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Identification of membrane-bound and secreted proteins from *Echinococcus granulosus* by signal sequence trap☆

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Abstract

The signal sequence trap technique was applied to identify genes coding for secreted and membrane bound proteins from *Echinococcus granulosus*, the etiologic agent of cystic hydatid disease. An *E. granulosus* protoscolex cDNA library was constructed in the AP-PST vector such that randomly primed cDNAs were fused with a placental alkaline phosphatase reporter gene lacking its endogenous signal peptide. *E. granulosus* cDNAs encoding a functional signal peptide were selected by their ability to rescue secretion of alkaline phosphatase by COS-7 cells that had been transfected with the cDNA library. Eighteen positive clones were identified and sequenced. Their deduced amino acid sequences showed significant similarity with amino acid transporters, Krebs cycle intermediates transporters, presenilins and vacuolar protein sorter proteins. Other cDNAs encoded secreted proteins without homologues. Three sequences were transcribed antisense to *E. granulosus* expressed sequence tags. All the mRNAs were expressed in protoscoleces and adult worms, but some of them were not found in oncospheres. The putative *E. granulosus* secreted and membrane bound proteins identified are likely to play important roles in the metabolism, development and survival in the host and represent potential targets for diagnosis, drugs and vaccines against *E. granulosus.*

Keywords: Signal sequence trap; Echinococcus granulosus; Secreted proteins; Membrane bound proteins; Developmental expression

1. Introduction

Echinococcus granulosus is a dog tapeworm that causes cystic echinococcosis in humans and infects domestic and wild animals (McManus et al., 2003). Cystic echinococcosis is endemic in many countries around the world, especially in rural areas with poor economic and sanitary conditions. *E. granulosus* has an indirect two-host life cycle, involving a histotrophic metacestode stage, the hydatid cyst, which develops from an invasive larva in the intermediate host (mostly ungulates or humans). Tissues within the cyst give rise by asexual proliferation to immature worms, named protoscoleces, which are infective to the definitive canid hosts. The hydatid cyst can survive in the host for long periods of time but the mechanisms that allow this persistence are poorly understood. Therefore, a better understanding of the molecular mechanisms of host-parasite interactions may provide new

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insights into identifying novel vaccine and drug targets that may facilitate improved control of cystic echinococcosis, an important aim from human health and economic perspectives.

Many of the proteins that are secreted or membrane bound (S/M) of parasites are involved in host-parasite interactions (Nene and Bishop, 2001; Fernández et al., 2002). These molecules participate in a wide range of parasite functions, including penetration and establishment in host tissues, modulation of the host immune response and incorporation of metabolites from the host. Because of their potential exposure to the host immune system, S/M components are candidates for improved diagnostic tests, as well as new drug and vaccine targets.

Secreted and type I membrane proteins have a signal peptide sequence at the N-terminus which directs transport to the secretory pathway (Gierasch, 1989; von Heijne, 1990; Rapoport, 1992). The common structure of signal peptides is conserved among distant phyla and consists of a positively charged N-region, followed by a hydrophobic H-region and a neutral but polar C-region (Nielsen et al., 1997). Protein trafficking is a complex process that is regulated at many steps (Martoglio and Dobberstein, 1998; Hegde and Lingappa, 1999) and the diversity of trafficked components is great. Nevertheless, the presence of a signal peptide has been used successfully to detect S/M proteins from many organisms (Klein et al., 1996; Jacobs et al., 1997; Arca et al., 1999; Kojima and Kitamura, 1999; Moffat et al., 2002; Smyth et al., 2003; Pearson et al., 2005).

One of the strategies to identify S/M proteins, the signal sequence trap (SST) technique, is based on heterologous gene expression systems that test the ability of possible signal sequences to rescue secretion of a reporter protein lacking its endogenous signal peptide by screening randomly primed cDNAs libraries. A yeast SST method has been used to test the functionality of predicted signal sequences from *Theileria parva* proteins (Nene and Bishop, 2001) and more recently, an SST method that uses human placental alkaline phosphatase (AP-PST) as a reporter (Chen and Leder, 1999) was used for the isolation of cDNAs from *Schistosoma mansoni* coding secreted and membrane bound proteins (Smyth et al., 2003; Pearson et al., 2005).

Here, we used the AP-PST technique to identify a subset of *E. granulosus* S/M proteins that could be involved in host-parasite interactions and may represent targets for improved control of cystic echinococcosis.

2. Materials and methods

2.1. Parasite material

Echinococcus granulosus protoscoleces were aspirated from hydatid cysts collected from sheep at an abattoir in Urumqi, Xinjiang, China. Adult worms and eggs were collected from dogs infected experimentally with viable protoscoleces. The eggs were treated with pepsin, pancreatin and sheep bile to obtain activated oncospheres (Li et al., 2004).

2.2. Nucleic acids

Total RNA from *E. granulosus* was extracted from protoscoleces that were crushed under liquid nitrogen and processed using Trizol reagent (Invitrogen). Poly A^+ RNA was purified using the MicroPoly (A) Purist™ mRNA Purification kit (Ambion). DNA was extracted from the interphase layer of the Trizol reagent and used to genotype the *E. granulosus* samples using sequence analysis of the mitochondrial *atp*6 gene (Le et al., 2002). All the RNA used was from sheep–dog (G1) strain.

2.3. cDNA synthesis and SST library construction and screening

cDNA synthesis and cloning were performed according to Chen and Leder (1999) with some minor modifications as outlined below. Protoscolex cDNA was synthesised using the OrientExpress Random Primer cDNA synthesis kit (Novagen), 4 μg of Poly (A^+) RNA, and random primers (TTN_6) as described elsewhere (Smyth et al., 2003). Double-stranded cDNA was size selected by agarose gel electrophoresis into two populations containing sequences of 300–550 bp and 550–700 bp; the rationale behind this size separation was to maximise fragment (and hence, open reading frame (ORF)) length while minimising the chances of generating fragments containing stop codons (Chen and Leder, 1999). *Eco*R I/*Hin*d III linkers were ligated to the cDNA for directional cloning in the AP-PST vector that contains a signalless placental alkaline phosphatase (PLAP) fusion molecule (Chen and Leder, 1999). The resulting *E. granulosus* protoscolex cDNA libraries were transformed into OneShot TOP10/P3 competent *Escherichia coli* (Invitrogen) and plated onto ampicillin (50 μg/ml)/tetracycline (10 μg/ml) LB-agar plates. Screening was performed using a sib-screen approach as described (Smyth et al., 2003). Briefly, 52 individual colonies were transferred to one LB agar plate containing ampicillin and tetracycline and were assigned a pool number. Bacteria from each pool were grown together in a liquid culture and plasmid DNA was extracted with a Qiagen mini prep kit. 1 μl of plasmid DNA was used for transfecting 1×10^6 COS-7 cells using Genejammer transfection reagent (Stratagene) in a 35 mm diameter well. After 24 h incubation at 37 °C, transfected cells were fixed using 4% paraformaldehyde, washed and screened for surface PLAP activity as described (Chen and Leder, 1999). Each positive pool was divided into 16 sub-pools that were screened in the same way until single positive clones were identified. PST and PST–PLAP plasmids were used as negative and positive controls, respectively.

2.4. Sequence analysis

Isolated cDNA clones were sequenced from plasmid DNA with T7 and T3 vector-derived primers using an Applied Biosystems Big Dye Terminator kit (Applied Biosystems) on an ABI 377 automated DNA sequencer. Nucleotide sequences