatergic transmission in glutamatergic synapses (Gegelashvili and Schousboe, 1997) and transport of neutral amino acids in the placenta (Jansson, 2001), eyelid, retina, lung and colon (Hatanaka et al., 2004). Recently, the crystal structure of a glutamate transporter homologue and member of the DAACS family, from the archeal organism *Pyrococcus horikoshii* was solved (Yernool et al., 2004). We report here the sequencing, bioinformatic analysis and histochemical localization of a new member of the DAACS family of amino acid transporters transcribed in all life cycle stages of the parasite (Rosenzvit et al., 2006). This represents the first report and partial characterization of a putative amino acid transporter member from the DAACS family in *Echinococcus* sp. The characterization of surface-expressed proteins regulating membrane amino acids transport may lead to new therapeutic targets or improved means of drug-delivery against cystic echinococcosis.

2. Materials and methods

2.1. Parasite materials and fixation technique

E. granulosus protoscoleces were aspirated from hydatid cysts collected from livers or lungs from sheep, cattle and pigs at abattoirs from Argentina, Chile and Uruguay. Protoscoleces and brood capsules were thoroughly washed with PBS. The parasite material was either fixed for histological studies in freshly prepared 4% (w/v) paraformaldehyde in PBS for 1 h or stored in liquid nitrogen for RNA or protein extraction. The fixed parasite material was washed several times in PBS, dehydrated through ethanol series in PBS, and stored in absolute ethanol at 4 °C until use.

2.2. RNA extraction and cDNA synthesis

Total RNA from *E. granulosus* was extracted from protoscoleces that were crushed under liquid nitrogen and processed using Trizol reagent (Invitrogen). The RNA obtained was treated with RQ1 RNAse-Free DNAse (Promega), ethanol precipitated and reverse transcribed using Superscript™ II reverse transcriptase (RT) (Invitrogen) and the GeneRacer™ RNA Oligo (to synthesize 3' cDNA ends) from the GeneRacer™ kit (Invitrogen) or random primers (to synthesize 5' cDNA ends).

2.3. Rapid amplification of cDNA ends (RACE) and sequence analysis

Amplification of cDNA ends was performed for EgP3G2, one of the previously isolated cDNA fragments (Rosenzvit et al., 2006) that showed amino acid identity with members of the DAACS family. The GeneRacer kit (Invitrogen) was used with minor modifications. Briefly, 3' terminal sequences were obtained using the oligo-dT GeneRacer primer provided with the kit to synthesize 3' Race cDNA followed by PCR amplification with Platinum Taq High Fidelity (Invitrogen) using the GeneRacer™ 3' primer and a gene-specific forward primer (5'-GAATTCTGTTATCATCGGTTTCGGTG-3'). For 5' terminal sequences, cDNA was synthesized using random primers. The PCR was preformed as before but with GeneRacer™ 5' primer as the forward primer and a gene-specific reverse primer (5'-ATGGTCACCACCATCATCGGTGTTA-3'). Amplification products were visualized by agarose gel electrophoresis and ethidium bromide staining and the bands of interest were extracted from the gel using the PureLink TM Quick Gel Extraction Kit (Invitrogen), cloned into pGEM®-T Easy Vector (Promega) and sequenced using an Applied Biosystems Big Dye terminator kit (Applied Biosystems) on an ABI 377 automated DNA sequencer. The full-length cDNA obtained was named *egat1* for *E. granulosus* amino acid transporter. Nucleotide sequence comparisons were performed using the Washington University Basic Local Alignment Search Tool (WU-Blast2) software on the EMBL nucleic acid database collection available under http://www.ebi.ac.uk/blast2/nucleotide.html. The signalP V3.0 server (http://www.cbs.dtu.dk/services/SignalP3.0) was used to screen the nucleotide sequences for predicted signal

sequences. The PSORT and PSORTII programs were used to screen for sub-cellular targeting signals (Expasy, www.expasy.org). The search of conserved domains was performed with the Conserved Domain Search service available under http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi. The presence of internal transmembrane domains was analyzed using the TMpred server (http://www.ch.embnet.org/software/TMPRED_from.html). The search of the protein with the most similar hydropathy plot and the corresponding alignment was carried out with the Hydrophilicity/Hydrophobicity Search and Comparison Server available under http://bioinformatics.weizmann.ac.il/hydroph/ using a window size of 19. The search of conserved motifs within proteins was performed with the fingerPRINTScan package, available under http://www.bioinf.man.ac.uk/fingerPRINTScan/.

