IDENTIFYING A ROLE FOR HEAT SHOCK PROTEINS IN

SCHISTOSOMA MANSONI

by

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List of Abbreviations

°C degrees Celsius
17-DMAG 17-dimethylaminoethylamino-17-demethoxygeldanamycin
A alanine (amino acid)
aa amino acid
ADE2 phosphoribosylaminoimidazole carboxylase (gene, "ADEnine requiring")
ADP adenosine diphosphate
AMP adenosine monophosphate
AMP-PNP adenyl-imidodiphosphate
AP affinity purification
ATP adenosine triphosphate
AWA adult worm antigen
BLAST basic local alignment search tool
BLASTP basic local alignment search tool-protein
bp base-pair
BRI Biomedical Research Institute
CAA circulating anodic antigen
CBP cyclic adenosine monophosphate response element-binding protein(CREB)-binding protein
CCA circulating cathodic antigen
CCD charge-coupled device
cDNA complementary deoxyribonucleic acid
cm centimeter
CoIP complex-immunoprecipitation
<table>
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<tr>
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<tr>
<td>COPT</td>
<td>circumoval precipitin test</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>CTF</td>
<td>cercarial transformation fluid</td>
</tr>
<tr>
<td>D</td>
<td>aspartic acid (amino acid)</td>
</tr>
<tr>
<td>DALYs</td>
<td>disability-adjusted life years</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylinodole</td>
</tr>
<tr>
<td>DBD</td>
<td>deoxyribonucleic acid-binding domain</td>
</tr>
<tr>
<td>DIC</td>
<td>differential inference contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOI</td>
<td>digital object identifier</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>g</td>
<td>acceleration due to gravity at the Earth's surface</td>
</tr>
<tr>
<td>G</td>
<td>glycine (amino acid)</td>
</tr>
<tr>
<td>GA</td>
<td>geldanamycin</td>
</tr>
<tr>
<td>Gal4</td>
<td>deoxyribonucleic acid-binding transcription factor required for activating GAL genes (protein, &quot;GALactose metabolism&quot;)</td>
</tr>
<tr>
<td>GAL4</td>
<td>deoxyribonucleic acid-binding transcription factor required for activating GAL genes (gene, &quot;GALactose metabolism&quot;)</td>
</tr>
<tr>
<td>HHF</td>
<td>heat shock protein 70 (Hsp70) honing factor (protein, putative)</td>
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<td>HIS3</td>
<td>imidazoleglycerol-phosphate dehydratase (gene, &quot;HIStidine&quot;)</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Hs</td>
<td>Homo sapiens</td>
</tr>
<tr>
<td>Hsc</td>
<td>heat shock cognate (protein)</td>
</tr>
<tr>
<td>HSE</td>
<td>heat shock deoxyribonucleic acid (DNA) binding element</td>
</tr>
<tr>
<td>HSF</td>
<td>heat shock factor (gene)</td>
</tr>
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<td>Hsf</td>
<td>heat shock factor (protein)</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein (gene)</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein (protein)</td>
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<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
</tr>
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<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>IHT</td>
<td>indirect hemagglutination test</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>INF</td>
<td>interferon</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>L</td>
<td>leucine (amino acid)</td>
</tr>
<tr>
<td>LAMP</td>
<td>loop-mediated isothermal amplification</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mA</td>
<td>milliAmpere</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>MEL1</td>
<td>secreted alpha-galactosidase (gene)</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mJ</td>
<td>milliJoule</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>Msg1</td>
<td>melanocyte specific gene (protein)</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>N</td>
<td>asparagine (amino acid)</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NEF</td>
<td>nucleotide exchange factor</td>
</tr>
<tr>
<td>NIAID</td>
<td>National Institute of Allergy and Infectious Diseases</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NMRI</td>
<td>Naval Medical Research Institute</td>
</tr>
<tr>
<td>P</td>
<td>proline (amino acid)</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBSTw</td>
<td>phosphate buffered saline-Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PES</td>
<td>2-phenylethynesulfonamide</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination-activating gene</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>synthetic dextrose medium</td>
</tr>
<tr>
<td>SD-Ade</td>
<td>synthetic dextrose medium lacking adenine</td>
</tr>
<tr>
<td>SD-His</td>
<td>synthetic dextrose medium lacking histidine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SD-Trp</td>
<td>synthetic dextrose medium lacking tryptophan</td>
</tr>
<tr>
<td>SEA</td>
<td>soluble egg antigen</td>
</tr>
<tr>
<td>Sj</td>
<td>Schistosoma japonicum</td>
</tr>
<tr>
<td>Sm</td>
<td>Schistosoma mansoni</td>
</tr>
<tr>
<td>Smad</td>
<td>small body size (SMA)-mothers against decapentaplegic (MAD) (protein)</td>
</tr>
<tr>
<td>SODD</td>
<td>silencer of death domain</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-boric acid-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>X-α-gal</td>
<td>5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
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Identifying a Role for Heat Shock Proteins in *Schistosoma mansoni*

**Abstract**

by

KENJI ISHIDA

Parasitic schistosome worms infect more than 240 million people worldwide, causing the debilitating disease, schistosomiasis. The primary drug for treatment, praziquantel, has been used for decades, with rising concern for drug resistance. Therefore, a better understanding of the biology of schistosomes is necessary to identify targets for the development of novel chemotherapeutics.

