RNA interference in schistosomes: machinery and methodology

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SUMMARY

RNA interference (RNAi) is a potent gene silencing process that is playing an increasingly important role in investigations of gene function in schistosomes. Here we review what is known about the process in these parasites and provide an update on the methodology and machinery of RNAi. Data are presented to demonstrate that: (1) not all schistosome genes can be suppressed to the same extent, using the methods employed here; (2) while there is variation in the level of suppression achieved for one target gene (SmAP) in adult parasites, all individuals exhibit robust (>80%) suppression; (3) short interfering RNAs (siRNAs) can effect suppression when delivered by soaking (and not just via electroporation, as reported previously); (4) Male/female adult pairs need not be separated prior to siRNA delivery by electroporation for effective gene suppression in both genders and (5) electroporation of siRNAs in medium is as efficient as in commercial electroporation buffer. Regarding the machinery of RNAi in schistosomes, a homologue of the *C. elegans* multi-membrane spanning, RNA importing protein SID-1 is identified *in silico*. The gene encoding this protein contains 21 exons and spans over 50 kb to potentially encode a 115,556 Mr protein (SmSID-1). These analyses, and a review of the literature, permit us to derive and present here a draft of potential RNAi pathways in schistosomes.

Key words: Gene suppression, Dicer, RISC, siRNA.

INTRODUCTION

RNA interference (RNAi) can be defined as a gene silencing process that is triggered by double-stranded RNA (dsRNA). By delivering gene-specific dsRNA to a competent cell, the RNAi pathway is engaged leading to suppression of target gene expression. As a molecular tool for analyzing gene function, RNAi has permeated all fields of eukaryote biology. This is particularly so in organisms such as schistosomes that are not amenable to classical genetic approaches. The technology has already provided valuable insight into diverse areas of schistosome biology including digestive pathways (Correnti *et al*. 2005; Delcroix *et al*. 2006; Krautz-Peterson and Skelly, 2008*b*; Morales *et al*. 2008), water movement (Faghiri and Skelly, 2009) and development (Dinguirard and Yoshino, 2006; Freitas *et al*. 2007; Pereira *et al*. 2008; Rinaldi *et al*. 2009). Given the abundance of schistosome transcriptome and genome sequences now available (Hoffmann and Dunne, 2003; Oliveira, 2007; van Hellemond *et al*. 2007; Verjovski-Almeida *et al*. 2003; Wilson *et al*. 2007; Berriman *et al*. 2009; Liu *et al*. 2009) RNAi technology has the potential to revolutionize investigation of the roles and importance of the genes of this globally significant parasite.

In addition to acting as a key tool for molecular studies, RNAi plays an important role in regulating normal gene expression. Endogenous short non-coding RNA substrates such as microRNAs (miRNAs) engage the RNAi pathway leading to gene silencing either through mRNA destruction or the suppression of protein synthesis (Chua *et al*. 2009; Liu *et al*. 2008). Five miRNAs have been identified in *S. japonicum* (Xue *et al*. 2008).

A simple model for RNAi is based on two steps, each centering on a ribonuclease enzyme. First, the trigger RNA (either dsRNA or miRNA primary transcript) is processed into a short inhibitory RNA (siRNA) by RNaseIII enzymes called Dicer and Drosha, with dsRNA binding domain (dsRBD) proteins acting as cofactors. In the second step, siRNAs are loaded into the effector protein complex called the RNA-induced silencing complex (RISC) (Pratt and Macrae, 2009). The siRNA is unwound in a strand-specific manner during RISC assembly. This single-stranded siRNA locates its cognate mRNA target by Watson–Crick base pairing.

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Gene silencing results from the nucleolytic degradation of the targeted mRNA by the RNaseH enzyme Argonaute (also called Slicer) (Pratt and Macrae, 2009). If the siRNA/mRNA duplex contains mismatches at the scissile site, as is often the case for miRNAs, the mRNA is not cleaved and gene silencing results from translational inhibition (Siomi and Siomi, 2009). In addition, small RNAs targeted to gene promoters can control gene expression not only through transcriptional suppression but also, in some cases, by activation (Janowski *et al*. 2007; Morris *et al*. 2008).

From gene suppression work, it is clear that schistosomes possess the molecular machinery that mediates RNAi. Furthermore, a number of potential protein participants in the RNAi pathway in schistosomes have been identified from analysis of transcriptome and genomic sequences. For instance, a single Dicer homologue (SmDicer) has been characterized from *S. mansoni* (Krautz-Peterson and Skelly, 2008*a*). At an estimated 2641 amino acids in size, it is the largest Dicer protein yet described. SmDicer contains all domains that are characteristic of metazoan dicers including an amino terminal helicase domain, DUF283, a PAZ domain, two RNAse III domains and an RNA binding domain (Krautz-Peterson and Skelly, 2008*a*). A Drosha gene has been identified in the *S. mansoni* genome which, through alternative splicing, is predicted to generate 2 protein homologues (Gomes *et al*. 2009). These proteins, SmDrosha1 & 2, comprise 1531 and 1577 amino acids, respectively, and possess conserved endonuclease domains and double-stranded RNA binding motifs (Gomes *et al*. 2009). Four Argonaute homologues have been identified (SmAgo1–4) with SmAgo 3 and 4 being splice variants (Gomes *et al*. 2009). Each SmAgo is ~900 amino acids in size and all possess conserved PAZ and Piwi domains (Gomes *et al*. 2009). Finally, analysis of the schistosome transcriptome has revealed several expressed sequence tags with homology to components of the RNAi pathway of other organisms (Verjovski-Almeida *et al*. 2003). These analyses permit us to derive here a draft of potential RNAi pathways in schistosomes.