2.4. In situ hybridization studies

A DNA sequence spanning nt 433 to 666 (233 bp coding for amino acids 132–209) of *egat1* cDNA was cloned in the pGEM-T vector (Promega) and sense and antisense digoxygenin-labelled riboprobes were generated. Histological processing and probe hybridization were performed as previously described (Freire et al., 2004). Digital images of parasite sections were captured under identical conditions by an Olympus BX40 microscope equipped with a Samsung SDC-310 digital camera. Contrast and brightness were adjusted using the Image Pro Plus Software (Media Cybernetics).

2.5. Preparation of monospecific polyclonal antibodies

The *egat1* cDNA region encoding the hydrophilic C-terminal fragment of EgAT1, was PCR-amplified from the recombinant pGEM®-T Easy Vector (Promega) with Platinum Taq High Fidelity (Invitrogen), using the primers forward (5'-GGTACCTACATCATCAATCCAGGAAAG-3') and reverse (5'-TTCGAATCCCGACACAGCCCTAAGCTT-3'). The PCR product was cloned into pBAD/Thio-TOPO expression vector (Invitrogen) for expression as a fused protein to *E. coli* thioredoxin (Trx). *E. coli* TOP10 cells were transformed with the recombinant plasmids obtained and the orientation of the insert was checked by colony PCR. The recombinant proteins were expressed by induction with 0.02% arabinose, purified with Ni^{2+}-nitrilotriacetic acid columns under denaturing conditions according to manufacturer instructions (Qiagen) and used to prepare rabbit antiserum. Additionally, one plasmid bearing only the Trx gene was used to transform *E. coli*. After induction, the Trx protein was purified and injected into a rabbit to obtain a control serum. Immunizations of rabbits were performed by an intramuscular priming with 200 μg protein in complete Freund's adjuvant (Sigma, USA), followed by five boosters of 200 μg protein in incomplete Freund's adjuvant (Sigma, USA) every 3 weeks. The rise in serum title was checked one week after each injection by ELISA using the corresponding protein as antigen. Three weeks after the last booster, the serum was collected, aliquoted and stored at −20 °C until use.

2.6. Affinity purification of antibodies

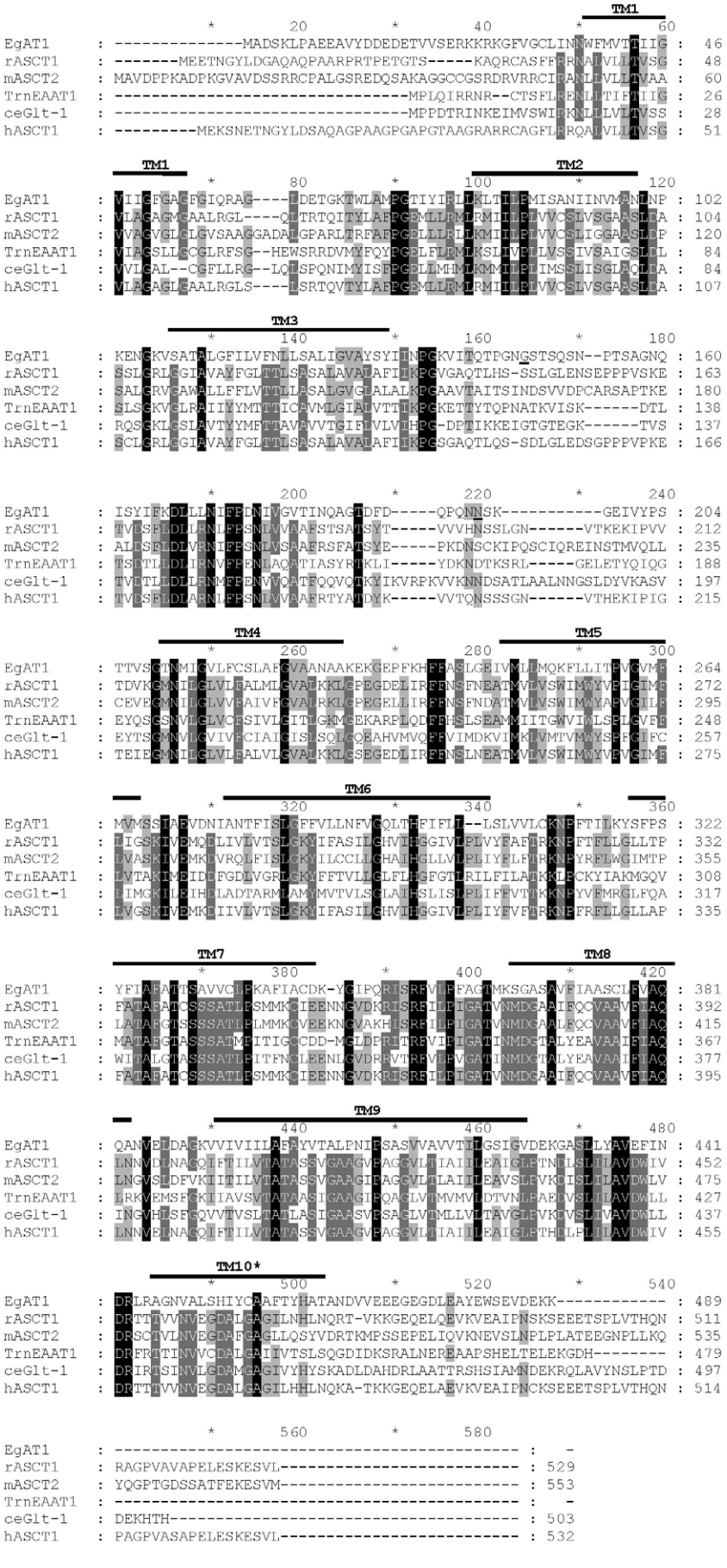
Antisera were purified on affinity columns, where Trx or TrxEgAT1 was coupled to Affi-Gel 15® (Bio-Rad, Richmond, CA). Trx and TrxEgAT1 antisera were purified in affinity columns containing the Trx and TrxEgAT1 fusion proteins respectively. Four milliliters of serum were applied per milliliter of resin and the eluate was repassed three more times through the column. After washing, the antibodies were finally eluted with 2 ml of glicyne 100 mM, NaCl 150 mM, pH 2.4. The eluate was collected in fractions of 0.5 ml, immediately neutralized and stored at −20 °C until use.

