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Protocols for Gene Silencing in Schistosomes

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Abstract

Schistosomes are parasitic platyhelminths that infect over 200 million people globally. In recent years there have been many advances in schistosome genomics and proteomics and in the development of molecular tools for use with these parasites. Among the more promising methodologies is RNA interference (RNAi) which is a mechanism by which gene-specific double-stranded RNA (dsRNA) triggers degradation of homologous mRNA transcripts. We aim to develop effective protocols utilizing RNAi for use in the intra-mammalian life stages of *Schistosoma mansoni*. In this work, the gene encoding alkaline phosphatase (SmAP) was targeted by exposing the parasites to dsRNA encoding part of the SmAP coding region. SmAP is known to be expressed in a variety of parasite tissues. We report that both long dsRNAs as well as synthetic short inhibitory RNAs (siRNAs) are effective at eliciting SmAP gene suppression in cultured schistosomula and in adult males and females. Electroporation as a mode of dsRNA delivery is more efficient than simply soaking the parasites in an equivalent dose. Relative SmAP RNA levels >90% lower than controls were routinely detected, when measured 2 days after treatment by electroporation, using quantitative real time PCR. Commensurate with this decline in SmAP RNA, relative alkaline phosphatase enzyme activity levels >70% lower than controls were detected, 5 days after treatment. Protocols described here that result in the robust suppression of target genes in intravascular schistosomes may have wide applicability and promote functional schistosome genomics.

Keywords

Schistosoma mansoni; gene silencing; RNAi; double-stranded RNA; alkaline phosphatase

Schistosomes are parasitic platyhelminths that infect over 200 million people worldwide and can cause debilitating disease. In recent years there have been remarkable advances in the analysis and understanding of the schistosome genome and proteome (Hoffmann et al., 2002; Verjovski-Almeida et al., 2003; Wilson et al., 2004). Techniques to capitalize on this knowledge by efficiently manipulating the expression of specific parasite genes are now being developed (Wipperfsteeg et al., 2002; Boyle et al., 2003; Rossi et al., 2003; Skelly et al., 2003; Correnti and Pearce, 2004; Wipperfsteeg et al., 2005; Dillon et al., 2006). Among the more promising methodologies is RNA interference (RNAi) which is a mechanism by which gene-specific double-stranded RNA (dsRNA) triggers degradation of homologous mRNA transcripts. The process can result in effective, sequence specific, post-transcriptional gene silencing (Tabara et al., 1998; Hunter, 1999; Grishok and Mello, 2002; Dykxhoorn et al., 2003). RNAi was first discovered in the free-living nematode, *Caenorhabditis elegans*, and has since been found in many organisms of diverse phylogeny where it has proven to be potent

and, in some instances, long lasting (Fire et al., 1998; Grishok and Mello, 2002). Not all organisms display RNAi, including the unicellular parasites *Leishmania major*, *Trypanosoma cruzi* and the yeast *Saccharomyces cerevisiae* (Robinson and Beverley, 2003; DaRocha et al., 2004; Ullu et al., 2004). In the case of the phylum platyhelminthes (to which schistosomes belong), RNAi has been documented in both free-living and parasitic forms (Sanchez Alvarado and Newmark, 1999; Boyle et al., 2003; Sanchez Alvarado, 2003; Skelly et al., 2003).

The process of gene silencing through RNAi first involves the recognition of dsRNAs by an RNaseIII ribonuclease designated Dicer that converts them into 20–26 nucleotide-long RNAs called small interfering RNAs (siRNAs) (Carthew, 2001; Cook and Conti, 2006). siRNAs then join with an effector nuclease complex, known as the RNA-induced silencing complex (RISC), where the siRNAs are unwound. The siRNAs next associate with homologous mRNA substrates and these are destroyed by the RISC endonuclease, Slicer (Grishok et al., 2001; Grishok and Mello, 2002; Ketting, 2006). Cleavage requires extensive complementarity between the target mRNA and an antisense siRNA and occurs near the middle of the region bound by the siRNA strand (Schwarz et al., 2003).