Beyond the basic pathway leading to mRNA cleavage or translational inhibition, additional molecular components bear upon how efficient the process is in different organisms or tissues. Foremost among these is the ability of the RNAi effect to spread from the point of first engagement with dsRNA. In the nematode *Caenorhabditis elegans* RNAi is systemic (Whangbo and Hunter, 2008). The effect can spread from a site of RNA introduction to suppress a targeted gene at a different site or throughout the animal and, in some cases, in the animal's progeny (Whangbo and Hunter, 2008). Furthermore, RNAi can take effect when worms are simply soaked in solutions of dsRNA or if they are fed bacteria that express dsRNA (Conte and Mello, 2003). It is clear therefore that dsRNA can enter *C. elegans* from the environment and spread internally. In *C. elegans*, mutants that were defective for spreading of the RNAi effect were identified (and designated systemic RNAi defective (Sid)) (Winston *et al*. 2002). This led to the identification of the *sid-1* gene that encodes a multiple membrane-spanning protein (Winston *et al*. 2002). This protein has been shown to be required for uptake of dsRNA in *C. elegans* and is expressed in all cells sensitive to systemic RNAi (Winston *et al*. 2002; Feinberg and Hunter, 2003). Furthermore, expression of SID-1 in *Drosophila* cells results in marked potentiation of dsRNA soaking-induced RNAi by facilitating dsRNA uptake from the growth medium (Shih *et al*. 2009). Current data suggest that SID-1 functions as an energy-independent channel capable of importing dsRNA into cells (Shih *et al*. 2009). RNAi triggered by environmental exposure to dsRNA has also been documented in several other invertebrates including schistosomes (Skelly *et al*. 2003; Skelly, 2006; Ndegwa *et al*. 2007). This suggests that these parasites also possess mechanisms to transport dsRNA into and between cells. An examination of the *S. mansoni* sequence database revealed a clear SID-1 homologue which we describe in this work.

To promote the use of RNAi in schistosome research, we also present recent work on the methodology and efficiency of RNAi in these parasites including data on the variation observed in the level of gene suppression for different target genes in schistosomes and for the same target gene in different individual worms.

MATERIALS AND METHODS

Parasites

The Puerto Rican strain of *Schistosoma mansoni* was maintained at the Biomedical Research Institute, Rockville, MD, and obtained from Dr. Fred Lewis. Cercariae were obtained from infected *Biomphalaria glabrata* and isolated parasite bodies were prepared as described previously (Skelly *et al*. 2003). Parasites were cultured in complete RPMI culture medium which is RPMI medium supplemented with 10 mM Hepes, 2 mM glutamate, 5% foetal calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C, in an atmosphere of 5% CO_2. Adult male and female parasites were recovered by perfusion from BALB/c mice that were infected with 125 cercariae, 7 weeks previously. Parasites were subjected to RNAi after 6–8 days in culture.

Preparation and delivery of dsRNA

Small inhibitory RNAs (siRNAs) targeting specific schistosome genes were synthesized commercially by

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Table 1. List of siRNAs, primers and probes. A. siRNA designations and sequences. SmAQP, *S. mansoni* aquaporin; SmAP, *S. mansoni* alkaline phosphatase; SPRM1hc, *S. mansoni* permease heavy chain. B. Oligonucleotide sequences of primers used to generate an SPRM1hc long double stranded RNA *in vitro*. The underlined sequences are promoters for T7 (top) and T3 (bottom) RNA polymerase. C. Gene specific primer sets and reporter probes labeled with 6-carboxyfluorescein (FAM) used in quantitative real time PCR TaqMan assays

	Gene	siRNA	Target sequence
A.	SmAQP	#1	CATGCTCATGGAACATTCATTTCAG
	SmAQP	#2	CTGTAATCCAGCTGTAACATTGGCA
	SmAP	#1	CCACAAGCATGTTCTCTTACATACA
	SmAP	#2	CAACTACACAGCAGAATCATTGGTG
	SmAP	#3	CAACTACTAACTAACGCTTCTCATG
	SmAP	#4	GAAATCAGCAGATGAGAGATTTAAT
	SPRM11hc	#1	GAAGGAGTTGGGTTCGAAATGGTTT
	SPRM1hc	#2	GGATCAATCTGGAGCTGGTTTCTCG
	SPRM1hc	#3	GAATCAGAAAGGTTGTCAATGAAGT
	Negative control		CTTCCTCTCTTTCTCTCCCTTGTGA
B.	SPRM1hcT7	GGTAATACGACTCACTATAGGATGAGTTCGAGCGGTACCAATGG	
	SPRM1hcT3	AATTAACCCTCACTAAAGGCGTTTAGGTATATCTGCGATGACGGG	
	Gene	Primer name	Sequence
C.	SmAQP	Smaqua-aqu1F	GTGATTTAGGACCCAGACTCATGAT
	SmAQP	Smaqua-aqu1R	GTTTGCTCCACTGAATGCTTTGTT
		probe Smaqua	FAM-ACCCCAACCGAATATAAA
	SmAP	Taq-siteF,	GCCATCCGACAAGGAATATAAGTGT
	SmAP	Taq-siteR,	GGTCCATTGAAAAAGGAGGATATGAGA
		probe SmAP	FAM-ATCTCCTTTTGCAGTATTATC
	SPRM1hc	3PM-HCP3F	GCTTTGGCTTCCACGTTTCTG
	SPRM1hc	3PM-HCP3R	CGTTTCCTCATTTAACTCCGAACCA
		probeSPRM1hc	FAM-CTTCCAGGCACTTCTC
	α tubulin	Tubulina2-F	GGTTGACAACGAGGCCATTTATG
		Tubulina2-R	TGTGTAGGTTGGACGCTCTATATCT
		probeTubulina	FAM- ATATTTGTCGACGGAAT