2.7. Immunoblot analysis

A protoscoleces lysate was prepared by ultrasonication in lysis buffer, containing Tris–HCl 50 mM, pH 8; NaCl 150 mM; Triton X-100 1%; EDTA 0.037% (w/v); EGTA 0.038% (w/v); PMSF 200 μM; Aprotinin 2 μg/ml. The destruction of the protoscolex structure was checked by visual inspection under the light microscope. The lysate was centrifuged briefly and dosed for protein concentration by Bradford assay (Bio-Rad). Three hundred micrograms of parasite protein per well were separated on 10% polyacrylamide gels and electrotransferred to nitrocellulose membranes (BioRad). The nitrocellulose strips were incubated with unpurified rabbit polyclonal antibodies diluted at 1:100 or 2 μg/100 μl of purified rabbit polyclonal antibodies. A peroxidase-conjugated anti-rabbit IgG (Sigma) diluted at 1:1000 was used as secondary antibody. The bands were visualized using diaminobenzidine reagent (ImmunoPure DAB, PIERCE).

2.8. Immunohistochemistry

The fixed parasite material stored in ethanol was embedded in paraplast plus (Kendall) at 62 °C. The blocks were cut with a microtome (Leica, Mod. RM2125RT) and slices of 3 μm were obtained and mounted. Tissue sections were rehydrated and treated with 25 mM ammonium chloride to eliminate autofluorescence. The sections were blocked overnight at 4 °C with the CAS-BLOCK™ solution (Zymed®), then incubated in a humid chamber for 2 h with the primary antibody diluted in blocking solution [BSA 1% (w/v), Saponin 0.1% (v/v) and calf serum 3% (v/v)]. Serum raised against EgAT1 (see Section 2.5) (1:100 dilution of anti-TrxEgAT1 or anti-Trx or preimmune serum in blocking solution) or purified polyclonal antibodies (see Section 2.6) (2 μg/100 μl of anti-TrxEgAT1 or anti-Trx) respectively, were used as primary antibodies. Sections were washed three times with PBS 1X and then incubated in humid chamber for 1 h at 37 °C with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Invitrogen) diluted 1/400 in blocking solution. After repeated washes, the slices were mounted with Hoescht (1 μg /ml in glycerol 70%) and immediately observed under the fluorescence microscope (Olympus BX41). The images were digitally captured (Qimaging MicroPublicher 3.3 RTV) and processed with the Qcapture Pro program (5.1.1.14 version). Controls consisted of: a) omission of primary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mASCT2
TrnEAAT1
ceGlt-1
hASCT1

antibodies, b) omission of secondary antibodies, c) preimmune serum and d) hyperimmune serum raised against thioredoxin.