A variety of functions have been proposed for the natural role of the RNAi machinery within eukaryotic cells. These include a role in the destruction of viral RNAs (Silhavy et al., 2002), or to suppress the expression of potentially harmful segments of the genome, such as transposons (Hannon, 2002). The RNAi machinery may also function in several pathways of gene regulation (Djikeng et al., 2001; Allshire, 2002; Hannon, 2002; Hu et al., 2002; Zilberman et al., 2003; Shi et al., 2004). In addition to these endogenous roles for RNAi, it is clear that the phenomenon is an extremely valuable molecular tool for analyzing gene function. An effective means to examine the biological role of a protein is to determine the phenotype of organisms that have none, or have reduced levels, of that protein. In many cases, this is achieved by mutating the gene encoding the protein with the aid of chemical mutagens or using molecular genetics. Alternative methods for silencing genes, such as RNAi, have more recently proved very powerful and this is especially true in those organisms (like schistosomes) that are not amenable to classical genetic methodologies.

In recent years, RNAi has become a specific and potent approach for loss-of-function studies in a wide variety of eukaryotes. We have attempted to optimize RNAi in schistosomes using the cathepsin B (SmCB1) gene as our target (Krautz-Peterson et al., 2007). SmCB1 is a major papain-like cysteine proteinase that is expressed largely in the schistosome gut and functions in hemoglobin digestion following the ingestion of red blood cells by intravascular parasites (Dalton et al., 1996; Caffrey et al., 2004). Double stranded RNA introduced by electroporation into schistosomula that have been cultured for 7 or more days is delivered to the parasite gut and this efficient delivery may account for the resulting pronounced suppression of this gene (Skelly et al., 2003; Correnti et al., 2005; Krautz-Peterson et al., 2007). In this paper we extend analysis to a second target that is more widely expressed in schistosomes to test the robustness of our RNAi protocols. We choose the schistosome alkaline phosphatase (SmAP) gene in this work and, while this gene is expressed widely within intravascular parasites, over 70% of alkaline phosphatase activity is associated with the tegument of male and female worms (Cesari, 1974). The distribution of alkaline phosphatase in schistosomes therefore differs markedly from that of our previous target, cathepsin B. Suppression of the alkaline phosphatase gene in this work can be easily measured at the RNA level using quantitative real-time PCR (qRT-PCR) and at the protein level by alkaline phosphatase enzyme activity assay (Dusanic, 1959; Halton, 1967; Morris and Threadgold, 1968).

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 (Skelly et al., 2003). Parasites were cultured in complete RPMI culture medium which is RPMI medium supplemented with 10mM Hepes, 2mM glutamate, 5% fetal calf serum and antibiotics (100U/ml penicillin and 100 µ/ml streptomycin) at 37°C, in an atmosphere of 5% CO₂. Culture samples were withdrawn periodically to assess parasite viability using the Trypan blue exclusion method. Adult parasites were recovered by perfusion from Balb/c mice that were infected with 125 cercariae, 7 weeks previously.

Preparation of dsRNA

A 928 bp fragment of the SmAP coding DNA was generated by PCR using gene specific primers flanked by either T7 or T3 RNA polymerase promoter sequences (underlined), (SmAP1-T7: 5'-GGTAATACGACTCACTATAGGGATGCTTCCAAGTGTCTTATCGACAGC-3' and SmAP1-T3: 5'-GGAATTAACCCTCACTAAAGGGGATTTTCTAGTCGGTAAGCCAATGG-3'). The GenBank accession number for SmAP is EU040139. A 990 bp irrelevant, non-schistosome control dsRNA, derived from the yeast expression vector pPIC9K, was similarly generated as previously described (Skelly et al., 2003). The PCR fragments were used to generate RNA by *in vitro* transcription with T7 or T3 RNA polymerases, as appropriate, using MEGAscript RNA transcription kits (Ambion, TX). All RNAs were purified using the RNeasy mini elute cleanup system (Qiagen, CA) following the manufacturer's instructions and recovered samples were quantified by spectrophotometry. Equimolar amounts of single stranded RNAs, in 300mM NaCl, 50 mM Tris (pH7.5), 1mM EDTA, were mixed and annealed by incubation at 68°C for 45 min followed by 10 min at room temperature. dsRNA annealing and integrity was confirmed by agarose gel electrophoresis.

