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RNA interference targeting leucine aminopeptidase blocks hatching of *Schistosoma mansoni* eggs

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ABSTRACT

Schistosoma mansoni leucine aminopeptidase (LAP) is thought to play a central role in hatching of the miracidium from the schistosome egg. We identified two discrete LAPs genes in the *S. mansoni* genome, and their orthologs in *S. japonicum*. The similarities in sequence and exon/intron structure of the two genes, LAP1 and LAP2, suggest that they arose by gene duplication and that this occurred before separation of the mansoni and japonicum lineages. The *Sm*LAP1 and *Sm*LAP2 genes have different expression patterns in diverse stages of the cycle; whereas both are equally expressed in the blood dwelling stages (schistosomules and adult), *Sm*LAP2 expression was higher in free living larval (miracidia) and in parasitic intra-snail (sporocysts) stages. We investigated the role of each enzyme in hatching of schistosome eggs and the early stages of schistosome development by RNA interference (RNAi). Using RNAi, we observed marked and specific reduction of mRNAs, along with a loss of exopeptidase activity in soluble parasite extracts against the diagnostic substrate L-leucine-7-amido-4-methylcoumarin hydroxide. Strikingly, knockdown of either *Sm*LAP1 or *Sm*LAP2, or both together, was accompanied by ≥80% inhibition of hatching of schistosome eggs showing that both enzymes are important to the escape of miracidia from the egg. The methods employed here refine the utility of RNAi for functional genomics studies in helminth parasites and confirm these can be used to identify potential drug targets, in this case schistosome aminopeptidases.
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1. Introduction

Schistosomiasis is endemic to 76 developing countries and affects an estimated 200 million people in impoverished areas of Africa, Central and South America and East Asia [1,2]. The adult blood flukes locate in mesenteric blood vessels (in *Schistosoma mansoni*, *S. japonicum*, *S. intercalatum* and *S. mekongi*) or the venous plexuses around the urinary bladder (in *S. haematobium*) and after sexual maturation release several hundreds to thousands of eggs a day during their long lifespan (up to 10 years). While some of these eggs reach the lumen of the intestines or the bladder, depending of the infecting species, and are discharged with the feces or urine, many are either trapped in the host tissues or transported by the circulation to the liver and other distant sites [3]. The host inflammatory reaction to the secretions produced by the embryonated eggs leads to formation of granulomas, which evolve in severe cases to portal fibrosis, hepato-splenomegaly and gastrointestinal varices [2–4].

The complement of antigens secreted by schistosome eggs includes protease activities [5–8]. Leucine aminopeptidase (LAP) activity was detected in schistosome eggs more than 30 years ago [9,10], and infected humans and rodents elicit antibody responses to the enzyme [11–13]. Histochemical studies localized the enzyme to eggs and surrounding liver tissue in experimentally infected mice [14]. Recent proteomic and glycomic approaches identified a LAP amongst a complex suite of proteins and glycoproteins within the schistosome egg [15–17]. Because bestatin, an inhibitor of aminopeptidase activity, markedly decreased the escape of miracidia from the eggs it was suggested that LAP activity was critical to the hatching process [18]. LAP activity was also demonstrated in the gut of adult schistosomes by McCarthy et al. [19] who considered that the enzymes play a downstream role in catabolism of ingested hemoglobin and serum proteins.

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In this study, we identified a second LAP gene in *S. mansoni* that is distinct but closely related to the transcript characterized previously [19]. Both LAP enzymes, here termed *Sm*LAP1 [19] and *Sm*LAP2, belong to Clan MF, family M17 metalloproteases [20] according to the MEROPS classification which includes aminopeptidases from archaea, bacteria and eukaryotes that function in protein turnover and biogenesis [21]. The two LAPs exhibit distinct temporal expression profiles during the life cycle of the schistosome. They are both expressed at the egg stage, although *Sm*Lap2 has an elevated expression in eggs and intra-snail stages. Using RNA interference (RNAi) targeting both *Sm*LAP1 and *Sm*LAP2, we now present evidence that proves the two aminopeptidases are required for hatching of eggs of *S. mansoni*.

2. Materials and methods

2.1. Schistosome eggs

Eggs of *S. mansoni* were isolated from livers of mice 7 or 8 weeks after experimental infection, as described [22]. Briefly, three to five livers were chopped finely with a scalpel blade, and then blended to a smooth consistency in 50 ml of phosphate-buffered saline, pH 7.4 (PBS), 5 ml of 0.5% clostridial collagenase (Sigma) and 500 μl of polymyxin B. The mixture was incubated with shaking at 37 °C overnight, after which the contents were subjected to centrifugation at 400 × *g* for 5 min. The supernatant was discarded and the pellet resuspended in 50 ml PBS. This wash procedure was repeated twice more, with the exception that after the final centrifugation the pellet was resuspended into 25 ml of PBS. The resuspended mixture was passed sequentially through 250 and 150 μm sieves. The filtrate was centrifuged at 400 × *g* for 5 min, the supernatant discarded and the pellet resuspended in 3 ml of PBS. This was applied to a column of Percoll, prepared by mixing 8 ml of Percoll (GE Healthcare Bio-Science AB) with 32 ml of 0.25 M sucrose in a 50 ml tube. The tube was centrifuged at 800 × *g* for 10 min. Liver cells that remained on the top of the Percoll were removed with a Pasteur pipette. The schistosome eggs, which pelleted tightly at the bottom of the tube, were washed three times with PBS and any residual liver cells were removed by discarding the supernatants. Further purification of eggs was achieved by resuspension in 0.5 ml of PBS and application on to a second Percoll column, prepared by mixing 2.5 ml of Percoll with 7.5 ml of 0.25 M sucrose in a 15 ml polypropylene tube. The eggs were pelleted and then washed as before. The eggs were resuspended in 6 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 U of penicillin and streptomycin (Invitrogen, Carlsbad, CA), split into 2 ml aliquots in a six-well plate and cultured at 37 °C, under 5% CO_2. Other developmental stages of the *S. mansoni* were obtained as described [23,24].