3. Results

3.1. Nucleotide sequence analysis

The entire *egat1* cDNA sequence comprised 1627 bp and showed an initiation codon within a Kozak context (Kozak, 1987), a polyA tail, and a hypothetical polyadenylation signal sequence (data not shown). The open reading frame is flanked by 39 nucleotides of 5'-untranslated region (UTR) and 106 nucleotides of 3'-UTR. In nucleotide database searches, the nucleotide coding region showed 85% of nucleotide identity to clone UNAM-cd2_larva in *Taenia solium* (EL748305; nucleotides 463–1067), 82% to clone HC7-A4 encoding for the solute carrier family 1-like protein in *Taenia asiatica* (EF201854; nucleotides 20–413), 54% to ASCT2 or neutral amino acid transporter B0 [ATB(0)] in rabbit (U75284; nucleotides 245–1025) and 54% to glutamate transporter (REAAC1) in rat (X94255; nucleotides 655–1376). BLASTN search in the *Echinococcus* full-length cDNA database (http://fullmal.hgc.jp/em/index.html), detected the *E. multilocularis* clone XvEMa19150 with 95% identity (nucleotides 1–848) *to egat1*. Both sequences are almost identical, even in their 5'-UTR region.

3.2. Deduced amino acid sequence analysis

The *egat1* cDNA contained one single open reading frame which encodes a 489 amino acids polypeptide (Fig. 1). The presence of an in frame TGA stop codon, 28 bp upstream of the first ATG codon of this ORF confirmed that the full-length ORF of *egat1* has been determined. The SignalP program did not predict a signal sequence within the first 70 amino acids of EgAT1. However, it was predicted as a plasma membrane protein by the PSORT and PSORT II programs. A SDF (Sodium Dicarboxilate Family) domain (which also could be termed DAACS) was predicted by the Conserved Domain Search service (CD-Search, NCBI) with an *E* value of $3e^{-44}$. The alignment of the deduced amino acid sequence EgAT1 with neutral amino acid transporters, ASCT1 and ASCT2; and excitatory amino acid transporters, TrnEAAT1 and Glt-1 is shown in Fig. 1. EgAT1 shared 30% of amino acid identity with ASCT1 from rat, 31% to ASCT2 from mouse, 28% to TrnEAAT1 from caterpillar, 25% to Glt-1 from nematode and 30% to ASCT1 from human.

In coincidence with members of the DAACS family, 9 to 10 transmembrane segments and a large hydrophilic loop between the third and fourth transmembrane domains were predicted for EgAT1 (Fig. 1). We also searched for putative N-glycosylation sites and found that the putative second extracellular loop of EgAT1 contains two consensus sequences for N-linked glycosylation (Fig. 1), in concordance with some mammalian members of the glutamate transporter family that showed N-linked glycosylated residues in the second extracellular loop (Trotti et al., 2001). We used an experimental web based server to search a database for hydropathy profiles matching EgAT1 sequence (Bioinformatics Unit, Weizmann Institute of Science). The search in SwissProt database showed that the human amino acid transporter (ASCT1) is highly similar in its hydropathy profile to EgAT1. All the peaks and valleys were almost coincident and only minor differences were seen (data not shown). Very similar results were obtained when other members of the DAACS family were analyzed. High degree of similarity between EgAT1 and ASCTs or EAATs hydrophobic profiles probably reflects that EgAT1 and DAACS protein family members present very similar folding. Another tool used to determine whether EgAT1 could be a member of the DAACS family was the FingerPRINTScan program. EgAT1 matched with EDTRNSPORT (PR00173, according to PRINTS database) which is a 9 element fingerprint that provides a signature for the glutamate–aspartate transporters. All members sharing EDTRNSPORT fingerprint also belong to DAACS family. EgAT1 matched with EDTRNSPORT with an *E* value of $2.3e^{-38}$. Eight motifs with significant match and in a strict order with EDTRNSPORT were found in EgAT1 (data not shown). At the end of the eighth membrane-spanning domain (Fig. 1), we found the sequence AASCLFVAQ, which is similar (but not identical) to the putative substrate binding domain AA(I,V) FIAQ. This latter motif is conserved in all known members of the glutamate transporter gene family (Slotboom et al., 1999) and in agreement with our results it was proposed to be located at the end of the eighth transmembrane segment (Kanai and Hediger, 1992).