A small inhibitory RNA (siRNA), designated siRNA-124 (spanning SmAP coding DNA positions 124–150) with the following sequence: 5'-AAGAAATCAGCAGATGAGAGATTTAAT-3', was synthesized commercially by 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>). The off-the-shelf "DS Scrambled Neg" negative control siRNA 5'-CTTCTCTCTTTCTCTCCCTTGTGA-3' was obtained from IDT, Inc. This sequence does not match any in the *S. mansoni* genome assembly (version 3) as assessed by using the Basic Local Alignment Search Tool (BLAST) at http://www.sanger.ac.uk/Projects/S_mansoni/ (Altschul et al., 1990).

dsRNA delivery

Using the soaking method, 25 µg dsRNA in RPMI medium without serum, was added to 1,000 schistosomula or 10 adult male or female parasites in 150 µl complete RPMI medium. Schistosomula were cultured in complete RPMI medium for 7 days before treatment. Using electroporation, 1,000 schistosomula or 10 adult male or female worms in 50µl electroporation buffer (Ambion, TX) containing 25 µg long dsRNA or 2.5 µg siRNA were electroporated in a 4mm cuvette by applying a square wave with a single 20 ms impulse, at 125V and at room temperature, as described (Correnti et al., 2005). Parasites were then transferred to 150 µl complete RPMI. After overnight culture, medium was replaced with 300 µl of fresh complete

RPMI culture medium. Gene suppression was measured at the RNA level 2 days after treatment and at the protein level 5 days after treatment.

Gene expression analysis

To monitor the expression of the SmAP gene in treated parasites, RNA was first extracted 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 (qRT-PCR) was performed using custom TaqMan Assays using primer sets and reporter probes labeled with 6-carboxyfluorescein (FAM), obtained from Applied Biosystems (Foster City, CA).

The following primers and probe were selected to detect alkaline phosphatase expression: SmAP1Taq-siteF, 5'-GCCATCCGACAAGGAATATAAGTGT-3'; SmAP1Taq-siteR, 5'-GGTCCATTGAAAAAGGAGGATATGAGA-3' and probe, SmAP1Taq-siteM2, 5'-FAM-ATCTCCTTTTGCAGTATTATC-3'. To detect expression of the endogenous control tubulin gene, the following primers and probe were used: Tubulina2-F, 5'-GGTTGACAACGAGGCCATTTATG-3'; Tubulina2-R, 5'-TGTGTAGGTTGGACGCTCTATATCT-3' and probe Tubulina2-M2, 5'-FAM-ATATTTGTCGACGGAAT-3'. 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 $\Delta\Delta C_t$ 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 $\Delta\Delta C_t$ values were normalized to controls and expressed as percent difference (Livak and Schmittgen, 2001).

Alkaline Phosphatase enzyme activity assay

Schistosomula and adult female worms were homogenized at 4°C in Cell Disruption Buffer (Ambion, TX). The homogenate was kept on ice for 1 h to maximize protein solubilization and was then centrifuged at 12,000 rpm for 10 min at 4°C in a microfuge. The protein concentration in the soluble fraction was determined using the QuantiPro bicinchoninic acid assay (Pierce, IL). Enzyme activity was measured using an alkaline phosphatase substrate kit (Bio-Rad, CA). In this assay soluble protein from each group was added in duplicate to 200 µl diethanolamine buffer containing p-nitrophenylphosphate substrate, according to the manufacturer's instructions (Bio-Rad, CA). Cleavage of substrate was measured at 405 nm, following incubation at room temperature for two hours. Results were expressed as relative percentage of alkaline phosphatase activity in test groups compared to controls.

Statistical analysis

All data are presented as the mean ($\pm$ standard error) of at least 3 replicates. Statistical analysis of qRT-PCR data were carried out using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Differences between all other groups were assessed for statistical significance using the student's t test. P values of ≤ 0.05 were considered significant.

Results

We are developing protocols for optimal gene suppression in schistosomes to facilitate molecular and cellular biological studies on these important parasites. In this work we focus

on intra-mammalian stage parasites, (schistosomula and adult males and females), with alkaline phosphatase (SmAP) as the target of suppression. Figure 1 shows the relative expression of the SmAP gene in cultured schistosomula treated with long SmAP dsRNA or control dsRNA by soaking (left panel) or by electroporation (right panel). Both delivery methods lead to significant suppression of SmAP gene expression relative to controls. However, the suppression achieved following electroporation is greater than that seen using the soaking method. Figure 2 extends these data to adult parasites and shows the relative expression of the SmAP gene in separated adult male or adult female worms treated with SmAP dsRNA or control dsRNA by soaking or electroporation. Again, both methods lead to significant gene suppression and, as for schistosomula, electroporation of adult worms results in greater suppression of the target gene compared to soaking.