In this thesis, we describe experiments involving heat shock genes and proteins performed in *Schistosoma mansoni*, one of the main species responsible for human schistosomiasis. From the understanding that schistosomes undergo many environmental transitions during their six-stage, two-host lifecycle, we can reason that heat shock response may play a vital role in helping the parasite survive these transitions. The studies here focus on the role of the heat shock transcription factor and heat shock proteins in cercariae, the infective stage for the mammalian host.

First, we characterized the heat shock transcription factor (*SmHSF1*) using several
Abstract

methods. A modified yeast 1-hybrid assay verified the ability of SmHsf1 to activate transcription. In a bandshift DNA-binding assay, recombinant purified SmHsf1 demonstrated specific binding to DNA oligonucleotides containing sequences corresponding to those from the region upstream of schistosome heat shock protein 70 (gene, HSP70) and of the heat shock consensus sequence. Transcript analysis by quantitative PCR of cDNA from several parasite stages showed constitutive expression of SmHSF1. Using a custom-raised antibody, Western blotting showed bands specific for SmHsf1, and immunohistochemical staining of cercaria stage parasites showed a novel localization of SmHsf1 to the acetabular glands.

In the subsequent study, we focused on the role of heat shock protein 70 (protein, Hsp70), which is a downstream effector protein of the Hsf1 transcription factor, in cercaria stage parasites. Treatment of cercariae with an Hsp70 inhibitor led to a shift in swimming behavior, similar to the treatment of cercariae with human skin lipid, indicating that Hsp70 may be a key mediator of the host sensing and invasion process.

Taken together, the studies presented here implicate for the first time, to our knowledge, an important role for heat shock response-associated proteins during the development of the infective cercaria stage and mammalian host invasion in schistosomes.
Chapter 1: Introduction

1.1 Purpose

This chapter aims to provide the reader with 1) a brief background in schistosome biology and associated disease, 2) a review of the current understanding of the behavior of infective larval schistosomes in the scope of mammalian host invasion, and 3) a primer on heat shock response and its associated genes and proteins: in general, then more specifically in schistosomes. The subsequent chapters of this thesis present observations that identify a novel role for schistosome heat shock response-associated proteins in host invasion. In the final chapter, we summarize and interpret the findings of the studies here and discuss future experiments to identify other molecular components involved in host invasion.

1.2 Schistosomiasis

Over 240 million people worldwide have schistosomiasis, a disease resulting from infection with parasitic trematodes of the Schistosoma genus [1]. Five species cause human schistosomiasis: Schistosoma mansoni, S. japonicum, S. haematobium, S. mekongi, and S. intercalatum [2]. Infection with S. haematobium leads to the urogenital form of the disease, while infection with any of the other species leads to the gastrointestinal form of the disease [3]. Some estimates suggest that nearly 800 million people are at risk of infection [4] and that over 250,000 people die each year from associated organ dysfunction [5]. Schistosomiasis also causes significant morbidity, with the loss of 1.7 million to 56 million disability-adjusted life years (DALYs), depending on the method of
calculation [6], placing it among the most important parasites in socioeconomic terms [7].

The disease pathology results not from the adult parasites directly, but from the eggs, which can become lodged in the bladder (\textit{S. haematobium}) or liver (\textit{S. mansoni}, \textit{S. japonicum}), activating the host immune response to form granulomas, ultimately leading to obstructive disorders of the respective organs, including bloody urine, calcification of the bladder and bladder cancer; and portal hypertension, liver fibrosis, and enlarged liver [2,3].

The primary treatment for schistosomiasis is the drug praziquantel. It has been used for several decades, and lack of equally effective alternative drugs [8], reports of reduced susceptibility [9–11], and demonstration of resistance in the laboratory [12,13] all raise concern for the continued efficacy of praziquantel. Cases of reduced susceptibility could be explained by heavy infection or high re-infection rates [14,15], as praziquantel does not prevent re-infection. Alternatively, the reason could be the parasite stage-specific efficacy of praziquantel: it can kill adult worms but not the juvenile human infective stages [16,17]. In any case, the need remains for the identification of new treatment strategies. While the findings presented in this thesis focus more on improving our knowledge of the molecular biology of schistosomes, such basic research provides the foundation for the identification of drug targets.

### 1.3 Geographic distribution

An estimated 78 countries worldwide are endemic for schistosomiasis [1], with the
majority in sub-Saharan Africa (Figure 1.1). *S. haematobium* and *S. mansoni* are responsible for most of the infections in Africa and Arabian peninsula [2,3], while *S. japonicum* resides exclusively in Asia [18]. Other schistosome species also have specific geographic restrictions; for example, *S. mekongi* exists mostly in Laos and Cambodia [19].