3.3. In situ hybridization studies

The antisense digoxygenin-labelled riboprobe generated from the coding sequence of the hydrophilic loop of the hypothetical transporter, *egat1*, showed a particular pattern of labeling being the tegument the structure more intensively stained (Fig. 2A, B and D). The cell territory immediately below was apparently only faintly stained (Fig. 2D).

3.4. Analysis by Western blot

A single 60 kDa band was identified in *E. granulosus* extract (Fig. 3, lanes 3 and 4) using either the unpurified or the purified serum against TrxEgAT1 fusion protein. The expected size for the deduced protein was 52.6 kDa. The difference may well be attributable to posttranslational modifications such as

Fig. 1. Alignment of the deduced amino acid sequence of EgAT1 with several homologues of the DAACS family. Indicated are the amino acid sequences from the rat *Rattus novergicus* (rASCT1; Yamamoto et al., 2004), the mouse *Mus musculus* (mASCT2; Utsunomiya-Tate et al., 1996), the caterpillar *Trichoplusia ni* (trnEAAT1; Donly et al., 1997), the nematode *Caenorhabditis elegans* (ceGlt-1; Kawano et al., 1997) and human (hASCT1; Shafqat et al., 1993) DACCS family transporters. Amino acid residues which are identical in all the aligned sequences are printed in white on black background, residues identical in at least five of the aligned sequences are printed in white on gray background, and finally, residues identical in four of the aligned sequences are printed black on gray background. Transmembrane domains (TM1–TM10) relative to EgAT1 are shown. Consensus sites for N-glycosylation are underlined. *The sequence indicated as transmembrane segment 10 is not recognized as a transmembrane segment by the alternative model (not preferred) of the TMpred program.

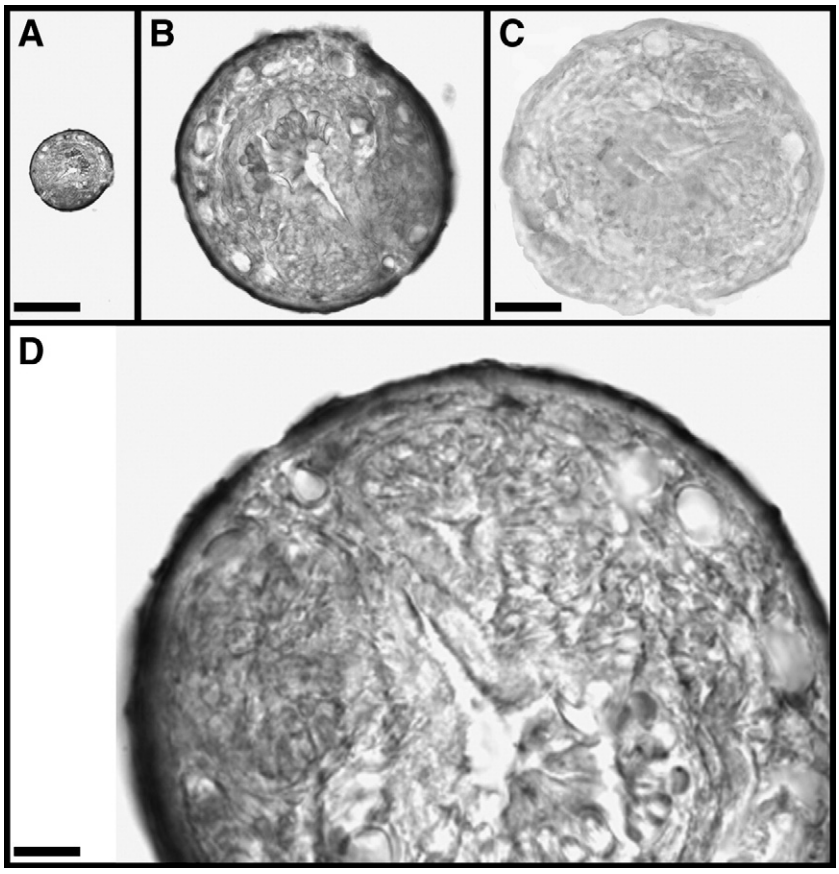

Fig. 2. Expression of *egat1* in *E. granulosus* larval stage: *in situ* hybridization analysis. Protoscolex sections were hybridized with digoxygenin-labelled antisense RNA probe (A, B and D); the reaction was developed using an alkaline phosphatase-conjugated anti-digoxygenin antibody. In all cases, the strongest signal was confined to the tegument region. A protoscolex section assayed in parallel with a sense probe (C) showed no reactivity. Scale bars: (A), 100 μm; (B, C), 30 μm; (D), 15 μm.