Integrated DNA Technologies (IDT, Coralville, IA) with the help of the on-line IDT RNAi Design Tool (https://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx). Table 1A lists the siRNAs used in this work. The primers shown in Table 1B were used in a PCR with *S. mansoni* adult cDNA to generate a 943 bp fragment of the SPRM1hc coding DNA with flanking T7 and T3 polymerase promoter sites (underlined). This purified PCR fragment was then used to generate RNA by *in vitro* transcription with T7 and T3 RNA polymerase (Megascript kits, Ambion). Equimolar amounts of complimentary, single stranded RNAs were mixed and heated to 68 °C for 15 min for annealing. A negative control, long dsRNA was generated in a similar manner using a 990 bp PCR fragment derived from a yeast expression vector, as previously described (Skelly *et al*. 2003).

To deliver the siRNAs, parasites (1000 schistosomula or 8 adults/group), in 100 μl electroporation buffer (Ambion, TX), or RPMI medium (as indicated), containing 2·5 μg siRNA (unless otherwise specified), or 20 μg long dsRNA, were electroporated in a 4 mm cuvette by applying a square wave with a single 20 ms impulse, at 125 V and at room temperature, as described (Correnti *et al*. 2005). Parasites were then transferred to 1 ml complete RPMI. After overnight culture, medium was replaced with 2 ml of fresh complete RPMI culture medium. Suppression was monitored by quantitative real-time PCR (qRT-PCR) 2 days after siRNA treatment.

Gene expression analysis

Gene expression was monitored by qRT-PCR. RNA was first extracted from the parasites using the Trizol method (Invitrogen, CA) following the manufacturer's instructions. Any residual DNA remaining in the RNA preparations was removed by DNase digestion using a TurboDNAse kit (Ambion, TX). cDNA was synthesized using 1 μg RNA and an oligo $(dT)_{20}$ primer and Superscript reverse transcriptase (Invitrogen, CA). Quantitative real-time PCR was performed using custom TaqMan Assays with primer sets and reporter probes labeled with 6-carboxyfluorescein (FAM), obtained from Applied Biosystems (Foster City, CA). Table 1C lists the primers & probes used in this work.

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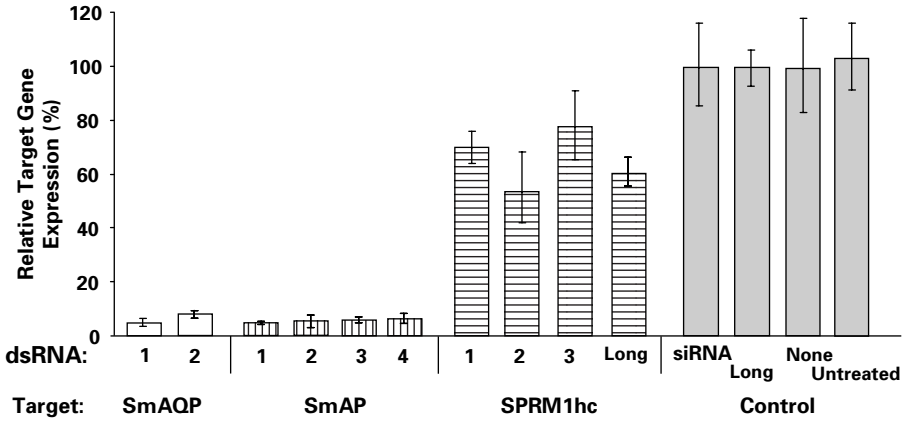


Fig. 1. Relative expression of 3 *S. mansoni* target genes, SmAQP (aquaporin), SmAP (alkaline phosphatase) and SPRM1hc (amino acid permease, heavy chain), following RNAi treatment of schistosomula using electroporation, as determined by quantitative real-time PCR. Numbers refer to different siRNAs targeting each gene, delivered at 2·5 μg (for SmAQP and SmAP) and at 10 μg (for SPRM1hc). 'Long' refers to long dsRNA, delivered at 20 μg. The controls (grey bars) include parasites treated with an irrelevant siRNA (10 μg, set at 100%), an irrelevant long dsRNA or no dsRNA. The final control (far right) represents parasites that were not exposed to dsRNA and were not electroporated. Sequences of all siRNAs are shown in Table 1.

Each real-time TaqMan PCR was performed using cDNA equivalent to 10 ng total parasite RNA according to the manufacturer's universal conditions PCR protocol, in a final volume of 25 μl. All samples were run in triplicate and underwent 45 amplification cycles on a 7500 ABI PRISM® Sequence Detection System Instrument. For relative quantification, the $\Delta\Delta$Ct method was employed, using alpha tubulin as the endogenous standard for each sample. Results obtained from parasites treated with irrelevant dsRNA were used for calibration (Livak and Schmittgen, 2001). For graphical representation, the $\Delta\Delta$Ct values were normalized to controls and expressed as percent difference (Livak and Schmittgen, 2001).

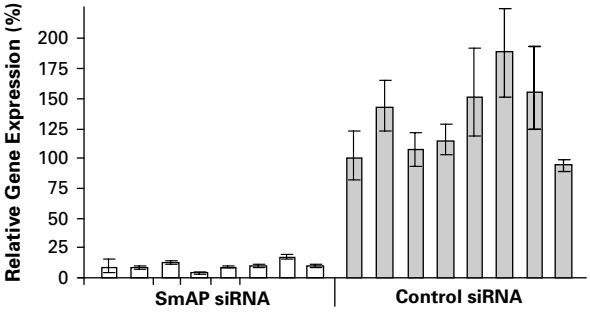


Fig. 2. Relative expression of the SmAP gene in 8 individual adult female parasites treated via electroporation with SmAP siRNA (white) versus 8 individual parasites treated with an irrelevant control siRNA (grey). The value achieved by control individual 1 is arbitrarily set at 100%.