![Map of Schistosomiasis Distribution](Image)

**Figure 1.1. Geographic distribution of schistosomiasis.** (From [3]).

### 1.4 Parasite lifecycle

Schistosomes have a complex lifecycle that includes six distinct stages and a molluscan intermediate host and mammalian definitive host (Figure 1.2). Free-swimming freshwater cercariae (singular: cercaria) infect their host via skin penetration. They lose their tails during the skin invasion process, and the heads transform into schistosomula (singular: schistosomulum), enter the blood circulation, and pass through the lungs into the liver...
and mesenteric veins of the intestine, developing into sexually dimorphic adult worms in 4-6 weeks. The adult worms pair and lay eggs: the retained eggs cause the disease, while the excreted eggs, either through urine (S. haematobium) or stool (other schistosome species) reach freshwater and hatch into miracidia (singular: miracidium). Miracidia infect the molluscan host, in which they develop into mother and daughter sporocysts. Asexual reproduction in the daughter sporocysts gives rise to cercariae, which exit from the molluscan host, in search for a mammalian host [3,20].

Figure 1.2. Schistosome lifecycle. (From [21]).
1.5 Clinical manifestation and pathophysiology

Human schistosomiasis begins with the cercaria stage invading and passing through the skin of the host. At the site(s) of entry, localized inflammation, or cercarial dermatitis, may occur, similar to the “swimmers’ itch” resulting from accidental infection of a human host with avian schistosomes [3,22]. In the acute phase of schistosomiasis, Katayama fever, or syndrome, whose symptoms can include fever, muscle pain, joint pain, diarrhea, may appear between 2 and 12 weeks following infection with cercariae, often coinciding with the deposition of eggs by the adult worms [23]. The host immune system responds to antigens released from the eggs, resulting in granuloma formation. The inflammatory granulomatous reaction can lead to hematuria and ulcer formation in the bladder and intestine and hepatomegaly in the liver [3].

Continued infection leads to the chronic phase of the disease, which can include bladder dysfunction and cancer, and continued polyp formation and diarrhea in the intestine, as well as the more serious periportal fibrosis and portal hypertension in the liver [24]. In this chronic hepatic form of the disease, the increased backpressure results in esophageal bleeding, which can cause anemia, growth stunting, or death in severe cases [3,24].

While the bladder, intestine, and liver represent the main organs affected by schistosomiasis, ectopic lesions in the lungs, genitals, and kidney may also occur. Most notably, neuroschistosomiasis occurs as a result of unusual migration of adult worm pairs to the central nervous system and subsequent inflammation in response to oviposition,
and it can cause myelitis, paresthesia, lower limb weakness, headache, dizziness, and seizures [25,26].

1.6 Diagnosis

Several methods can be used to detect schistosome infection (for review, [17,27]). The predominant method involves the direct detection of parasite eggs in urine or stool samples [3]; in particular, the Kato-Katz stool smear microscopy method remains the long-standing standard for intestinal schistosomiasis because it does not require extensive equipment or labor [17,28,29]. While the Kato-Katz test works well for high-load infections, it may fail to detect infections with low egg density, uneven physical egg distribution, or variable temporal egg release/distribution [6,17].

As an alternative to direct detection of schistosomes, other detection methods test for the presence of schistosome-specific antigens, antibodies or nucleic acids. These tests usually offer improved sensitivity and specificity at the cost of expense and infrastructure requirements and have been recommended for application in areas that have progressed beyond morbidity and prevalence stages (into transmission, surveillance, and elimination stages) of helminth control programs [30].

In antigen tests, patient serum or urine is used with an enzyme-linked immunosorbent assay (ELISA) or antigen capture assay to detect known schistosome antigens from egg (soluble egg antigen, SEA) and adult (adult worm antigen, AWA) stages [17]. Circulating
cathodic antigen and circulating anodic antigen (CCA and CAA, respectively) derived from adult parasites have been used with success in a rapid detection dipstick format [31]. The method of detecting circulating antigens, however, has not demonstrated a significant improvement over microscopic detection methods [32,33].

Antibody detection methods test for the presence of host antibodies sensitive to schistosome products by exposing patient serum to known antigens. These tests include the circumoval precipitin test (COPT), indirect hemagglutination test (IHT), immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA) [17]. In COPT, serum is mixed with schistosome eggs; serum that forms a precipitate the around eggs indicates a positive test result. Similarly, in IHT, serum is mixed with red blood cells coated with schistosome antigen (usually an adult worm antigen preparation); again, a precipitate indicates a positive result [34]. In IFA, serum is used to probe cercaria, adult, and egg stage parasites; a secondary antibody conjugated with a fluorophor is used to detect the presence of schistosome-reactive antibodies. In ELISA, an antigen of choice, such as SEA, is immobilized on the surface of a test plate, followed by the addition of serum and secondary antibody conjugated with a chromogenic enzyme; a change in color indicates a positive result. Cercarial transformation fluid (CTF) has also been used in place of SEA for ELISA with comparable results and the advantage of lower cost of production [35]. While these antibody detection methods can give higher sensitivity results compared to microscopy detection methods, they also have the disadvantages that antibodies may still exist after infection has resolved, leading to false-positive readings,
and that they often require specialized equipment and trained personnel, decreasing their practical use in large surveys [17].