Next we compared the ability of the long SmAP dsRNA versus a synthetic, small inhibitory dsRNA (siRNA) to effectively suppress alkaline phosphatase gene expression in schistosomula and adult parasites, using electroporation as the delivery method. Figure 3 shows the results for schistosomula and demonstrates that both forms of dsRNA produce significant suppression. Controls treated with irrelevant long dsRNA or control siRNA do not differ significantly in their level of SmAP gene expression. Nor is there any significant difference between these controls and control parasites subjected to electroporation without any dsRNA (electroporation control, figure 3) and control parasites placed in culture and subject to no treatment (untreated control, figure 3). Figure 4 shows comparable results for mature adult male and female parasites; delivery of long dsRNA or siRNA by electroporation leads to robust suppression (>85%) in each case, while control long dsRNA or siRNA has no impact. We next set out to determine if the observed suppression of SmAP gene expression resulted in any change in alkaline phosphatase enzyme levels. Protein preparations were obtained from schistosomula and adult parasites 5 days after treatment with dsRNA and alkaline phosphatase activity was measured. Figure 5 illustrates the results and shows that, commensurate with the decline in SmAP gene expression, there is a significant decrease in alkaline phosphatase activity in schistosomula (Figure 5A) and adult female (left, figure 5B) and male (right, figure 5B) parasites that were treated with SmAP dsRNA but little change in controls treated with an irrelevant dsRNA or in untreated controls.

Discussion

RNAi has proven to be a powerful molecular tool for analyzing gene function in a wide variety of experimental systems. We aim to develop robust RNAi protocols for use in genomic research involving intra-mammalian stage schistosomes. Our previous work focused on silencing the cathepsin B gene whose expression is largely confined to the schistosome gastrodermis (Skelly et al., 2003; Krautz-Peterson et al., 2007). The cathepsin B protein is a papain-like cysteine proteinase that participates in the catabolism of hemoglobin and other proteins ingested by blood-feeding life stages (Dalton et al., 1996; Caffrey et al., 2004). Since protocols to suppress cathepsin B expression may not work on other targets, in this report we choose a second molecule - alkaline phosphatase, as our target. The alkaline phosphatase gene is expressed in a variety of schistosome tissues, including the tegument, gastrodermis and reproductive structures of adult worms (Dusanic, 1959; Halton, 1967; Morris and Threadgold, 1968). Therefore by developing procedures to silence such a widely expressed gene, our hope is that these methods may be used by members of the research community to silence any schistosome gene.

Protocols described here lead to substantial suppression of the schistosome alkaline phosphatase gene. Our results show that the SmAP gene is very susceptible to RNA interference and that the effect is specific, since parasites treated with control, irrelevant dsRNA exhibit no significant suppression of this target gene. All intravascular forms tested (schistosomula,

mature adult male and adult female worms) are susceptible to RNAi-induced SmAP gene suppression. The dsRNA can be delivered to the parasites as a long molecule or in the form of siRNA to effect suppression. In addition, dsRNA can be delivered by simply soaking the parasites in medium containing it or by electroporation. While both protocols lead to substantial gene suppression, electroporation is superior in that significantly greater suppression is achieved using this method. This is the case for all life stages examined and likely results because of the more efficient entry of dsRNA into the parasites by electroporation. We previously obtained similar results using cathepsin B as the target; that is, both long and short dsRNAs elicited suppression of the cathepsin B gene in the same intravascular life stages examined in this study and electroporation proved more efficient as a delivery method (Krautz-Peterson et al., 2007). These new results targeting a second gene that yield a similar potent suppression suggest that these protocols have wide applicability.

At this time, our recommended protocol for suppressing gene expression in intravascular schistosomes is to first obtain synthetic, gene specific siRNAs. Several companies now synthesize siRNAs and the cost is comparable to that for the commercial *in vitro* RNA synthesis kits that are required in order to most efficiently generate long dsRNAs. Next, relatively low doses of the siRNAs should be delivered to cultured parasites by electroporation. We routinely test for suppression at the RNA level by qRT-PCR 48 hours after treatment and at the protein level using enzyme activity assays at least 5 days following treatment.