glycosylation which increases the molecular weight in around 5–9 kDa (Pow et al., 2003). This band was not seen when the strips were incubated with the unpurified or purified serum against Trx or preimmune serum (Fig. 3, lanes 1, 2 and 5).

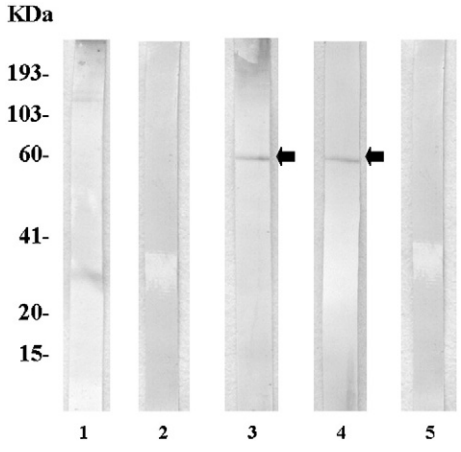

Fig. 3. Western blot detection of EgAT1. Proteins from protoscoleces lysate were subjected to Western blot analysis with: 1, unpurified sera against recombinant thioredoxin; 2, purified antibodies against recombinant thioredoxin; 3, unpurified sera against recombinant thioredoxin-EgAT1 fusion protein; 4, purified antibodies against recombinant thioredoxin-EgAT1 fusion protein; and 5, preimmune sera.

3.5. Analysis by immunohistochemistry

Immunolocalization studies of EgAT1 were performed on protoscoleces and associated germinal layers. A clear staining was observed all along the subtegumental region of the scolex and body of the invaginated (Fig. 4D, M and N) and the evaginated (Fig. 4G) protoscolex. The fluorescence was particularly intense around the suckers (Fig. 4D and G), and rostellum (Fig. 4D). This pattern of fluorescence was not observed when parasite sections were incubated in the same conditions with antibodies against thioredoxin alone (Fig. 4A) or preimmune serum (data not shown). Interestingly, a very intense fluorescence was observed associated with brood capsules layers (Fig. 4J, M and N).

4. Discussion

In this study, we have isolated and partially characterized an *E. granulosus* cDNA, termed *egat1*, encoding a putative amino acid transporter. To our knowledge, this is the first amino acid transporter characterized at the molecular level in cestodes. Amino acid transporters from the DAACS family play important roles in a variety of cellular processes in all living organisms. Although we still lack functional information on this molecule, it is likely that *egat1* is involved in the transport of