2.2. Bioinformatics characterization of schistosome leucine aminopeptidase

The protein sequences of *S. mansoni* (P91803) [19] and *Fasciola hepatica* (AY064459) [25] LAPs described previously were used as queries for blast searches using the Omniblast interface available at the *S. mansoni* Genome Project page at the Sanger Institute, www.sanger.ac.uk/cgi-bin/blast/submitblast/s_mansoni/omni. Only two genomic regions produced significant hits with the queries, one being identical to the P91803 query termed *Sm*LAP1 (GeneDB Smp_030000), and the other termed *Sm*LAP2 (Smp_083870). The genomic scaffolds containing the hits were visualized in the genome browser available at the *S. mansoni* GeneDB, www.genedb.org/genedb/smansoni/. A similar search was performed on *S. japonicum* using as database the 95,389 contigs of the assembly release 2 available from the Shanghai Centre for Life Sciences & Biotechnology Information, http://lifecenter.sgst.cn/sjschistosoma/en/schistosomaDispatch.do?disName=intro. The contigs or scaffolds including the putative LAP loci from both species were retrieved and further analysed using Artemis; synteny was evaluated by comparison using the Artemis Comparison Tool, ACT [26]. The deduced amino acid sequences of the *S. mansoni* and *S. japonicum* LAPs were aligned with reference sequences (accessions provided in figure legends) using ClustalX [27]. A bootstrapped neighbor joining tree was generated based on the more conserved carboxy terminal domain, using the aminopeptidase A from *Escherichia coli* as outgroup. Based on the sequences of *Sm*LAP1 and *Sm*LAP2 appropriate specific primers for their amplification were generated (see below for sequences).

2.3. Leucine aminopeptidase expression analysis

RNA was extracted from different *S. mansoni* stages using the RNAqueus-Micro Kit (Ambion, Austin, TX). Residual DNA contaminating the RNA was removed by DNase digestion (TurboDNase, Ambion). cDNAs were synthesized using 300 ng of total RNA, using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA). Leucine aminopeptidase 1 (*S. mansoni* Gene DB Smp_030000) (*Sm*LAP1) and leucine aminopeptidase 2 (*S. mansoni* Gene DB Smp_083870) (*Sm*LAP2) expression was investigated by RT-PCR using gene specific primers (F: 5′-CAT TCC CAG AAC ATC CAT CAA A-3′; R: 5′-CAT TAT CCA TCA CAG CCG TGA A-3′ spanning 1020 bp for *Sm*LAP1, and F: 5′-TAC AAG AAG CAT CAC AGG TGA A-3′; R: 5′-CAG AGT AGC GAT TGT CAT TAG TC-3′ spanning 1070 bp, for *Sm*LAP2). *Sm*LAP1 and *Sm*LAP2 were confirmed by cloning the PCR products in pCR4.0 TOPO TA vector (Invitrogen, CA, USA) and sequencing. Expression of *S. mansoni* actin gene (GenBank U19945) [28,29] was used as an internal control using the primers, F: 5′-CAG TGT TCC CTT CCA TCG TT-3′; R: 5′-GGA CAG GGT GTT CTT CTG GA-3′, spanning 224 bp. PCR conditions included an initial denaturation at 94 °C for 30 s followed by 35 cycles of 30 s at 94 °C, 60 s at 52 °C, 90 s at 72 °C and a final extension at 72 °C for 10 min. Images of PCR products in ethidium-stained gels were documented using a Versadoc imaging system and Quantity One software (BioRad). Densitometric measurements of ethidium-stained PCR product bands were obtained using Image J, http://rsbweb.nih.gov/ij/.

2.4. Synthesis of dsRNA

dsRNAs were transcribed *in vitro*, from template PCR products, using gene specific primers tailed with the T7 promoter sequence. Irrelevant control firefly luciferase dsRNA (dsLUC) template encoding the full length 1672 kb was generated using the pGL3-basic plasmid (Promega, Madison, WI) as template and primers F: 5′-*TAA TAC GAC TCA CTA TAG GG* T GCG CCC GCG AAC GAC ATT TA-3′ (T7 promoter residues indicated in italics); R: 5′-*TAA TAC GAC TCA CTA TAG GG*G CAA CCG CTT CCC CGA CTT CCT TA-3′. *Sm*LAP1 and *Sm*LAP2 clones were employed for synthesis of *Sm*LAP1 dsRNA (dsLAP1) and *Sm*LAP2 dsRNA (dsLAP2) using primers that included the T7 promoter sequence (F: 5′-*TAA TAC GAC TCA CTA TAG GG*A CGA ACA TTA GCA CGA GAT ATT-3′; R: 5′-*TAA TAC GAC TCA CTA TAG GG*C ATA ACC ATT CTA CCT TCA GCA-3′ spanning coding DNA positions 543–1077 for dsLAP1; and F: 5′-*TAA TAC GAC TCA CTA TAG GG*G CTG AAG TCC TGG GTT GGT T-3′; R: 5′-*TAA TAC GAC TCA CTA TAG GG*C CAT TCG ACC TTC AGC ATC A-3′ spanning coding DNA positions 502–1117 for dsLAP2). dsRNA was synthesized and purified using the Megascript RNAi kit (Ambion). dsRNAs were precipitated with one volume of 5 M ammonium acetate and 2.5 volumes of 95% ethanol after which the RNA pellet was dissolved in water. Integrity of the dsRNAs was verified by non-denaturing 1% agarose gel electrophoresis,

and concentration and purity determined with a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE).

2.5. *Delivery of dsRNA*

Immediately after the isolation, eggs were cultured in 1,000 μl of DMEM complete media as described above, at 37 °C under 5% CO_2 in 24 wells plate (∼5000 eggs per well). The eggs were soaked in 20 μg/ml of either ds*Sm*LAP1, ds*Sm*LAP2, ds*Sm*LAP1 and ds*Sm*LAP2 together, or dsLUC (the latter serving as a control, irrelevant dsRNA). Another group of eggs was cultured without dsRNA, as a mock-treatment control. The media and the dsRNA were changed every other day, with frequent microscopic examination to identify visual phenotypic effects. The eggs were harvested after 7 days in culture, for subsequent RNA and protease activity analyses, and in addition an aliquot of eggs from each treatment group was induced to hatch (below).