RESULTS

Fig. 1 shows the expression level of 3 different target genes (encoding an *S. mansoni* aquaporin (SmAQP), alkaline phosphatase (SmAP) and an amino acid permease heavy chain (SPRM1hc)) following RNAi treatment of schistosomula using electroporation (Faghiri and Skelly, 2009; Krautz-Peterson *et al.* 2007*a*; Ndegwa *et al.* 2007). SmAQP was targeted with 2 distinct siRNAs, SmAP was targeted with 4 distinct siRNAs and SPRM1hc was targeted with 3 distinct siRNAs (Fig. 1). Sequences of each siRNA are given in Table 1. The levels of suppression achieved with each siRNA for its specific target are comparable. Both siRNAs targeting SmAQP suppress the expression of this gene >90%. Likewise all 4 of the siRNAs targeting SmAP yield >90% gene suppression (Fig. 1). However, none of the 3 different siRNAs targeting SPRM1hc (here used at a four fold higher concentration than the siRNAs targeting SmAQP and SmAP) gives rise to more than 50% suppression of this target gene expression. Delivering long dsRNA (at the relatively high dose of 20 μg) targeting SPRM1hc, does not improve on the level of suppression achieved using siRNAs. The controls (Fig. 1, grey) include parasites treated with an irrelevant siRNA (10 μg, set at 100% in Fig. 1), the irrelevant long dsRNA (20 μg) or no dsRNA. The final control (Fig. 1, far right) represents parasites that were not exposed to dsRNA and were not electroporated. These data demonstrate that not all schistosome genes can be suppressed to the same extent, using the methods employed here.

Fig. 2 examines the variation in the level of suppression achieved in individual adult female parasites following RNAi treatment using SmAP siRNA #1 by electroporation. The level of expression of the SmAP gene in 8 individual adult female worms treated with control siRNA (Fig. 2, grey bars) is

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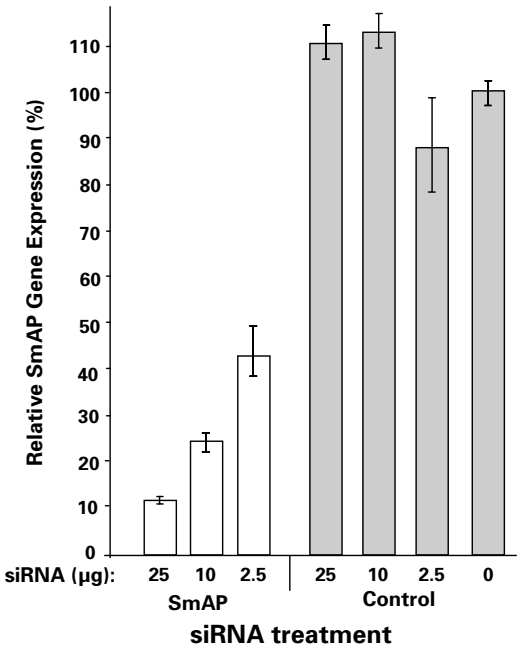


Fig. 3. Relative expression of the SmAP gene in parasites treated with different concentrations of SmAP siRNA by soaking (white) versus controls (grey).

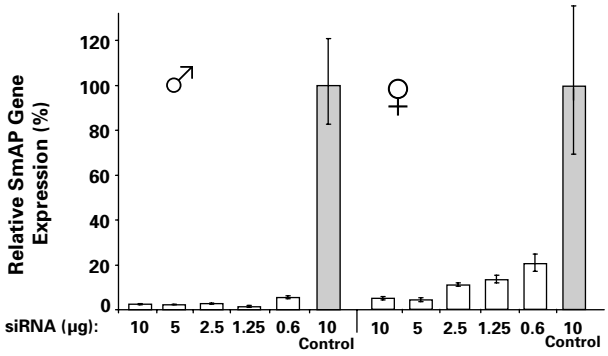


Fig. 4. Relative expression of the SmAP gene in male (left) and female (right) parasites treated while *in copula* with different concentrations of SmAP siRNA by electroporation (white) versus controls (grey).

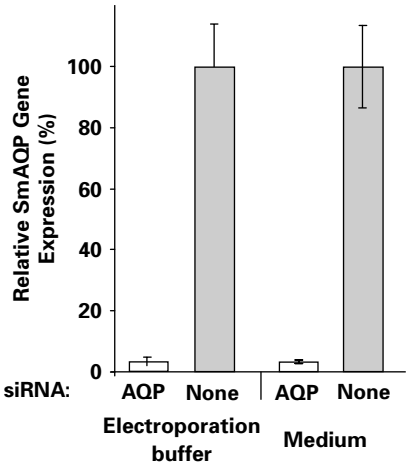


Fig. 5. Relative expression of the SmAQP gene in untreated control parasites (grey) and parasites treated with SmAQP siRNA (white) by electroporation in commercial electroporation buffer (left) versus in RPMI medium.

compared with SmAP gene expression in 8 parasites treated with SmAP siRNA (Fig. 2, white bars). It is clear that there is a large variation in relative SmAP gene expression (as much as two fold) between different individual control worms. While there are likewise differences in the levels of gene suppression achieved in individual worms treated with SmAP siRNA, it is clear that all SmAP siRNA treated worms exhibit robust (>80%) gene suppression.