Schistosome infection can also be detected using nucleic acid methods, namely, PCR and its derivatives. PCR amplification of a 121 base-pair (bp) tandem repeat DNA fragment from stool samples has been shown to yield greater sensitivity compared to the Kato-Katz stool examination method [36]. Quantitative PCR extends on ordinary PCR in that it can be used to detect infection intensity and eliminates the need for electrophoresis [37]. These PCR methods, however, require costly equipment and reagents to perform. A low-cost alternative, loop-mediated isothermal amplification (LAMP), replaces the thermal cycler and electrophoresis with a water bath and dye. It has been tested using stool and serum samples from rabbits and humans infected with *S. japonicum*, showing a lower detection limit by orders of magnitude compared to conventional PCR in the range of sub-femtogram amounts of DNA [38].

### 1.7 Treatment

Praziquantel remains the drug of choice for schistosomiasis treatment since its introduction in the 1970s, owing to its efficacy in treating infections of all schistosome species, mild side effects, and low cost [39,40]. Upon exposure to praziquantel, adult worms undergo muscle contraction, tegumental disruption, and metabolic disruption, ultimately leading to detection and removal by the host immune system [41,42]. The exact mechanism of action of praziquantel still eludes researchers even after decades of
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An increase in intracellular calcium ions following praziquantel has been observed and is thought to play a role in muscle contraction, implicating calcium ion channels as the target of praziquantel [43]; however, pretreatment with cytochalasin D prevented praziquantel-mediated death while increasing calcium ion influx, suggesting that praziquantel acts through other mechanisms to induce parasite death [44].

Praziquantel treatment with a dose of 40 mg/kg is effective in causing 80-95% reduction in egg excretion; the inability to completely eliminate the infection may be the result of the stage-specific efficacy of praziquantel (effective against adult worms but not schistosomula), prompting repeated treatments [45,46]. For the concurrent elimination of the immature infection stage schistosomes, the anti-malarial artemisinin and derivative compounds have demonstrated efficacy; however, co-treatment with artemisinins has not become common practice because their increased use may lead to rise of resistant malaria strains [47]. Other drugs used for treatment include oxamniquine for S. mansoni and metrifonate for S. haematobium; unfortunately, these compounds do not affect S. japonicum infections, and, in the case of oxamniquine, drug resistant infections requiring treatment with a dosage several hundred-fold higher compared to susceptible infections have been demonstrated [45,47].

1.8 Immunology

Schistosomes have been of much interest immunologically because of their ability to evade the host immune response and live for up to several decades [48,49]. Over the
course of infection, the host immune system adapts to the different phases of parasite development (Figure 1.3). The interaction of the parasite and host immune system begins with the acute stage of infection, during which a T helper 1 (Th1)-type immune response is observed. Th1 responses typically result in elevated serum levels of several cytokines, including tumor necrosis factor alpha (TNFα), interleukin (IL)-1, and IL-6. As the infection continues and adult worms deposit eggs, a Th2-type immune response, associated with the fibrogenic cytokines IL-4 and IL-13, becomes apparent [48].

![Figure 1.3. Development of the host immune response to schistosome infection. (From [48]).](image)

Interestingly, schistosomes require a mammalian host with a competent immune system to thrive [50,51]. Experimental schistosome infection of immune-compromised RAG-1 knockout mice showed slowed growth of parasites compared to those infecting normal
mice [50]. Similarly, elevated temperature treatment of snail hosts has been shown to result in increased susceptibility to infection by schistosome miracidia, and that inhibition of heat shock protein 90 (Hsp90) reverses this effect [52], suggesting that schistosomes have the ability to sense the health of the host and adjust their development.

1.9 Host invasion by schistosome cercariae

1.9.1 Host identification and cercarial honing

In this thesis, we define the term, cercarial honing, as the change in cercarial swimming behavior following the identification of the host. With respect to host identification, infective schistosome cercariae respond to a variety of stimuli, including changes in light, heat, and components of host skin, such as fatty acids and L-arginine [53–58]. While the physiology of the molecular sensing is not well understood, exposure to such host molecules results in a change in swimming behavior (cercarial honing), which involves a shift in buoyancy and reduction in upward swimming motion [57]. After making contact with the host, the cercariae crawl along the surface of the skin for a suitable place to penetrate and increase the release of acetabular gland contents, which include mucins to help adhere to the skin and elastases to help penetrate the skin [59,60].

Many studies investigating the chemical requirements that stimulate a cercarial response have come from the Haas group [54,55,57,61]. Based on these in vitro studies, cercarial honing has been traditionally thought to be a highly chemotaxis-driven process, in which cercariae actively swim toward the source of host molecules. The question remains of
whether and to what degree cercariae exhibit the same chemotactic behavior toward a host in the field, which typically features a larger distance between the parasite and the host.