2.6. *RNAi effects*

2.6.1. *Hatching of eggs*

Eggs from each group were washed three times in 1 × PBS, added to 500 μl of distilled water and exposed to bright light. Micrographs of the cultured eggs were taken, 30 and 60 min later, using an inverted microscope fitted with a digital camera. The ratio of non-hatched to total eggs in each group was determined from visual inspection of the images; 80–100 eggs were inspected in each treatment group in each experiment. Another group of eggs was incubated with the aminopeptidase inhibitor, bestatin, following the protocol of Xu and Dresden [18]. Briefly, 5 μl of bestatin (Sigma–Aldrich, St. Louis, MO) (10 mg/ml in DMSO) was added to the eggs (∼5000 eggs) in 45 μl of 0.2 M of NaCl, and the eggs incubated for 60 min at 23 °C. Subsequently, 950 μl dH_2O was added to the eggs to stimulate hatching of the eggs, after which hatching was monitored and documented as above. In addition, a control group of eggs without the inhibitor bestatin, but with the DMSO carrier, was included. These assays were repeated twice.

2.6.2. *RNA level: gene expression analysis*

Eggs were harvested 7 days after treatment with dsRNA. Total RNA was extracted using the RNAqueus-Micro Kit (Ambion). Residual DNA contaminating RNA preparations was removed by DNase digestion (TurboDNase, Ambion). cDNAs were synthesized using 10-fold serial dilutions of egg RNA, starting with 100 ng RNA, using the iScript^{TM} cDNA Synthesis Kit (BioRad, Hercules, CA). Primers targeting the LAP and actin transcripts, and PCR cycle conditions, are described above.

2.6.3. *Protein level: LAP enzyme activity assay*

Soluble protein extracts from eggs were prepared by sonication-induced lysis (5 × 5 s bursts on ice, output control value 3, model W-220F Sonicator, Heat Systems-Ultrasonics, Inc., Plainview, New York) in 100 mM glycine, pH 8.5 containing 1 mM $MnCl_2$. After centrifugation of the lysate for 10 min at 4 °C at 14,000 rpm, the supernatant was employed as soluble schistosome eggs extract. A total of 2 μg of soluble protein from each group was added in triplicate to a 100 μl reaction mixture of 100 mM glycine, pH 8.5 containing 1 mM $MnCl_2$ and the fluorogenic substrate L-leucine-7-amido-4-methylcoumarin hydroxide (Bachem) at a final substrate concentration of 50 μM, and incubated at 37 °C for 30 min. Fluorescence from substrate hydrolysis was measured in a fluorometer (BioTek Synergy HT, Winooski, VT) at 430 nm with excitation at 360 nm. To confirm that LAP activity was being measured, the egg extracts were pre-incubated for 10 min with 50 μM concentration of the broad-spectrum aminopeptidase inhibitor, bestatin (Sigma–Aldrich), prior to the addition of the substrate. Protein concentrations of soluble egg extracts were determined using the bicinchoninic acid assay (BCA kit, Pierce, Rockford, IL).

2.7. *Statistical analysis*

Levels of statistical significance among and between treatments were determined using Analysis of Variance (ANOVA) and Student's *t*-test. *P*-values of ≤0.05 were considered to be significant.

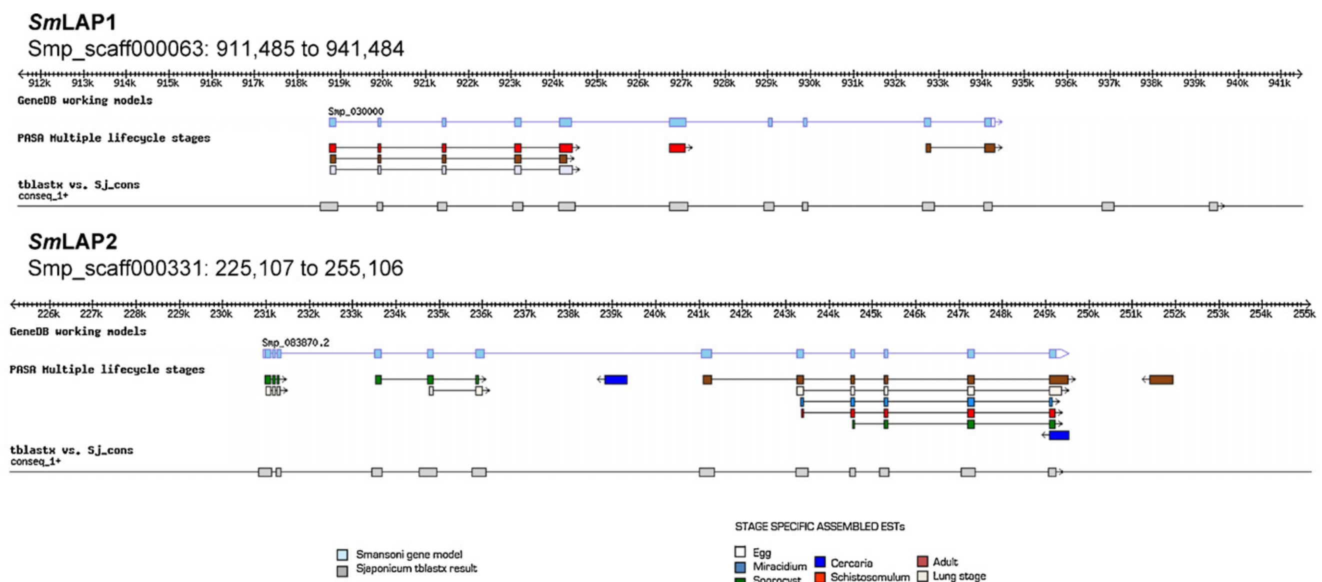

Fig. 1. A schematic view of the genome loci encoding the *Schistosoma mansoni* leucine aminopeptidase (LAP) genes. The genome browser view, obtained from www.genedb.org/schistosoma/, indicates the position of the exons of *Sm*LAP1 and *Sm*LAP2 (coding portions in light blue boxes, 3′ untranslated regions in white boxes). High scoring regions on tblastx comparisons against the *S. japonicum* genome draft are indicated (grey boxes). Stage specific assembled ESTs (color coded) mapped with the PASA package [60] on the genomic sequence and their orientations are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