Fig. 3 shows the level of suppression of the SmAP gene that is achieved after adult female parasites are soaked in siRNA. Greater suppression is achieved following exposure to higher levels of siRNA and a clear dose response is recorded. However the level of suppression achieved is lower than that seen when siRNAs are delivered by electroporation; soaking parasites in 2·5 μg siRNA leads to ~60% SmAP gene suppression (Fig. 3), whereas electroporating the parasites with this amount routinely yields >80% suppression (Fig. 2).

Male/female parasite pairs were electroporated with increasing amounts of siRNA while the parasites were *in copula*. Fig. 4 shows the level of SmAP gene suppression achieved in separated male and female parasites 8 days later. It is clear that robust suppression (>80%) is seen in all cases. However, at lower doses of siRNA (e.g. 1·25 and 0·6 μg) the level of gene suppression, while still substantial, declines in females.

Fig. 5 compares the level of SmAQP gene suppression achieved following schistosomula electroporation with siRNA either in electroporation buffer (Fig. 5, left) or in RPMI medium (Fig. 5, right). Comparable levels of suppression (both ~95%) are seen using both approaches.

An examination of the predicted *S. mansoni* protein database at *S. mansoni* genedb (http://www.genedb.org/genedb/smansoni/) for homologues of the *C. elegans* RNA channel SID-1 revealed a match coded on sequence Smp_152020. This sequence potentially encodes a 1018 amino acid protein, here designated SmSID-1, with a predicted size of 115 556 and pI of 8·3. The SmSID-1 sequence is shown in Fig. 6A along with an alignment of this protein with diverse members of this protein family generated using ClustalW. Conserved motifs are shaded. SmSID-1 exhibits high sequence similarity (81%) with its counterpart from *S. japonicum* (Genbank accession number BAH22347), moderate similarity (40%) with a human homologue (NP_060169) and lower similarity (19%) with *C. elegans* SID-1 (AF478687) (Fig. 6A). Amino acids 1–21 are predicted to constitute a signal peptide

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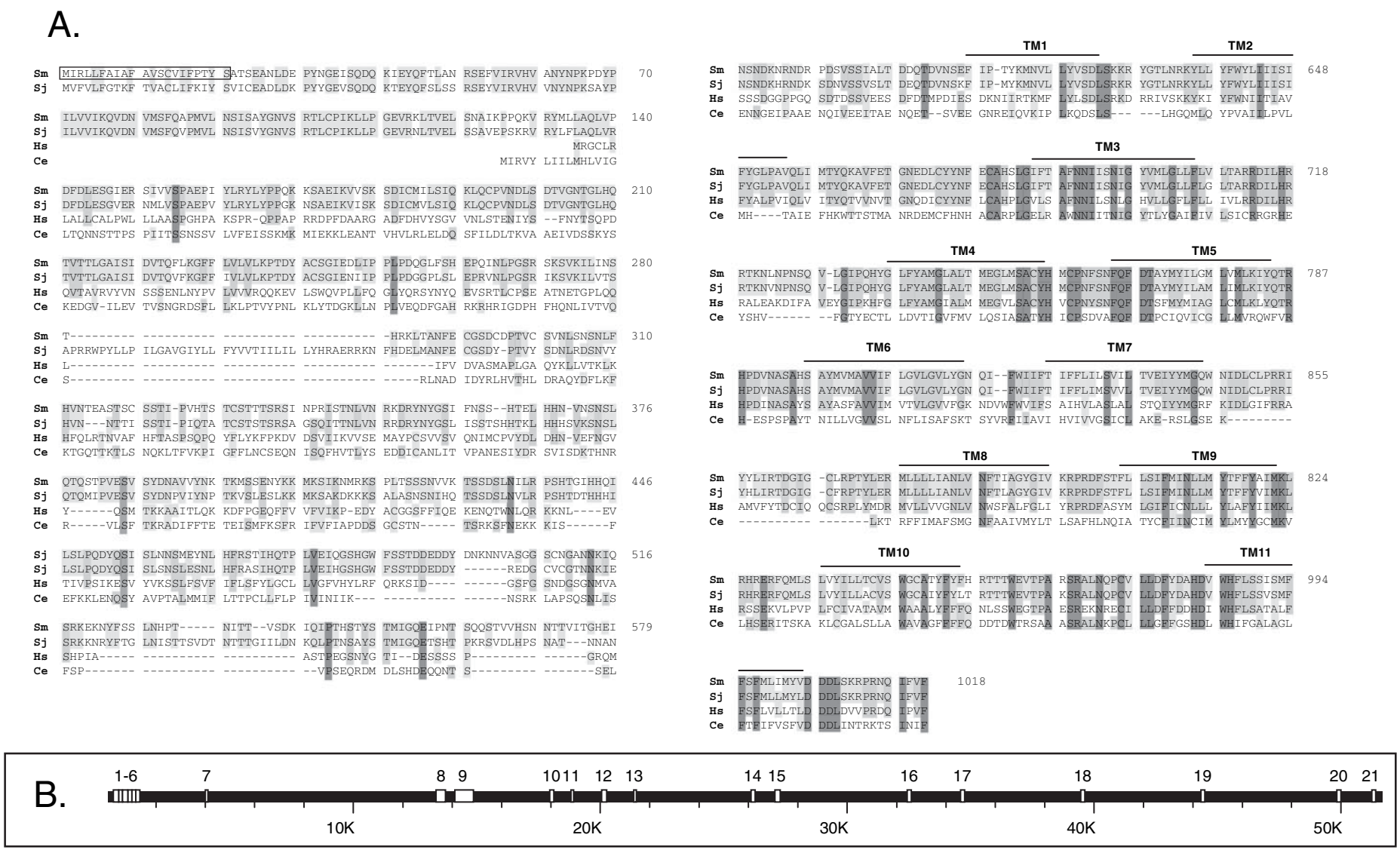


Fig. 6. Alignment of SmSID-1 with other members of the SID protein family. Sm, *Schistosoma mansoni*; Sj, *Schistosoma japonicum* (GenBank accession number BAH22347); Hs, *Homo sapiens* (NP_060169); Ce, *C. elegans* SID-1 (AF478687). Predicted transmembrane domains are indicated, TM1-11, and a predicted signal sequence is boxed. Shading highlights similarity between family members. B. The *S. mansoni* SID-1 (SmSID-1) gene. Exons 1 to 21 are indicated by white boxes 'K' indicates kilobase pairs.