Our observations following cercariae treatment with skin lipid have suggested a different behavior, in which cercariae that sense host molecules do not swim toward the source, but instead decrease their upward swimming motion, while, at the same time, increase their overall swimming motion. In a larger body of water, as in the wild, this new description of cercarial response to host molecules agrees with the reasoning that because cercariae typically cannot swim faster than a moving host, a chemotactic response would not necessarily increase the chance of catching their host. A binary, rather than a continuous or concentration gradient-dependent, determination of host presence or absence that controls the initiation of host contact behavior seems more plausible.

1.9.2 Penetration of host skin and key morphological features of cercariae

After exiting the snail host, cercariae have only several hours during which they must successfully invade a mammalian host [62,63]. The cercarial body is therefore equipped for rapid and efficient penetration of host skin. It has a total length of about 500 μm, and its tail features a bifurcation (Figure 1.4). To facilitate penetration into the host skin, the head contains several pairs of pre- and post- acetabular glands, whose respective bases extend from an anterior or posterior region around the acetabulum (ventral sucker) to the very anterior end, and which function as storage and release vessels for components
necessary for host invasion such as mucins (for attachment to skin) and elastases (for degradation of skin) [64]. In contrast to a typical gland, which consists of a series of epithelial cells secreting substances into a lumen, each “gland” in cercariae actually represents an elongated individual cell filled with vesicles that are released intact and rupture after exiting the gland [60].

Penetration into the host skin involves the release of the contents from the acetabular glands and vigorous mechanical movement against the skin, eventually resulting in the separation of the head and tail. As the head penetrates through the skin, it loses its glycocalyx, a carbohydrate-rich coat that allows tolerance to the hypotonic freshwater environment, subsequently allowing the migration of multilaminate vesicles to the
surface and transforming the previously trilaminate (single-membrane) surface to a 
hepta-/multilaminate (double-/multi-membrane) one [66]. Activity of the proteases from 
the acetabular glands has been proposed to assist in the shedding of the glycocalyx, 
which has been demonstrated to activate the alternative pathway of complement innate 
immune response [67,68]. The shedding of the glycocalyx may therefore represent the 
first example of host immune system evasion over the course of infection. In addition to 
the separation of the head and tail, loss of the glycocalyx, and replacement of the single 
outer membrane with a double outer membrane, several other characteristics, including 
the loss of water tolerance, a negative Cercarienhüllen reaktion (a serodiagnosis method 
that results in pericercarial envelope formation), capability for cryopreservation, change 
from heterochromatic to euchromatic nuclei, and observation of migrating cyton granules 
or vesicles through cytoplasmic bridges in the tegument indicate that transformation of 
the cercarial head into a schistosomulum has occurred [69].

1.10 Heat shock response

Originally named for its discovery following elevated heat treatment, the heat shock 
response is a well-conserved response across many different organisms to cellular stress 
from a variety of sources such as heat, oxidative radicals, injury, and infection [70–72]. 
Such insults usually lead to proteotoxic conditions, under which proteins can no longer 
function properly and adopt a non-native folding state, often resulting in protein 
aggregation and cell cycle arrest or cell death [71]. The increased population of misfolded 
proteins prompts a stressed cell to upregulate the transcriptional activity of the master
regulator of the heat shock response, heat shock factor (Hsf), which, under normal (unstressed) conditions and depending on the organism, is held transcriptionally inactive, by sequestration in a complex with chaperone heat shock proteins such as Hsp70 and Hsp90, and/or by phosphorylation state. Activation of the heat shock response (stress) leads to the release of Hsf inhibition, resulting in elevated levels of heat shock protein transcripts and proteins (Figure 1.5).

Figure 1.5. Heat shock response. (From [71]).

These chaperone heat shock proteins function to bind and refold proteins in an ATP-dependent manner. For example, in the Hsp70 chaperone model, a complex forms
consisting of Hsp40, a misfolded client protein (alternatively called “S” for Substrate), and Hsp70 in the ATP-bound state (Hsp70*ATP), which has low affinity for the client protein (Figure 1.6). Hsp40 in this complex catalyzes the ATP hydrolysis activity of Hsp70, converting Hsp70*ATP to the ADP-bound state (Hsp70*ADP), which has much higher affinity for the client protein. A nucleotide exchange factor (NEF), often a member of a different heat shock protein family, replaces ADP with ATP, returning Hsp70 to its ATP-bound state and allowing the release of the (now properly folded) client protein [73,74].
1.11 Heat shock genes in schistosomes

Several heat shock response-related genes, including heat shock protein 40 (HSP40) [75,76], HSP90 [77], HSP70 [78], and heat shock factor (HSF) [79,80], have been identified in schistosomes. HSP40 was originally identified as a major 40 kDa protein in S. mansoni eggs (p40) that showed homology to Drosophila small heat shock proteins [75]. A subsequent study found that treatment of mice with either recombinant or native p40 resulted in a Th1-type immune response with elevated IL-2 and IFN-γ levels.
suggesting that egg deposition initially leads to p40-mediated Th1 response before progression toward a Th2 response [81]. Further studies have not yet been performed on schistosome \textit{HSP40} and \textit{HSP90}.