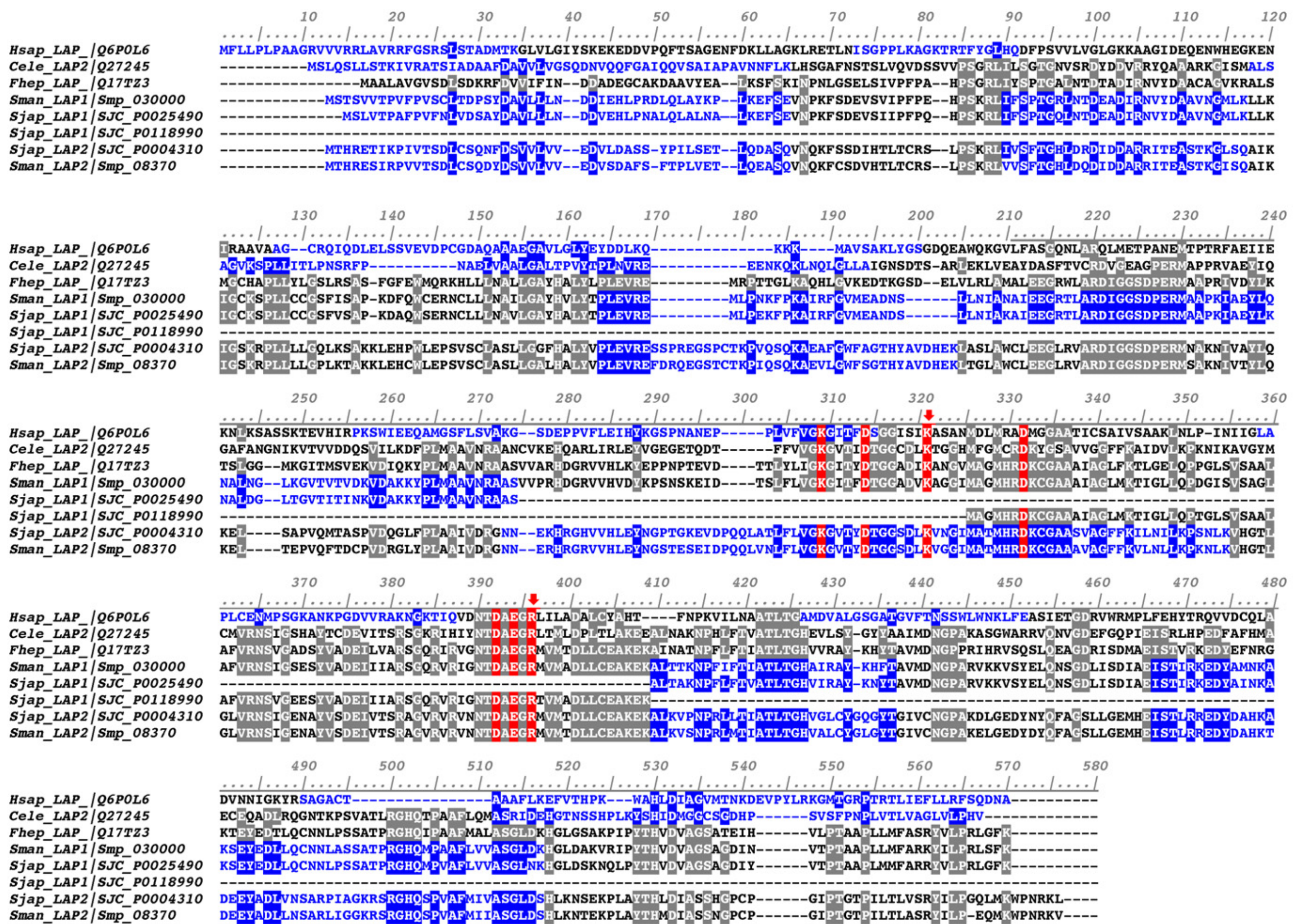

Fig. 2. Alignment of amino acid sequences of leucine aminopeptidases. Exon alternation in genomic sequences is indicated by alternation of blue and black type. Conserved regions are shown as shaded blocks. Residues that participate in coordination of metal ions are indicated in red and those involved in the catalysis are arrowed. (By way of reference, residues Asp 289, Asp 367 and Glu 369 [*Sm*LAP1 numbering] are involved in the coordination of a zinc atom, while Asp 289, Lys 284, Asp 307 and Glu 369 bind a second zinc ion. Lys 296 and Arg 371 are also involved in catalysis.) The conserved carboxy terminal domain is indicated by underlining in the alignment positions. The reference sequences are from *Fasciola hepatica* (accession Q17TZ3) *Caenorhabditis elegans* (Q27245) and *Homo sapiens* (Q6P0L6). The schistosome identifiers correspond to those provided in the genome assembly 4 of *Schistosoma mansoni* and assembly 2 of *Schistosoma japonicum*. The two contigs comprising *Sj*LAP1 are included. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results

3.1. Two leucine aminopeptidase genes in Schistosoma mansoni genome

To investigate the complement of LAP genes in the genome of *S. mansoni*, we searched the draft genome (version 4.0) available at the Sanger Institute, a seven-fold coverage that likely includes all the protein encoding genes of the schistosome. The search identified two genes; one corresponding to the cDNA reported previously, which we refer to now as *Sm*LAP1, encoding a protease of 523 deduced amino acid residues, and a novel gene, *Sm*LAP2, of 544 deduced amino acid residues (Fig. 1). A similar search of the draft of the *S. japonicum* genome located the putative orthologs of both genes. No other M17 aminopeptidase-like sequences were found in the schistosome genomes. An alignment of the deduced amino acid sequences of the *S. mansoni* and *S. japonicum* LAPs 1 and 2 with closely related enzymes from *Homo sapiens*, *F. hepatica* and *Caenorhabditis elegans* is presented in Fig. 2. The *Sm*LAP1 and *Sm*LAP2 sequences were 49% identical and 63% similar at the amino acid level, while *Sm*LAP1 and *Sj*LAP1 were 89% identical and 95% similar, *Sm*LAP2 and *Sj*LAP2 were 88% identical and 93% similar. *Sm*LAP1 and *Sm*LAP2 were 20–21% identical to human LAP, respectively. LAPs exhibit a two domain structure, a less conserved N-terminal domain and a conserved C-terminal domain that contains the catalytic residues. Residues Asp 289, Asp 367 and Glu 369 (*Sm*LAP1 numbering) are involved in the coordination of a zinc atom, while Asp 289, Lys 284, Asp 307 and Glu 369 bind a second zinc ion. Lys 296 and Arg 371 are also involved in the catalytic mechanism by acting as an electrophile and proton donor, respectively.