([1]MIRLLFAIAFAVSCVIFPTYS[21]) at http://www.cbs.dtu.dk/services/SignalP-3.0/ and this is boxed in Fig. 6A. In common with the other members of this protein family, SmSID-1 is predicted to contain 11 transmembrane domains (TM1-11, Fig. 6A) and to possess a large (here >600 amino acid) extracellular N-terminal domain.

The entire SmSID-1 gene was identified on contig Smp_scaff000150 at *S. mansoni* genedb. As illustrated in Fig. 6B, the gene is >50 kb and consists of 21 exons that range in size from 690 bp (exon 9) to 44 bp (exon 13). Introns range in size from 9284 bp (intron 7) to 43 bp (intron 3). All introns possess canonical GT:AG splice donor and acceptor sites.

DISCUSSION

In this work, we show that all genes are not susceptible to RNAi to the same degree in *S. mansoni*. Techniques that lead to the efficient suppression of some target genes (here SmAQP and SmAP) lead to very modest suppression of a third target – the SPRM1hc gene. The inability to suppress efficiently the SPRM1hc gene is not likely due to its expression in cell types that are restrictive for RNAi; SPRM1hc is widely expressed in intravascular schistosomes and this is the case for the highly suppressible SmAP too (Dusanic, 1959; Halton, 1967; Krautz-Peterson *et al.* 2007*a*). The lower susceptibility of SPRM1hc to RNAi may be due to the secondary structure of its mRNA. Perhaps this mRNA is less accessible to the RISC than are other targets. To ensure that the 3 siRNAs originally designed to target SPRM1hc did not derive from particularly inaccessible regions, a 943 bp long dsRNA was generated and parasites were electroporated at a high dose (20 μg) with this RNA. This long dsRNA should be processed by SmDicer to generate an array of siRNAs. The expectation was that some of these siRNAs would be able to reach the SPRM1hc mRNA to effect suppression but this was not observed. The result with SPRM1hc is unusual. In published and unpublished work, it is the only gene target that is recalcitrant to robust RNAi, of 12 targets tested so far in this laboratory (Faghiri and Skelly, 2009; Krautz-Peterson and Skelly, 2008*b*; Ndegwa *et al.* 2007). Note that very high levels of gene suppression may not be essential for a phenotype to emerge. Depending on the importance of each gene product, a modest diminution in expression of an important gene may be detrimental for the parasites and may be informative. For instance, suppressing the gene that encodes the schistosome TGF-β homologue SmInAct leads to a modest ~40% suppression at the RNA level, nevertheless eggs produced by SmInAct dsRNA–treated female parasites fail to develop (Freitas *et al.* 2007).

Following exposure of a schistosomula population to dsRNA, the outcome for each parasite is not uniform and this is likely due to differences in dsRNA uptake and/or RNAi pathway activation and/or target gene expression in different individuals. For instance, following cathepsin B gene suppression in a population of schistosomula, the level of cathepsin B protein present in individuals within the population was assessed after 6 days by immunostaining using anti-cathepsin B antibodies. While most parasites (63%) lacked detectable protein, a sizable percentage (37%) stained clearly and some of these (5%) very brightly (Skelly *et al.* 2003). To monitor individual variability directly in gene suppression achieved following dsRNA exposure, the level of SmAP gene expression was assessed in each of eight adult worms that had been subjected, as a group, to RNAi. This work reveals some variability (up to 2 fold) in normal SmAP gene expression in control parasites as well as a remarkably robust suppression of SmAP gene expression (by 80–95%) in *each* worm tested. This high level of gene suppression in each individual is encouraging and may lead to a more uniform and informative phenotypic manifestation following gene suppression in adult parasites.

In an effort to simplify the RNAi protocol and minimize excessive handling of the parasites, which can damage them, parasite pairs were not separated before treatment. Instead, worms *in copula* were subjected to RNAi by electroporation. Eight days later the level of gene suppression achieved was assessed in now separated males and females. It is clear that both genders are highly susceptible to RNAi following this protocol and that separation of females from their male partners is not essential for efficient gene suppression. However, at the lower doses of siRNA used, females do display some diminution in the effect suggesting that at these levels their residence within the gynaecophoric canal of their male partners lessens their exposure to sufficient siRNA for maximal gene suppression.

It has been reported that dsRNA can be delivered to schistosomes by electroporation in RPMI culture medium (Morales *et al.* 2008). In work described here, the efficiency of gene suppression was compared when siRNA was delivered by electroporation to parasites in RPMI medium versus in commercial electroporation buffer (from Ambion Inc). Very high and comparable levels of suppression of the SmAQP target gene were observed in both cases. The use of medium makes each experiment more economical given its considerably lower cost versus electroporation buffer. It remains possible that differences in efficiency, following the use of medium versus electroporation buffer, may be revealed at lower concentrations of dsRNA or for different gene targets.