Most studies on heat shock genes and proteins in schistosomes have centered on \textit{HSP70}. Recombinant Hsp70 protein from \textit{S. mansoni} has been used in ELISA assays to detect the presence of host antibodies in the serum of chronically, but not acutely, infected human and mouse subjects, suggesting a potential diagnostic use for Hsp70 [82]. Interestingly, the host immune system shows exquisite specificity against Hsp70 proteins from different schistosome species, as demonstrated by the lack of cross-reactivity of sera from \textit{S. japonicum}-infected human and mouse to in vitro translated \textit{S. mansoni} mRNA and vice versa [83]. Hsp70 has also been observed to be among the earliest synthesized proteins after host invasion, as measured by labeled methionine incorporation and two-dimensional gel electrophoresis [84].

A more recent series of studies on heat shock genes in schistosomes by the Schechter group began with the demonstration of the ability of schistosome protein extract to bind DNA sequences contained in the Hsp70 promoter region, or heat shock DNA binding element (HSE) [79]. In subsequent papers, the group compared the DNA sequence recognition of schistosome extract and recombinant Drosophila Hsf and schistosome Hsf [80,85,86], and showed the expression of different \textit{HSF} isoforms in different parasite stages [80,87,88].
In addition to their work on \textit{Sm}Hsf, the Schechter group also cloned the \textit{SmHSP70} gene [89] and promoter [90], and showed stage-specific expression of \textit{HSP70} transcript by Northern blot and the presence of Hsp70 protein in several stages by Western blot [91]. HSP70 has also been described in \textit{S. japonicum}, in which the Wu group cloned and characterized two genes belonging to the HSP70 family [92,93], and a further study identified \textit{Sj}Hsp70 as a potential vaccine candidate [94].

1.12 Thesis objective

This thesis has the following objective:
Characterize and identify a role for the heat shock response-related proteins 1) heat shock factor (\textit{Sm}Hsf1) and 2) heat shock protein 70 (\textit{Sm}Hsp70) in \textit{Schistosoma mansoni}, with a specific focus on the cercaria to schistosomulum stage transition.

1.13 Study rationale

We reasoned that heat shock response may play an important role during the transition of schistosome parasites from the cercaria stage to schistosomulum stage because of the drastic change in the environment during this transition. Such changes are associated with cell stress, which can activate the heat shock response.

The Jolly lab has expertise in studying transcription factors [95], and, therefore, we undertook the investigation of the schistosome heat shock transcription factor (\textit{Sm}Hsf1). Our findings from this study implicated a role for \textit{Sm}Hsf1 in the development of the
cercarial glands, which facilitate the invasion of the mammalian host. Because Hsf regulates downstream effector proteins of the heat shock response, such as Hsp70, we asked whether these heat shock effector proteins play a role in the cercaria stage, especially in the context of cercarial honing.

So far, studies have identified host molecules and other stimulatory factors that serve as the initial signals to activate cercarial honing. However, little is known about the downstream signals and proteins involved following the reception of these initial factors. The limited knowledge on this topic consists of some studies that suggest that protein kinase C (PKC) and other kinases may play a role during cercarial honing, since various stimuli, such as skin lipid and changes in light, have been observed to modulate the activity of these kinases [96,97]. We address this gap in understanding in this thesis by investigating and identifying a novel role for SmHsp70 in cercarial honing.

1.14 Summary of findings
With respect to the thesis objectives and study rationale above, this thesis demonstrates that 1) SmHsf1 is localized to the acetabular glands of cercariae and 2) SmHsp70 may act as a regulatory factor that controls cercarial honing during mammalian host invasion. These novel findings represent a major step forward in the understanding of the molecular components necessary during the host invasion process.
1.15 References


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Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator

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2.1 Abstract

Schistosomiasis is a chronically debilitating disease caused by parasitic worms of the genus *Schistosoma*, and it is a global problem affecting over 240 million people. Little is known about the regulatory proteins and mechanisms that control schistosome host invasion, gene expression, and development. Schistosome larvae, cercariae, are transiently free-swimming organisms and infectious to man. Cercariae penetrate human host skin directly using proteases that degrade skin connective tissue. These proteases are secreted from anucleate acetabular glands that contain many proteins, including heat shock proteins. Heat shock transcription factors are strongly conserved activators that play crucial roles in the maintenance of cell homeostasis by transcriptionally regulating heat shock protein expression. In this study, we clone and characterize the schistosome Heat shock factor 1 gene (*SmHSF1*). We verify its ability to activate transcription using a modified yeast one-hybrid system, and we show that it can bind to the heat shock binding element (HSE) consensus DNA sequence. Our quantitative RT-PCR analysis shows that *SmHSF1* is expressed throughout several life-cycle stages from sporocyst to adult worm. Most interesting, using immunohistochemistry, a polyclonal antibody raised against an Hsf1-peptide demonstrates novel localization for this conserved, stress-modulating activator. Our analysis suggests that schistosome Heat shock factor 1 may be localized to the acetabular glands of infective cercariae.