The *Sm*LAP1 gene spans 15.5 kb in the center of scaffold 000063 (1959 Mb), and is structured in 10 exons, encoding 523 deduced amino acid residues, and includes a short 101 bp 3′UTR. The *Sm*LAP2 gene covers 18.59 kb of scaffold 000331 (779 kb) comprising 12 exons, consisting of a 28 bp 5′UTR, 1635 bases of the CDS (544 deduced amino acid residues) and a 293 bp 3′UTR (Fig. 1). A similar search in the draft of the *S. japonicum* genome allowed us to identify the putative orthologs of both genes. *Sj*LAP2 maps to contig SJC_C002437 (included in the scaffold SJC_000012). The coding sequence (SJC_P0004310) is also organized in 12 exons identical to those of *Sm*LAP2, although the introns are bigger, giving the whole gene a length of 21.9 kb. There are two contigs with high homology to *Sm*LAP1; scaffold SJC_000149 covers the full length of the gene, comprising exons 1–5 and 7–10. The sixth exon is missing due to a sequencing gap included in the scaffold; a different small contig (SJC_010679, 3.4 kb) contains the last portion of the missing exon (see Figs. S1 and S2). No other relevant hits to LAPs were

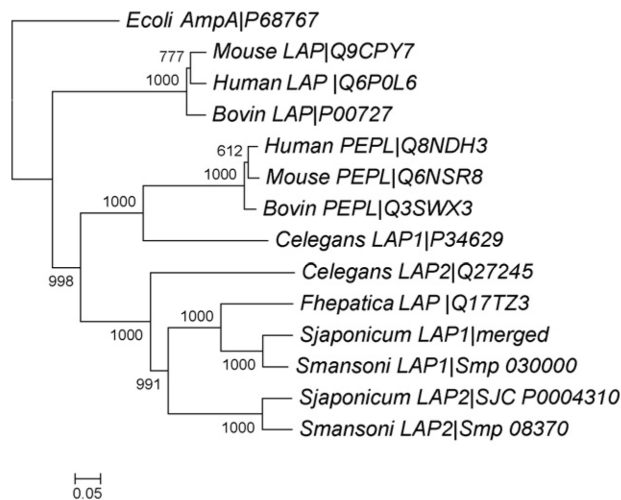

Fig. 3. Phylogenetic tree reveals relationships between schistosome leucine aminopeptidases and other family M17 LAP enzymes. Neighbor joining tree based on the conserved carboxy terminal domain of selected proteins. The aminopeptidase A from *Escherichia coli* served as the outgroup. Bootstrap values supporting the major clusters are indicated. Branch names indicate species or common name along with Uniprot accessions.

detected in the *S. japonicum* genome. The comparison of the coding sequences of the schistosome LAP1 and LAP2 genes indicated that they are closely related. The difference in their exonic structure is restricted to the fifth and sixth exons of LAP1 that are split in two exons each respectively in LAP2 genes. This structural arrangement is conserved between both schistosome species, suggesting that the homologues emerged by a duplication event previous to separation of the *mansoni* and *japonicum* species (Figs. 1 and 2). Phylogenetic analysis confirmed that schistosome LAP1 and LAP2 were closely related, and constituted a separate clade with other flatworm LAPs and *C. elegans* LAP2, discrete from the vertebrate LAPs (Fig. 3).

3.2. Differential developmental expression of the leucine aminopeptidases

Information provided at the schistosome GeneDB of ESTs mapping to the genomic sequences indicated that both *Sm*LAP1 and *Sm*LAP2 genes were expressed in different stages of the schistosome developmental cycle, with *Sm*LAP1 exhibiting a more restricted expression (Fig. 1). In order to confirm this observation and assess the expression level of the two LAPs during development of *S. mansoni*, we designed gene specific primes to clone *Sm*LAP1 and *Sm*LAP2. 300 ng of total RNA from different stages were used to perform RT-PCR using gene specific primers. Actin gene expression was used as a control. The identity of the PCR products as *Sm*LAP1 and *Sm*LAP2 was confirmed by nucleotide sequencing (not shown). Interestingly, whereas both LAPs are equally expressed in the blood dwelling stages (schistosomules and adult) *Sm*LAP2 expression was higher in free living larval (miracidia) and in parasitic intra-snail (sporocysts) stages (Fig. 4A). There were no apparent differences in the expression of the two LAPs in adult male and female blood flukes, while the absence of expression in cercarial stage was in concordance with the global reduction of gene expression in cercariae reported by Jolly et al. [30] (Fig. 4A). There were no apparent sex-related differences between male and female worms or combined adults for any of the three genes, including actin. Densitometric measurements of the relative levels of *Sm*LAP1 and *Sm*LAP2 measured across the different bands in Fig. 4A using actin as normalization control are shown in Fig. 4B. Interestingly, ESTs mapping data suggest that only a reverse transcript is found in this stage, which may indicate that anti-sense transcripts are involved in silencing gene expression in cercariae (Fig. 1).

3.3. RNAi targeting LAPs inhibited hatching of schistosome eggs

We employed RNAi to investigate the putative function of LAP activity in the egg hatching process. We soaked cultured eggs with ds*Sm*LAP1 RNA, ds*Sm*LAP2 RNA, or both, or with bestatin. Control eggs were cultured without dsRNAs or bestatin, while other eggs treated with bestatin served as a positive control. In addition, eggs treated with double stranded RNA specific for firefly luciferase (dsLUC), a gene not present in the schistosome genome, were included as a dsRNA treatment control. After 7 days in culture (during which time no phenotypic differences were apparent among the treatment groups), eggs were washed in PBS, transferred into water at 23 °C under bright light, and photographed 60 min later to quantify how many miracidia had hatched from the eggs. Representative micrographs of eggs during the hatching process 1 h after incubation in water are presented in Fig. 5, panel A. More specifically, soaking in dsRNAs targeting *Sm*LAP1, *Sm*LAP2, or both together, markedly inhibited the hatching process (Fig. 5A, panels C–E), quantified as the ratio between hatched eggs over total egg number. In contrast, the number of empty shells found in the control without treatment or in the irrelevant dsRNA treated group was significantly higher than in the other treated groups (Fig. 5A, panels A and B). Bestatin blocked hatching to similar levels of the dsRNA targeting the LAPs (Fig. 5A, panel F). Specifically, bestatin and the dsRNAs targeting LAPs inhibited hatching by ~80% compared to the control treatment groups and the group with the irrelevant dsRNA Luc (Fig. 5B). ANOVA demonstrated significant differences among the groups ($P < 0.01$); moreover, significantly fewer eggs hatched in each of the dsLAP groups and the bestatin group compared with the controls ($P < 0.01$). The inhibition experiments were repeated twice, with similar results (not shown).