The preferred protocol for RNAi in schistosomes that is followed in this laboratory involves treating parasites with relatively low doses (2·5 μg) of

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gene-specific siRNA by electroporation. This routinely leads to robust target gene suppression. In work described here, the level of suppression achieved by soaking parasites in siRNA was assessed. In a *Drosophila* cell line cultured in medium containing dsRNAs, it was reported that longer dsRNAs are preferentially taken up versus siRNAs (Saleh *et al*. 2006). Here we show that schistosomes can take up siRNAs in a dose-dependent manner. However, the level of suppression achieved is substantially lower than that seen using electroporation. Approximately 55% suppression of SmAP gene expression is achieved after soaking parasites in 2·5 μg siRNA versus >80% following electroporation. Electroporation likely delivers dsRNA more efficiently into the body of the parasite. Since schistosomes can take up siRNA by soaking this suggests that the parasites in the blood stream will be amenable to RNAi if they are exposed to a sufficiently high dsRNA dose. It has previously been shown that gene expression in schistosomes can be efficiently suppressed by soaking the parasites in long dsRNA (Ndegwa *et al*. 2007; Skelly *et al*. 2003). Long dsRNA cannot be used *in vivo* to suppress gene expression in intravascular parasites since nucleases in the blood are known to rapidly degrade any introduced long dsRNAs (Aagaard and Rossi, 2007). Additionally, long dsRNAs are rapid inducers of a systemic/nonspecific interferon response when delivered *in vivo*, resulting in cell apoptosis (Aigner, 2006).This implies that siRNAs will need to be used for use with blood-dwelling schistosomes and it was recently reported that injecting siRNAs into schistosome-infected mice could suppress gene expression in parasites in the blood (Pereira *et al*. 2008). Three siRNAs targeting the parasite hypoxanthine–guanine phosphoribosyltransferase (HGPRTase) gene were injected in phosphate buffered saline intravenously into schistosome-infected mice and 6 days later, parasites were recovered. The total number of parasites was reduced by ~27% after treatment and the level of HGPRTase gene expression was significantly reduced in the HGPRTase siRNA-treated group versus controls (Pereira *et al*. 2008).

By scanning the available schistosome DNA sequence databases, a number of groups have identified homologues of proteins in schistosomes that are known to be involved in the RNAi pathway in other organisms (Gomes *et al*. 2009; Lendner *et al*. 2008). This, coupled with our own work, has permitted the generation of a first draft of RNAi pathways in schistosomes that are represented in Fig. 7. The pathway engaged following the uptake of exogenous dsRNA is shown in Fig. 7A while that engaged by endogenous dsRNA is shown in Fig. 7B.

Exogenous dsRNA that is taken up (Fig. 7A (i)) engages SmDicer and an RNA-binding protein (Partner Dicer) (Fig. 7A (ii)). This interaction leads to dsRNA cleavage and siRNA generation. The siRNA duplex is unwound and one strand is preferentially loaded into the short interfering RNA induced silencing complex (siRISC) (Fig. 7A (iii)). The minimal RISC, sufficient for target RNA recognition and cleavage is simply an Argonaute protein bound to a small RNA (Rivas *et al*. 2005). However Argonaute proteins can have several associated binding partners (Pratt and Macrae, 2009). Here we postulate that in a schistosome RISC, Argonaute (SmAgo) associates with homologues of the RNA binding protein Fmr1 and the nuclease Tudor-SN (Fig. 7A (iii)); the *S. mansoni* genome is reported to contain homologues of these proteins and they exist in other invertebrate RISCs (Gomes *et al*. 2009; Lendner *et al*. 2008). The RISC, now loaded with a single stranded RNA (called the guide RNA), searches the transcriptome to find potential target mRNAs. Targets are engaged by Watson-Crick base pairing which directs the Argonaute endonuclease (SmAgo) to cleave them (Fig. 7A (iv)) (Siomi and Siomi, 2009). Many rounds of mRNA cleavage result in substantial suppression of target gene expression and effectively results in gene silencing.

In the case of the microRNA pathway (Fig. 7B), first, primary precursor miRNA transcripts (pri-miRNAs), generated in the nucleus, are cleaved by the RNaseIII SmDrosha (in association with the RNA binding protein Partner Drosha) (Fig. 7B (i)). These pri-miRNAs, containing stem-loop structures depicted in Fig. 7B, harbour the mature miRNAs in the 5′ or 3′ half of the stem. The Drosha-cleaved product is generally 60–70 nucleotides and is called a precursor miRNA (pre-miRNA). This pre-miRNA is then exported, via the carrier protein Exportin 5, to the cytoplasm. Here the SmDicer/Partner Dicer complex may engage it and cleave it to generate a mature miRNA (Fig. 7B (ii)). Next, one strand of the miRNA is loaded into the microRNA induced silencing complex (miRISC) whose composition may be similar, as depicted in Fig. 7B (iii), or different from the postulated siRISC. In other systems different proteins, including distinct Argonautes, comprise different RISCs (Okamura *et al*. 2004) and this may be the case for schistosomes too since miRNA engagement often results in gene suppression via translational repression (Fig. 7B (iv)) as opposed to target mRNA cleavage. In the *S. mansoni* genome 4 Argonaute homologues have been identified (Gomes *et al*. 2009) and it is possible that different forms occupy distinct RISCs, or perform other functions within the cell. It is noteworthy that of the 4 Argonaute proteins in humans only Ago2 is a catalytically active slicer (Liu *et al*. 2004).