2.2 Author Summary

Schistosome parasites are the cause of human schistosomiasis and infect more than 200
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million people worldwide. Schistosome larvae, termed cercariae, are a free-swimming mobile developmental stage responsible for host infection. These larvae produce enzymes that degrade human skin allowing them to pass into the human host. After invasion, they continue evade the immune system and develop into adult worms. The transition from free-swimming larvae in freshwater to invasion into a warm-blooded saline environment requires that the parasite regulate genes to adapt to these changes. Heat shock factor 1 is a well-characterized activator of stress and heat response that functions in cellular nuclei. Using immunohistochemistry, we observed non-nuclear localization for anti-Heat shock factor 1 signal in the secretory glands necessary for the invasive function of schistosome larvae. This observation expands the potential mechanistic roles for Heat shock factor 1 and may aid in our understanding of schistosome host invasion and early development.

2.3 Introduction

Schistosomiasis affects more than 240 million people worldwide, ranking second after malaria in the World Health Organization listing of neglected tropical diseases. [1-6]. Resistance to praziquantel, the primary therapeutic used for decades to treat schistosome infection has been reported [7] and partial efficacy is observed in some patients [8]. Thus, the development of novel drug strategies and alternative treatment options are pressing issues in schistosome research [9].

Understanding host-parasite interactions can lead to novel therapeutic strategies that can interfere with infection or eliminate established infections. For example, topical
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator application of inhibitors against the proteases of infective cercariae can block skin invasion [10, 11]. We have been exploring heat-shock pathway components as a potential essential pathway in larval schistosomes. In protozoan parasites, heat shock proteins are essential for mediating changes in morphology during stage differentiation that are often concurrent with stress-related transitions from insect to mammalian host, or extracellular to intracellular conditions [12-17]. In human cancers, the heat shock proteins chaperone function mediates oncogenic transformation and blocks apoptosis [18, 19]. In our Schistosoma mansoni system, heat shock proteins 70 and 89 were identified as abundant components of cercarial secretions used for host invasion [20, 21], suggesting a potential role in host-parasite interactions as well.

We have focused on characterizing Heat shock factor 1 in S. mansoni as a route to better understanding the role of heat shock pathway activation in infective larvae. Schistosome infections occur when the skin of a potential mammalian host is exposed to schistosome larvae in freshwater. The larvae penetrate the skin and begin development into adult worms. Identification of suitable targets via drug screening approaches are ongoing, but challenges remain to translate the results of these screens into useful anti-schistosomal drugs [22-26]. Therefore, a more thorough understanding of basic schistosome biology and schistosome infection strategies is necessary.

Schistosomes require both molluscan and mammalian hosts for parasite development. The free-swimming and infectious schistosome larvae, cercariae, penetrate human skin
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator with the aid of proteolytic enzymes [21, 27-29]. During penetration, cercariae lose their tails, while the cercarial head continues to develop, transforming into the next developmental stage, the schistosomula. After invasion, schistosomula immediately begin host immune system evasion strategies, elongate, and develop into male and female adults. Worms pair in the liver, then travel to the veins of the bladder or small intestine, where they produce eggs, the pathologic agent in schistosomiasis. Once the eggs are excreted and reach fresh water, the eggs hatch into transient and free-swimming miracidia, which invade a molluscan host, develop into mother and daughter sporocysts, and mass produce infectious cercariae [27], completing the life-cycle.

The transformation from infectious cercariae to schistosomula involves not only a morphological change, but also includes a change in temperature (from the temperature of the external water to that of the host body (37°C)) and osmolarity (from relatively hypotonic freshwater to a saline environment in the bloodstream of the human host). Before transforming into schistosomula, cercariae must first breach the host skin barrier. Cercariae penetrate human skin by releasing the contents of two sets of acetabular glands (preacetabular and postacetabular). These glands produce many substances, including the proteolytic enzyme cercarial elastase (which breaks down host skin) and mucins (which enable adhesion to the host skin) [11, 19, 29-33]. In addition, these glands contain a conglomerate of other proteins such as the heat shock proteins (HSPs) Heat shock protein 70 (Hsp70), Heat shock protein 90 (Hsp90), and Heat shock protein 60 (Hsp60) [20, 21, 28]. After the acetabular glands release their contents, they atrophy to make space for gut
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator and other organ development [34]. Since schistosomula effectively survive the transformation from cercariae, we reasoned that a heat shock response system could be involved in schistosome invasion, as well as adaptation to and survival in a warm-blooded human host.