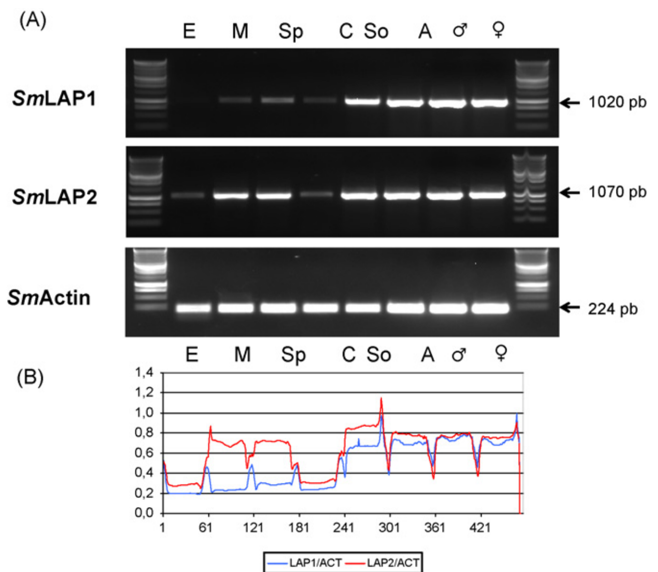

Fig. 4. Developmental stage specific expression of leucine aminopeptidases (LAP) in *Schistosoma mansoni*. (Panel A) cDNAs from eggs (E), miracidia (M), sporocysts (Sp), cercariae (C), schistosomules (So), mix sex adults (A), male adults (♂) and female adults (♀) were employed as templates for PCRs using primers specific for the *Sm*LAP1, *Sm*LAP2 and *S. mansoni* actin genes. (Panel B) Densitometric measurements (arbitrary units) across the different bands and relative levels of *Sm*LAP1 (blue) and *Sm*LAP2 (red) compared with levels for actin presented in panel A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

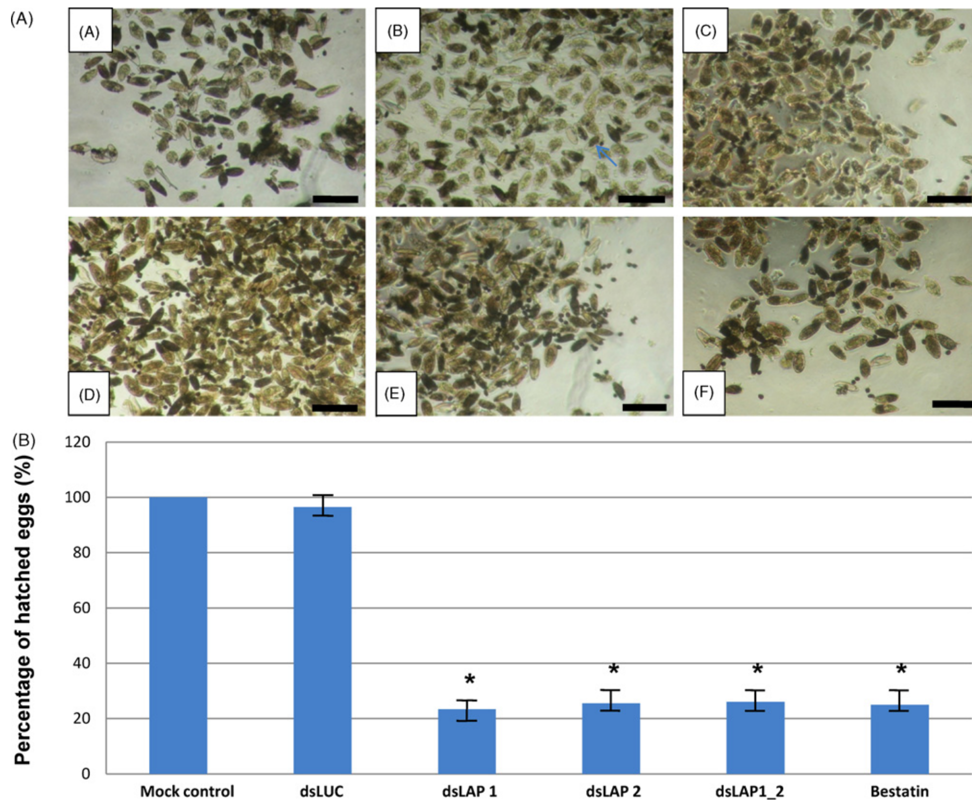

Fig. 5. Knockdown of egg hatching by RNA interference in *Schistosoma mansoni*. Hatching of eggs was blocked by dsRNA against *Sm*LAP1, *Sm*LAP2, both together, and bestatin. (A) Representative micrographs of eggs during the hatching process, 1 h after incubation in water. A: mock control, B: dsLUC treated eggs, C: dsLAP1 treated eggs, D: dsLAP2 treated eggs, E: eggs treated with dsLAP1 and dsLAP2, F: bestatin treated eggs. Bar, 200 μm. (B) Percentage of hatched eggs: 100%, control eggs. Statistically significant differences were evident among the dsLAP1 group, dsLAP2 group, dsLAP1 + dsLAP2 group, bestatin group and the controls mock and irrelevant dsRNA. The bars indicate standard deviation of mean for two experiments, and asterisks indicate significant differences ($P < 0.01$).

