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Characterisation of major vault protein during the life cycle of the human parasite *Schistosoma mansoni*

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ABSTRACT

Vaults are ribonucleoproteins (13 MDa) highly conserved among lower and higher eukaryotes. Their association produces a complex composed of three proteins named Major Vault Protein (MVP), vault (PolyADP-ribose) polymerase (VPARP) and Telomerase-associated protein (TEP1), plus a small untranslated RNA. The exact function of this complex is unknown, although the biological role of vaults has been associated with multidrug resistance phenotypes and signal transduction pathways. Genomic analysis showed that model organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, do not possess genes encoding vaults. However, we have found that vault-related genes are present in the *Schistosoma mansoni* genome. These observations raised questions on the involvement of vaults in mechanisms of adaptation of the parasite in its mammalian host. Therefore, molecular characterisation of the putative Major Vault Protein performed using bioinformatics tools showed that this vault component is highly conserved in *S. mansoni*. The MVP expression level was quantified by qRT-PCR using total RNA from susceptible (LE) and resistant (LE-PZQ) adult worm lineages, cercariae and mechanically transformed schistosomula (MTS) cultured for 3.5, 24, 48 and 72 h *in vitro*. Our results suggest a stage-specific expression in all developmental stages analysed. Western blotting has shown up-regulation of *Sm*MVP in the MTS-3.5, 72 h and resistant adult worms, and similar levels in all other stages. Furthermore, *Sm*MVP was found differentially expressed in adult males and females from the susceptible lineage. Further studies should clarify whether *Sm*MVP is somehow linked to drug resistance in *S. mansoni*.

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1. Introduction

Human schistosomiasis, a major parasitic disease that affects more than 200 million individuals in 76 tropical and subtropical countries, is the second most prevalent tropical disease worldwide, causing high morbidity and mortality. Currently, praziquantel (PZQ) is the drug of choice for the treatment of schistosomiasis due to its low toxicity and low cost [1,2]. Despite the advantages of PZQ, *Schistosoma mansoni* resistance to PZQ has been found in isolates from endemic areas and can also be induced in the laboratory [3].

The success of *S. mansoni* parasitism has a direct relationship with the parasite's adaptation to different environments and hosts during its life cycle. Therefore, genes involved with cellular differentiation are of particular importance to understanding parasite's biology. In this context, during a bioinformatic analysis using the transcriptome and genome databases, we have identified putative entries coding for a 100 kDa major vault protein (MVP), the predominant component of vaults which represents more than 70% of the vault complex mass, as well as the vault poly (ADP-Ribose) polymerase (VPARP - 193 kDa), a telomerase-associated protein 1 (TEP1 - 240 kDa) and a small untranslated RNA (vRNA, approximately 140 b).

Vault particles are identified as 13-MDa ribonucleoproteins, measuring approximately 70 nm × 40 nm × 40 nm [4]. They are abundant and extremely conserved across a large number of species [5]. Although the exact function of vault remains unknown, several studies have shown the potential involvement of this complex in multidrug resistance. MVP is identical to the human lung resistance protein and its expression is increased in tumours as well as in various multiple drug resistance models [6,7]. Vault is also involved in the regulation of PTEN (phosphatase and tensin homolog) and EGFR (epidermal growth factor)-induced MAPK pathway, the activation of COP1 (Constitutive Photomorphogenic 1) for the degradation of c-Jun, import and/or activation of nuclear estrogen receptors, in the activation of the expression of INF-γ and several other events of the immune

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response, especially in innate immunity [7,8]. Despite a huge amount of data from diverse species and systems, defining the precise functions of vaults is still highly complex and challenging.

This report shows the conservation as well the mRNA and protein levels of *Sm*MVP. We observed up-regulation of this protein during cercariae to early-schistosomula transition and in PZQ-resistant adult worms. In addition, increased expression of MVP in the adult male relative to the female was observed for the LE strain. Moreover, bioinformatics analysis provided support for the presence of other protein components of vaults in the *S. mansoni* genome. The presence of the vault complex in *S. mansoni* suggests a possible role during infection of the mammalian host.

2. Materials and methods

2.1. Ethics statement

All experiments involving animals were authorised by the Ethical Committee for Animal Care of Federal University of Ouro Preto (CEUA-UFOP protocol no. 2011/55). These were in accordance with national and international regulations accepted for laboratory animal use and care.

2.2. In silico analysis

The MVP was identified by mining the *S. mansoni* sequences at GeneDB (http://www.genedb.org/genedb/smansoni/) using Blastp and as queries known *Homo sapiens* and *Mus musculus* proteins. Reference proteins from other species were searched in NCBI database to obtain a full set of putative homologue proteins in order to compare with the *S. mansoni* proteins. The BLASTp algorithm, underpinned by Pfam (v26.0), allowed for the detection of conserved protein domains or motifs from *S. mansoni* sequences. The whole protein sequences were used to perform the alignments. Multiple sequence alignments were performed using the program ClustalX 2.0 with default settings [9]. Phylogenetic tree was inferred using the Neighbor-Joining method (NJ) and JTT model [10]. The bootstrap consensus tree inferred from 1000 replicates was used to represent the evolutionary history of the taxa analysed. Molecular phylogenetic analyses were conducted using MEGA 5 software [11,12].