One concern with this protocol is the increased handling of the parasites that is required when using electroporation. Adult worms occasionally adhere using their suckers to the walls of the electroporation cuvette and care must be taken when recovering these to decrease the risks of damaging the specimens. While we note no persistent, overt change in the morphology, behavior or gene expression patterns of parasites subjected to electroporation, it remains possible that electroporation may stress the parasites and result in as-yet undetected morphological alterations and/or alterations in gene expression. Clearly, soaking the parasites in dsRNA solution is a simpler alternative that minimizes handling but more dsRNA reagent is needed for robust suppression using this method. So far, this later approach has been adopted by most workers. For instance, in sporocysts (the larval schistosome stage that develops inside mollusks) a number of genes have been subject to RNAi-induced suppression by soaking cultured parasites in solution containing dsRNA encoding portions of these molecules (Boyle et al., 2003; Dinguirard and Yoshino, 2006). Targeted genes include the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glucose transporter protein (SGTP1), and a scavenger receptor class B homologue. Similarly, soaking schistosomula with dsRNA encoding the peroxiredoxin1 gene resulted in a decrease in total peroxiredoxin enzymatic activity and lowered survival of cultured parasites (Sayed et al., 2006). In addition, soaking adult parasites in medium containing dsRNA encoding several of the enzymes involved in hemoglobin digestion (cathepsin B, cathepsin L1, legumain and cathepsin D) all resulted in potent suppression of the specific target gene (Delcroix et al., 2006). The egg stage of the parasite life cycle has been similarly subjected to RNAi by soaking the eggs for 7 days in low doses (1 µg/ml) of dsRNA encoding a schistosome TGF-β homolog (SmInAct) and this resulted in inhibition of egg maturation (Freitas et al., 2007). One study adopted a different approach; long dsRNAs encoding a portion of a TGF-β type II receptor (SmTβRII) were digested with RNase III into corresponding siRNAs (Osman et al., 2006). These were coated onto gold particles that were introduced into 26- and 33-day old worm pairs by bombardment (Osman et al., 2006). Since this protocol is somewhat complex and requires a particle delivery system (gene gun) that may not be commonly available in most laboratories, it likely will not be very widely adopted. The schistosome Dicer gene has been identified and shown to be expressed throughout schistosome development (Krautz-Peterson and Skelly, 2007). Since the Dicer enzyme plays a central role in gene suppression, its widespread expression in schistosomes bodes well for the use of RNAi technologies in deciphering gene function in all

developmental stages of *S. mansoni*. In summary, we describe here straightforward protocols for the robust suppression of target genes in intravascular schistosomes and we anticipate that these will continue to help decipher gene function in *S. mansoni*.

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Figure 1. Relative SmAP expression (mean $\pm$ S.E.) in schistosomula treated with 25 μ g SmAP dsRNA (open bars) or control, irrelevant dsRNA (grey bars) by electroporation (left) or soaking (right).

Figure 2.

Relative SmAP expression (mean $\pm$ S.E.) in adult female (A) or male (B) parasites treated with either 25 μ g SmAP dsRNA (open bars) or control, irrelevant dsRNA (grey bars) by electroporation (left) or soaking (right).

Figure 3.

Relative SmAP expression (mean $\pm$ S.E.) in schistosomula treated by electroporation with either 25 μ g long dsRNAs (left, SmAP dsRNA (open bar) or control, irrelevant long dsRNA (grey bar) or with 2.5 μ g siRNA (center, siRNA-124 (open bar) or control, irrelevant siRNA (grey bar)). Additional controls (right) include untreated parasites and parasites subjected to electroporation only.

Figure 4. Relative SmAP expression (mean $\pm$ S.E.) in adult female (A) or male (B) parasites treated by electroporation with either 25 μ g long dsRNAs (left, SmAP dsRNA (open bar)) or control, irrelevant long dsRNA (grey bar) or with 2.5 μ g siRNA (right, siRNA-124 (open bar)) or control, irrelevant siRNA (grey bar).

Figure 5.

Relative SmAP enzyme activity (mean $\pm$ S.E.) in schistosomula (A) and adult schistosomes (B, females (left) and males (right)) 5 days after treatment with 25 μ g SmAP dsRNA (open bar) or control, irrelevant dsRNA (grey bar, center) or no dsRNA (grey bar, right).