3.4. Transcript levels and LAP activity in eggs knocked down by RNAi

To corroborate the egg-hatch data, we investigated leucine aminopeptidase transcription and translation in RNAi-treated and non-treated eggs. Total RNA isolated from representatives of the control and experimental treated groups was 10-fold diluted serially and these dilutions were employed as the template for RT-PCR. As illustrated in Fig. 6B, the inhibitory effect mediated by the dsRNA was specific for *Sm*LAP1 and *Sm*LAP2, since transcripts for schistosome actin (*Sm*Actin) appeared to be unaffected by dsLAP1, and dsLAP2 treatment, and demonstrated that RNAi successfully mediated blocking of LAP transcription. We observed that expression of *Sm*LAP1 was elevated in the experiment presented in Fig. 6 in comparison to the study described in Fig. 4. Whereas the reason for these differences in expression in these two discrete trials was not clear, within each experiment there was consistent evidence of specific RNAi-mediated knockdown of *Sm*LAP1.

We then assessed enzyme activity in soluble extracts of the eggs by employing the LAP-diagnostic peptide H-Leu-NHMec as the substrate, although it should be noted that this substrate would not distinguish between the two enzymes. LAP activity in eggs harvested 7 days after exposure to the dsRNAs was reduced in *Sm*LAP-dsRNA treated eggs with relative fluorescence units (RLUs)/μg schistosome protein of 14,585 RLUs/μg for the mock-treatment control, 20,841 RLUs/μg for the dsRNA irrelevant control (dsLUC), 8939 RLUs/μg for the dsLAP1 treated group, 9355 RLUs/μg for the dsLAP2 treated group, and 11,118 RLUs/μg for the dsLAP1 and dsLAP2 treated group (ANOVA, $F = 149$, df = 9, $P < 0.01$) between the dsLAP treated groups and both controls). The extracts of *Sm*LAP-dsLAP1 treated eggs exhibited only 42% as much activity as the dsRNA irrelevant control group ($P < 0.01$); similarly, while extracts of *Sm*LAP-dsLAP2-treated eggs exhibited only 44% as much activity as the control dsLUC treated eggs ($P < 0.01$) (Fig. 6A). The effect at RNA level in the irrelevant control is accompanied by an increase in LAP enzyme activity as indicated in Fig. 6A. A similar effect has been seen in other dsRNA silencing experiments by our group and others at RNA or protein levels [31–33]. Furthermore, this effect is clearly seen only when a mock group is included in addition to an irrelevant control, but not when the irrelevant is the only control present as happens usually in several reports.

Fig. 6A and B revealed a discrepancy with respect to the semi-quantitative RT-PCR and the enzyme assay. In the eggs treated with both *Sm*LAP1 and *Sm*LAP2 dsRNAs there was no product in RT-PCR compared to either *Sm*LAP1 or *Sm*LAP2 treatments alone. In contrast, when the enzyme activity was assayed, eggs treated with both *Sm*LAP1 and *Sm*LAP2 dsRNAs showed higher activity than the singly treated samples. One explanation of these contrasting findings is that H-Leu-NHMec may have been hydrolyzed by other, as yet uncharacterized, aminopeptidases or other proteases in the egg extracts.

4. Discussion

Leucyl aminopeptidases are hexameric metalloproteases found in archaea, bacteria and eukaryotes where they perform diverse physiological functions, including protein turnover, redox status, MHC antigen presentation, site specific recombination, bacterial transcription, and pathogenesis of cataract formation and other pathologies. In higher eukaryotes they are involved in post-proteasome processing of peptides, generation and/or elimination

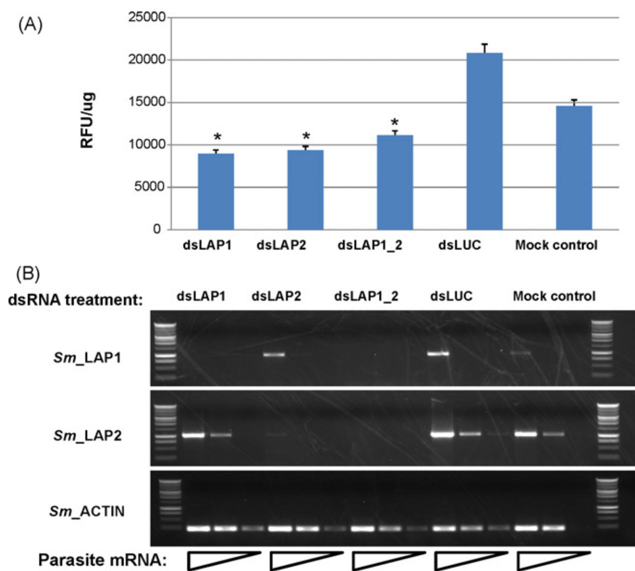

Fig. 6. RNAi targeting schistosome leucine aminopeptidases results in statistically significant knockdown of protease activities and mRNA levels. (Panel A) Leucine aminopeptidase (LAP) activity assay performed using the diagnostic substrate, L-leucine-7-amido-4-methylcoumarin hydroxide. LAP activity in relative fluorescence units per microgram (RLU/μg) of soluble schistosome protein presented on *Y*-axis. Groups of eggs were treated with double stranded RNA targeting *Sm*LAP1, *Sm*LAP2, both, firefly luciferase, and not treated with dsRNA (mock control). (Panel B) Semi-quantitative RT-PCR using LAP specific primers and actin as an internal control. Schistosome egg cDNAs were synthesized from serial dilutions of total RNAs (100, 10 and 1 ng) isolated from treatment group of eggs, soaked in doubled strand RNA targeting *Sm*LAP1, *Sm*LAP2, both, firefly luciferase, and not treated with dsRNA (mock control). The bars show the standard deviations for two experiments. Asterisks indicate significant differences ($P < 0.01$) between treated groups and both controls groups. The difference between the mock control and the irrelevant dsRNA control also was significant ($P < 0.01$).

of active biopeptides, and regulation of signal transduction pathways [34,35].