One factor determining the efficiency of RNAi is the ability of a cell to directly take up dsRNA. Recalcitrance to RNAi can perhaps be overcome by electroporation. In the case of schistosomes, it is clear that electroporation with gene-specific dsRNA leads to efficient target gene suppression in most

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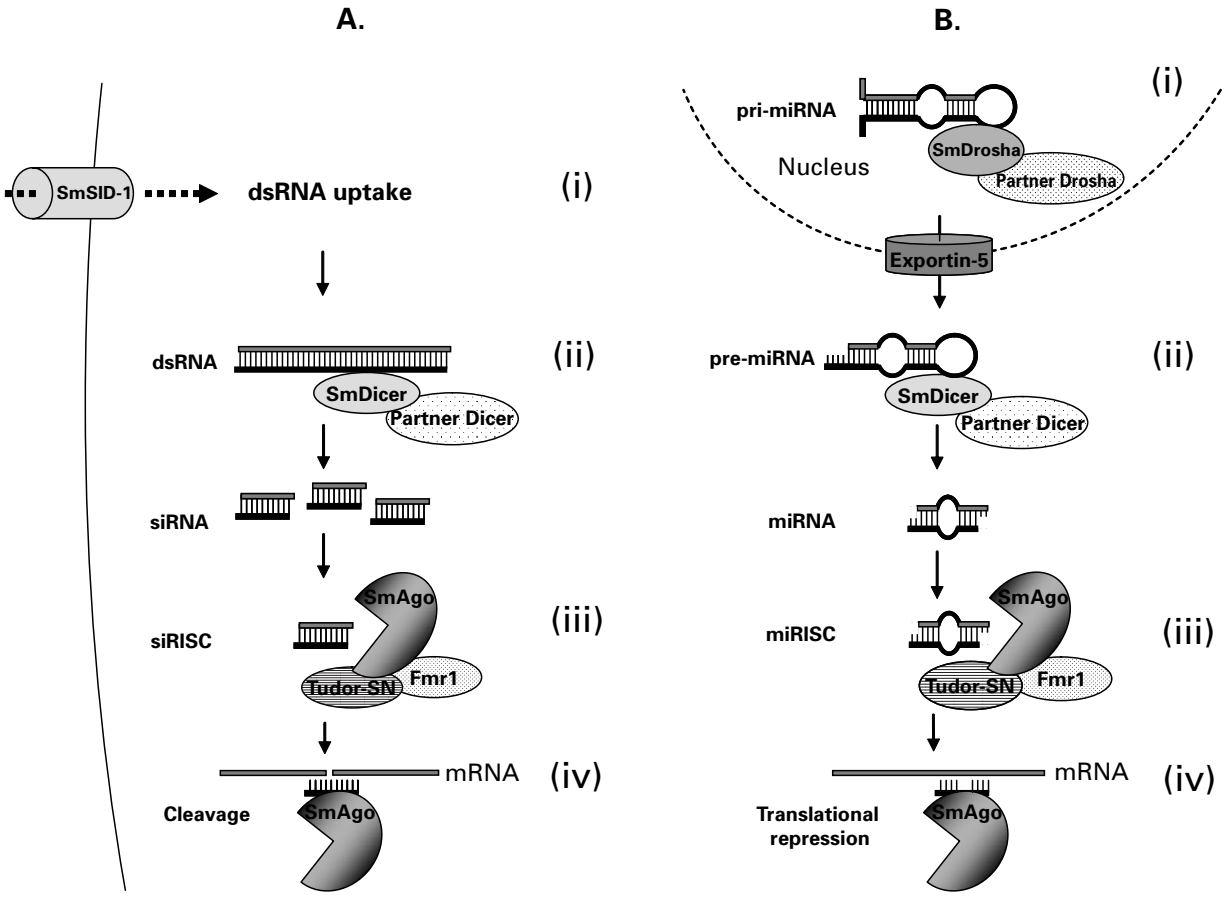


Fig. 7. Proposed RNAi pathways in *S. mansoni*. A: Double stranded RNA (dsRNA) taken in via SmSID-1 (i) engages SmDicer/Partner Dicer (ii) and is cleaved into short inhibitory RNAs (siRNAs). These engage the RNA induced silencing complex (RISC) containing an Argonaute (SmAgo) and other proteins (Tudor-SN & Fmr-1) (iii). A cognate mRNA is identified and cleaved to effect gene silencing (iv). B: Pri-miRNAs are processed in the nucleus by SmDrosha/Partner Drosha (i) and the resultant pre-miRNAs are exported to the cytoplasm via Exportin-5. Here they are acted upon by Dicer/Partner Dicer (ii) to generate miRNAs which enter the RISC (iii). This interaction generally results in translational repression (iv).

cases. It has been demonstrated here, and previously, that simply soaking schistosomes in dsRNA can also lead to efficient gene suppression (Krautz-Peterson *et al*. 2007*b*; Skelly *et al*. 2003). How dsRNA enters the soaked parasites remains unclear. In *C. elegans* the SID-1 (systemic RNAi defective-1) protein has been shown to be required for uptake of dsRNA (Feinberg and Hunter, 2003; Winston *et al*. 2002). In this work we describe the identification *in silico* of a schistosome homologue of this *C. elegans* protein. The schistosome protein, SmSID-1, has a close homologue in *S. japonicum* and both predicted schistosome proteins are substantially larger when compared to the SID-1 proteins of other organisms. All members of this family have 11 predicted transmembrane domains toward the carboxyl end. The distance from the initiator methionine to the first of these transmembrane domains is >600 amino acids in the case of the schistosomes. This amounts to >200 amino acids more than for the remaining homologues. Since both the *C. elegans* and human SID-1 proteins have been shown to be capable of effecting dsRNA uptake (Duxbury *et al*. 2005; Shih *et al*. 2009), it is reasonable to assume that SmSID-1 likewise acts as a channel to import dsRNA into schistosomes and is depicted as such in Fig 7A (i). It is noteworthy that many schistosome tissues are syncytial (Morris, 1968; Silk *et al*. 1969; Spence and Silk, 1970, 1971) so that, once dsRNA has entered a tissue, it may be able to traverse relatively large distances internally without the need to cross additional plasma membranes.

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