2.3. Parasites

The resistance in this isolate (LE-PZQ) was induced *in vivo* when infected *Biomphalaria glabrata* snails were submitted to three treatments with PZQ, each treatment administered on 5 consecutive days, with 1 week interval, for selection of less susceptible parasites to PZQ. It was passaged through mice and treated 45 days after infection with 400 mg/kg PZQ [13]. The LE (susceptible strain to PZQ) and LE-PZQ (resistant isolate to PZQ) adult worms were obtained from by liver perfusion of mice after 50 days of infection, washed in RPMI 1640 (Sigma Chemical Co.), and were quick-frozen in liquid nitrogen and stored at −80 °C until use.

The *S. mansoni* parasite (LE strain) [14] was maintained by routine passage through *B. glabrata* snails and BALB/c mice. The infected snails were induced to shed cercariae under light exposure for 2 h and the cercariae were recovered by sedimentation on ice. The mechanically transformed schistosomula (MTS) were prepared as previously described [15]. Briefly, cercariae were recovered and washed in RPMI 1640 medium (Invitrogen, Sao Paulo, Brazil) before vortexing at maximum speed for 90 s and immediately cultured for 3.5 h at 37 °C in a 5% CO_2 incubator. Then, the recovered schistosomula were washed with RPMI 1640 until no tails were detected. For subsequent incubations, the parasites were maintained in M169 medium supplemented with 10% FBS (Fetal Bovine Serum), penicillin (100 μg/mL), streptomycin (100 μg/mL) and 5% of Schneider's medium [16] at 37 °C in a 5% CO_2 incubator for 3.5, 24, 48 and 72 h.

2.4. Expression analysis of MVP by qRT-PCR

Total RNA from cercariae, schistosomula, adult worms and eggs was obtained using a combination of Trizol reagent (Sigma, Belo Horizonte, Brazil) and chloroform for extraction and column-purified using the "SV total RNA Isolation System" (Promega, Belo Horizonte, Brazil). The preparation was treated with RNase-free DNase I in 3 different rounds with decreasing enzyme concentrations (RQ1 DNase; Promega). RNA was quantified using a spectrophotometer and an aliquot containing 1 μg of total RNA was reverse transcribed using an oligodT primer from the Thermoscript RT-PCR System (Invitrogen), as described by the manufacturer. The efficiency of DNAse I treatment was evaluated by PCR amplification of the cDNA reaction mix without the addition of the Thermoscript enzyme. *S. mansoni* specific primers were designed using the program GeneRunner®. The primers for MVP were forward 5′-GAATGGGTGACGAGGAGTAC-3′ and reverse 5′-AGTCTGAGTGCCGA GTTTGG-3′ (GenBank no. 8346893). Reverse-transcribed cDNA samples were used as templates for PCR amplification using SYBR Green Master Mix UDG-ROX® (Invitrogen) and 7300 Real Time PCR System (Applied Biosystems; Rio de Janeiro, Brazil). Specific primers for *S. mansoni* EIF4E were used as an endogenous control (GeneDB ID: Smp_001500) (forward 5′TGTTCCAACCACGGTCTCG3′, reverse 5′TCGCCTTCCAATGCT TAGG3′) [17]. The efficiency of each pair of primers was evaluated according to the protocol developed by Applied Biosystems (cDNA dilutions were 1:10, 1:100 and 1:1000) [18,19]. For all investigated transcripts, three biological replicates were performed and their gene expression was normalised against the EIF4E transcript according to the $2^{-\Delta Ct}$ method using Applied Biosystems 7300 software [20].

2.5. Detection of SmMVP by Western blotting

Determination of MVP expression was performed by Western blot using the monoclonal anti-human MVP MAB4141 antibody (Millipore, Sao Paulo, Brazil). Briefly, total protein extracts from cercariae, MTS at 3.5, 24, 48 and 72 h, and susceptible and PZQ-resistant adult worms were prepared by sonication in 25 mMTris–HCl pH 7.5, 1 mMDTT, 1 mM EDTA, and 10 μM of the following protease inhibitors: TLCK, TPCK, NEM, and PMSF. After centrifugation at 10,000 ×g for 15 min, the soluble protein concentration was determined by QuantiPro™ BCA Assay Kit (Sigma Aldrich, Sao Paulo, Brazil). Twenty micrograms of total soluble protein was separated by 10% SDS–PAGE [21,22]. The gel was transferred to a PVDF membrane at 25 V for 2 h at 4 °C. After 16 h incubation in blocking solution, the membrane was washed and incubated with primary anti-human MVP antibody at a 1:500 dilution. Alkaline phosphatase-conjugated anti-mouse IgG (Sigma) was used as the secondary antibody at 1:2500 and the reactivity visualised by NBT/BCIP Western blot detection reagents (Amresco, Sao Paulo, Brazil) as per the manufacturer's instructions. Differences in protein loading among the extracts present in the membrane were registered through densitometric analysis of the total protein content in each lane [23]. Relative expression levels of *Sm*MVP were obtained by densitometric analysis of the reactive bands, taking into account and correcting for the observed differences in total protein loading, using the Quantity one® (Bio-Rad, Sao Paulo, Brazil) software.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (Irvine, CA, USA). Normality of the data was established using one-way analysis of variance (ANOVA). Tukey post-tests were used to investigate significant differential expression of transcripts throughout the investigated stages [24]. In all cases, the differences were considered significant when $p < 0.05$.

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