Dresden and co-workers [15,26,27] demonstrated that LAP like activity is present in schistosome eggs and was associated with the hatching of miracidia from eggs, since this activity was detected in hatching fluid and emergence of miracidia was inhibited by bestatin [18,36,37]. Bestatin, the dipeptide *N*-((2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl butanyl) L-leucine is an antibiotic of microbial origin with broad-spectrum activity against leucine aminopeptidases, aminopeptidases B and N and other metalloproteases [38–40]. Our results supports the findings of Dresden and co-workers, and indeed have extended them by employing RNAi in parallel with bestatin to definitively show that blocking schistosome LAP activity prevents the hatching process. Thus, soaking schistosome eggs in dsRNA targeting *Sm*LAP1, *Sm*LAP2 or both of them markedly blocked egg hatching and emergence of miracidia. The magnitude of egg-hatch inhibition was similar in range and amplitude to that obtained with bestatin [18]. The visible phenotype was accompanied by an almost complete ablation of the corresponding mRNA, and a marked diminution of LAP activity in soluble extracts of eggs against the diagnostic substrate H-Leu-NHMec. The disappearance of the mRNA indicated that the effect was due to mRNA degradation as expected for RNAi. Furthermore, although knockdown of either *Sm*LAP1 or *Sm*LAP2 elicited almost identical reductions in hatching, these were specific since the RNAi selectively reduced expression of the targeted LAP with no effect on the other aminopeptidase. The RNAi effects indicated that the LAPs either play a direct role in hatching or they provide metabolites essential for the process.

Although leucine aminopeptidase activity had been known from the egg and adult stages of both *S. mansoni* and *S. japonicum* [17,19], there was little information on the genes that encoded this activity. The availability of the draft genome of *S. mansoni*, the current draft of which has >7-fold genome coverage, facilitated targeted bioinformatics searches, from which we recognized a second M17 family leucine aminopeptidase gene, discrete from the original cDNA reported by McCarthy et al. [19]. A similar search in the genome draft of *S. japonicum* predicted two related genes, orthologous to those found in *S. mansoni*. The genomic structure of both schistosome LAP1 and LAP2 genes was similar, including the length, conservation of intron positions, and number of exons. The difference between LAP1 and LAP2 exon count, 10 and 12 exons respectively, could be accounted for by a split in two of the fifth and sixth exons of LAP1 in two exons each in LAP2. These data, the sequence similarity between them and the phylogenetic analysis support the hypothesis that they emerged by a duplication event. Because the genomic structure was conserved between the two schistosome species, it seems likely that the duplication occurred before speciation of Asian (*japonicum*) and African (*mansoni*) and schistosomes [41]. Whereas the two genes are closely related, the central position of both genes in the corresponding scaffolds of the *S. mansoni* genome draft and the differences in the surrounding genes make it unlikely that they are located in tandem in the genome. Furthermore, the phylogenetic analysis confirmed that trematode LAPs are dissimilar to the mammalian homologues which constitute separate clades (Fig. 3) [25].

The *S. mansoni* LAPs exhibited differences, compared to each other, in developmental expression as measured directly by RT-PCR, which was supported by the presence of stage specific ESTs. While both enzymes have similar strong expression in the mammalian stages, only LAP2 was expressed strongly in miracidia and sporocysts. Only modest expression of both LAPs was observed in cercariae, a situation that might be related to the anti-sense transcripts in ESTs from cercariae, and consistent with a general reduction of gene expression in this developmental stage [30]. Expression levels of the LAPs in the eggs were not high as indicated by RT-PCR signals despite their critical importance in hatching [14,18,36,37]. *Sm*LAP1 occurs in the gut of the adult fluke where it has been ascribed a role in terminal digestion of hemoglobin and other ingested host proteins [19].

LAPs have been characterized in several other trematodes, including *Paragonimus westermani* [42] and *F. hepatica* [25,43], although a role in egg hatching has not been determined definitely in these flukes. However, the relevance of LAP as a potential target for control of trematodiases at large was highlighted by experimental immunization of sheep with native *F. hepatica* LAP, which delivered 89% protection against challenge infection along with reduced disease [43,44]. Moreover, recombinant *F. hepatica* LAP tested as a vaccine antigen in rabbits elicited a strong antibody response and 78% reduction in worm burden after challenge [25]. RNAi directed towards LAP of *F. hepatica* induced specific mRNA degradation in juvenile *F. hepatica*, although morphological phenotypes were not apparent [33]. LAPs appear to play important roles in development and fecundity in other invertebrates. In the gastrointestinal nematode *Haemonchus contortus*, LAP has been associated with molting and egg hatching since both processes can be blocked with bestatin and induced by Zn^{2+} [45–49]. LAP activity is present in other parasitic nematodes [50,51]. In the free living *C. elegans* null mutants of LAP1 show reduced growth and retarded egg-laying [52]. Inhibition by RNAi of the cytosolic LAP of the tick *Haemaphysalis longicornis* resulted in a delay in oviposition and reduction in egg biomass [53].

The present investigation confirmed long held views that exopeptidase activity ascribable to leucine aminopeptidase was critical to the hatching of schistosome eggs. We have now definitively shown – using RNA interference targeting two schistosome LAP genes – that schistosome LAP appears to be necessary for hatching of the schistosome egg. Schistosome eggs are fully embry-

onated as when passed in the feces or urine and, as a consequence, miracidia can hatch immediately from the egg in the environment [54]. Osmotic changes that occur as the egg enters freshwater are the primary cue for emergence of the miracidia from the eggshell [55–57]. Downstream of the osmotic changes, physiological and molecular events leading to hatching include calcium ion fluxes and the activity of LAP [56–58]. Indeed, release of LAP immediately follows activation of *S. mansoni* eggs [18,57]. One possibility is that schistosome LAPs participate in the digestion of components of the inner membrane of the egg in the hatching process [54]. The identity of the natural substrate(s) for LAP from eggs has not been reported. However, potential roles for LAP include scission of the outer envelope-shell boundary, autolysis of the inner envelope, or/and degradation of proteins in the lacunae [54].

It has been reported that TGF-β signaling plays a major role in the embryogenesis of *S. mansoni*; RNAi-mediated knockdown of SmInAct expression in eggs aborts egg development [59]. Our data suggest that LAP acts at a later stage of development since the effect is seen on the emergence of fully differentiated miracidia. Immunological responses to eggs trapped in the livers and other organs of infected persons represent the root cause of the pathology of schistosomiasis. Accordingly, a deeper understanding of the role of LAP in development of eggs as well as in hatching, as of the receptors and other components of the schistosome egg [16,58] can be expected to have broad implications for novel treatment and control of this significant neglected tropical disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2009.05.002.

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