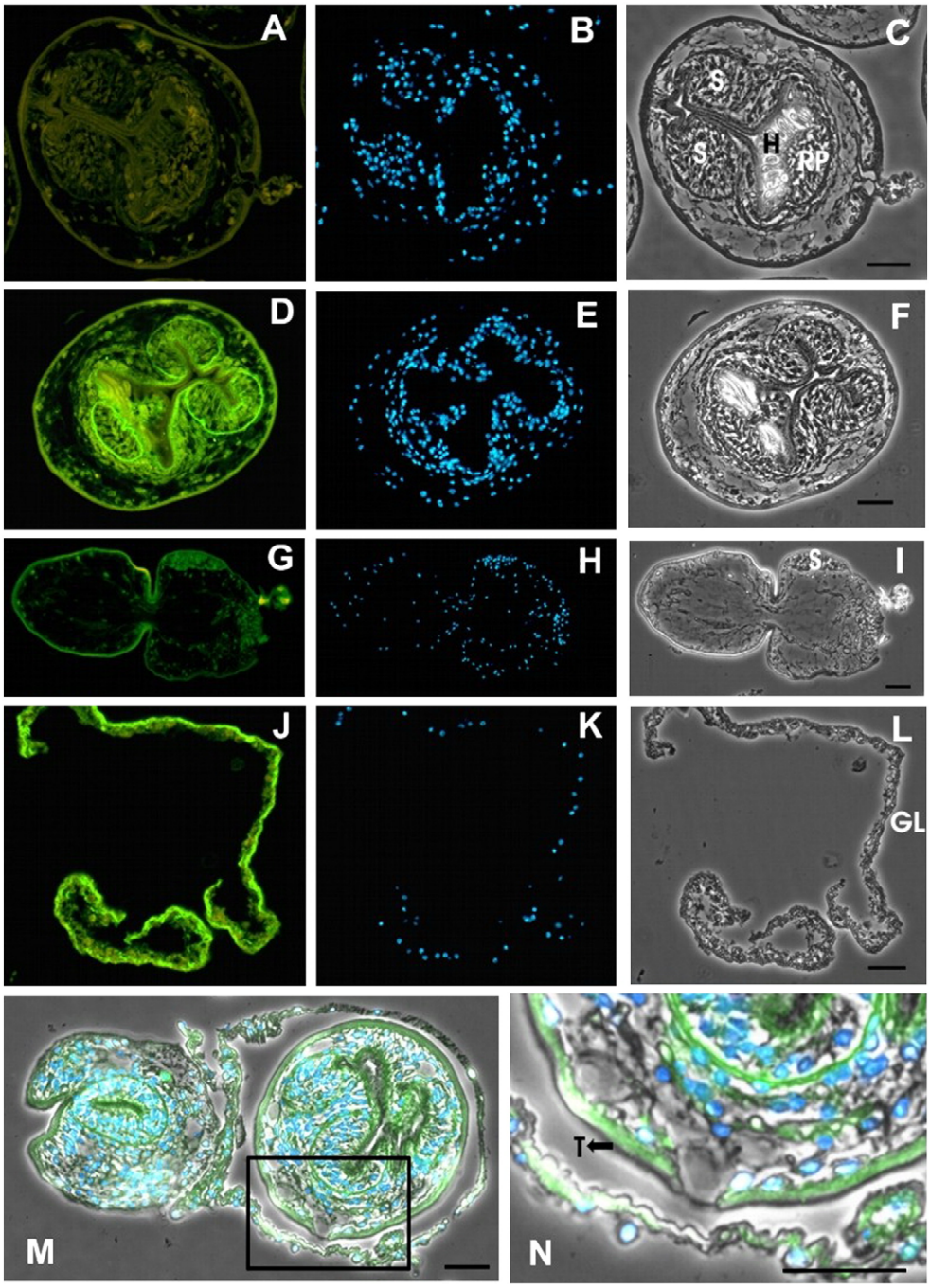

Fig. 4. Immunolocalization of EgAT1 on parasite sections. Protoscoleces and associated germinal layer were incubated with unpurified (D, G and J) or purified (M and N) sera against thioredoxin-EgAT1 fusion protein or purified (A) sera against thioredoxin alone and Alexa 488-conjugated secondary antibody. B, E, H and K: Hoechst nuclei staining. C, F, I and L: contrast phase image. Panel M shows contrast phase image merged with Hoechst nuclear staining (blue) and anti recombinant EgAT1 fluorescence (green). N: magnification of the square indicated in M. GL, germinal layer, H, hooks; RP, rostellar pad; S, sucker; T, tegument, bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

amino acids across parasite tissues which would be important for parasite survival in the host.

Several sequence features suggest that EgAT1 is an amino acid transporter belonging to the DAACS family: (1) identity at the nucleotide and amino acid levels with functionally characterized DAACS family members, (2) similar hydropathy profile with ASCT1 and other members of the family, (3) presence of 9–10 transmembrane domains, (4) a large hydrophilic loop with two consensus sequences for N-glycosylation between the third and fourth transmembrane domains, (5) high score with the SDF family (Sodium Dicarboxilate Family, also known as DAACS) and finally, (6) high score with EDTRNSPORT, a fingerprint of the excitatory amino acids belonging to the DAACS family. It has been noted that within a family of membrane proteins, the hydropathy profile of the amino acid sequences is much better conserved in the course of evolution than the sequences themselves (Lolkema and Slotboom,

1998). Based on their hydropathy profiles, DAACS family form a separate structural class, which is clearly distinct from any other family of secondary transporters (Lolkema and Slotboom, 1998, 2005). The high level of similarity observed among the profiles of EgAT1 and DAACS family members probably reflects structural and possibly functional similarities between them. However, some of the differences found between this protein and DAACS transporters from other taxa, for example in the putative amino acid binding domain, could be evaluated for the rational design of antiparasitic drugs.

A few *egat1* orthologous were reported in cestodes, which were not characterized so far. In *E. multilocularis* a clone of 95% amino acid identity with EgAT1 was found. The high degree of sequence conservation suggests an important function of these molecules in *Echinococcus* sp.

In a previous study it was showed by RT-PCR that *egat1* is transcribed in protoscoleces, adult worms and onchospheres (Rosenzvit et al., 2006), suggesting that it could play an important role all along the life cycle of the parasite. In the current study we confirmed the presence of the *egat1* transcripts by *in situ* hybridization analysis in protoscoleces, and showed expression at the protein level in protoscoleces and metacestode layers by immunohistochemistry and Western blot.

The localization of the putative amino acid transporter was analyzed by *in situ* hybridization and immunofluorescence in protoscoleces and associated brood capsule layers with probes directed to different regions of the protein. The results obtained were partially coincident. *In situ* hybridization studies showed that *egat1* is mainly expressed in the tegumental region. Immunolocalization studies showed that the EgAT1 protein appears to be localized in the subtegumental region all along the body and scolex of the protoscolex and layers from brood capsules. Suckers and rostellum were particularly stained in the scolex. In suckers, we could observe a very strong label by immunohystochemistry but not by *in situ* hybridization techniques. This result may be explained by different sensitivity levels of detection, by an absence of correlation between the mRNA and the corresponding protein level, and/or by the detection of alternatively spliced different isoforms in different regions of the protoscolex. Indeed, in a previous work (Rosenzvit et al., 2006), it was reported that two cDNA amplification products and only one genomic amplification product of *egat1* (EgP3G2) in protoscoleces were detected, raising the possibility of alternative splicing of this gene. Although the antibodies used for immunolocalization recognized a single band of the expected size in Western blot, strongly suggesting specificity, the possibility of recognition of a protein with unrelated function but with similar molecular weight cannot be ruled out.

The rol of EgAT1 could be nutritional or excitatory since amino acids are required as building blocks for proteins or energy supply but some of them are also involved in neurotransmission. In fact, pharmacological and immunohistochemical evidence has strong suggested that L-glutamate and L-aspartate are neurotransmitters in platyhelminths (Keenan and Koopowitz, 1982; Mendonça-Silva et al., 2002; Miller et al., 1996; Webb, 1987). The precise identity of EgAT1 inside the DAACS family could not be defined by bioinformatic analysis. However, the absence of staining of neuronal structures like nerve cell bodies or branching nerves fibers (Brownlee et al., 1994) suggests that EgAT1 is not a neuronal transporter and hence an EAAT member. Instead, the high level of staining at the tegumental level in the suckers, rostellum and rest of the body of the parasite, suggest an important role of these structures in the nutrition of the parasite. The tegument could act not only as an efficient barrier against the host, it could also satisfy the nutritional needs of the parasite and could facilitate the survival of the larval stage for long periods of time in a diversity of tissues of its intermediate hosts and perhaps in the intestine of the definitive hosts.

The close proximity to the surface of the larva, its expression all along the life cycle showed by RT-PCR (Rosenzvit et al., 2006), and particularly the intimate association with the attachment organs like suckers and rostellum, which are of major importance for the survival in the final host, could make of EgAT1 a particularly amenable target for antihelmintics. EgAT1 was also localized in the germinal layer of the metacestode, which probably reflects the biosynthetic requirements of this layer that continually undergoes cell division and protoscoleces formation. The localization of EgAT1 in the germinal layer could be exploited as a target for the generation of antiparasitic drugs or for its use as specific portals for the entry of drugs into the hydatid cyst.

The existence of a dicarboxilate amino acid (Na^+ or H^+) symporter (DAACS) member could be supported by the concomitant action a Na^+/K^+-ATPase discovered many years ago in protoscoleces of *E. granulosus* (McManus and Barret, 1985). The substrate specificity and sodium dependence of the putative transporter partially characterized on this study could not be determined by bioinformatics tools and should be determined by functional studies that are underway.

In summary, we have characterized a new *E. granulosus* molecule with several common features of amino acids transporters of the DAACS family which is expressed in particular regions of the metacestode of *E. granulosus*, suggesting an important role for the parasite biology. We are currently undertaking functional studies to determine the specificity and kinetics of transport.

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