The heat shock response pathway is a highly conserved and adaptive response system that has evolved to reduce stress-induced cellular damage (for review, see [35-37]). When cells are stressed by elevated temperature or by other cellular insults, HSPs such as Hsp70 bind unfolded proteins to prevent protein aggregation [38] and to maintain cellular integrity and organismal viability. A major regulator of the heat shock pathway is Heat shock factor 1 (Hsf1), a transcriptional activator that is critical for positive regulation of HSP transcript levels such as those of HSP70 [37, 39]. Under non-stress conditions, HSPs are thought to interact with Hsf1 and sequester its transcriptional activity [40, 41]. Under heat stress conditions, HSPs release Hsf1, allowing it to activate the transcription of HSP70 and other genes encoding HSPs.

Recently, the view that heat shock factors (HSFs) function solely to regulate the heat shock pathway has been changing [42]. Mounting evidence suggests that HSFs have complex roles in development. In *Drosophila*, the HSF gene functions in oogenesis and larval development [43], and in mice, mutations in the HSF gene result in developmental defects, infertility, retarded growth and lethality [44]. Similarly, HSF promotes an extended lifespan in *Caenorhabditis elegans* [45, 46], and is involved in the regulation of
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator apoptosis in cancer cell lines [47, 48].

Parasitic schistosomes have a gene encoding Hsf1 [49, 50] that responds to heat stress [51, 52]. A role for Hsf1 in the transformation from free-swimming cercariae to skin schistosomula has not been described. We reasoned that this transformation involves a heat shock response. To begin to address this question, we verified the existence of an HSF1 homolog in the infective stage of schistosomes, tested its ability to activate transcription, assessed its expression profile across different schistosome developmental stages, and examined its capacity to bind DNA. We report here the identification of an antibody, raised against a sequence from SmHsf1, which specifically labels the acetabular glands of invasive cercariae, an observation which may have potential implications for novel functions of SmHsf1 in schistosome host invasion and development.

2.4 Materials and Methods

2.4.1 Animals and parasites

Infected Biomphalaria glabrata snails (strain NMRI, NR-21962) were obtained from the Biomedical Research Institute (BRI, Rockville, MD). Schistosoma mansoni cercariae were shed from B. glabrata snails as previously described [53]. Sporocyst stage parasites were dissected from these snails.

2.4.2 Preparation of schistosomal RNA

Sporocyst, cercaria, and 4-hour schistosomulum, S. mansoni were each suspended in
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TRIzol reagent (Invitrogen, Carlsbad, CA) and homogenized by Dounce homogenization. As per manufacturer’s instructions for the PureLink RNA Mini kit (Invitrogen), the samples were then centrifuged, and a phenol-chloroform extraction was performed on the supernatant, followed by DNase I treatment. The eluted RNA was quantitated on a ND-8000 spectrophotometer (Thermo Scientific, Waltham MA). Adult stage and uninfected $B.\ glabrata$ snail RNA was obtained from the BRI.

2.4.3 Cloning

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using mixed schistosome RNA (from sporocyst, cercaria, schistosomulum, and adult stages) and Superscript III/Platinum Taq RT-PCR kit (Invitrogen) with forward primer oKI001 (5’-CATATGATGTATGGTTTCACATCTGGACCTGTA-3’) and reverse primer oKI002 (5’-GAATTCTCATTCCAATTCTTCCTCACAAAAATCAGG-3’) (Integrated DNA Technologies, Coralville, IA) for the schistosome gene Smp_068270 (www.genedb.org) and with cycling conditions: single cycle of (45°C for 30 min, 95°C for 2 min) and 25 cycles of (94°C for 30 sec, 50.4°C for 30 sec, 72°C for 2.5 min) with a final extension at 72°C 10 min. The RT-PCR product was subcloned into the SmaI restriction site of the pGBK7 vector (Clontech) to make plasmid pKI003, and sequenced (Elim Biopharmaceuticals, Hayward, CA) for verification.

2.4.4 Yeast transformation and modified yeast one-hybrid

$Saccharomyces\ cerevisiae$ yeast strain AH109 was transformed for a modified yeast
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator one-hybrid experiment (with pKI003 in this study) as previously described [54, 55]. Yeast cells were plated on synthetic dextrose medium lacking tryptophan (SD-Trp). Transformed colonies were patched onto SD-Trp plates overlaid with 1000 μg 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) and incubated at 30°C for 1 day. The yeast cells were used for a serial dilution growth test, for which the cells were grown to saturation in SD-Trp medium, diluted to a 600 nm absorbance (A₆₀₀) value of 0.85 (“1” in the dilution series), serially diluted from 1 to 10⁻⁵, and grown on synthetic dextrose medium lacking histidine or adenine (SD-His or SD-Ade) plates at 30°C for 3 and 4 days, respectively.

2.4.5 Electrophoretic mobility shift assay (EMSA)

Biotin-labeled and non-labeled oligonucleotide probes containing DNA binding sequences were designed and obtained from Integrated DNA Technologies. The double-stranded, biotin-labeled oligonucleotide oKI068(ds)(5’-Biotin-ttagaagccgccgagatct[aGAAagTTCtaGTAc]agatctagcgaagactctcct-3’) contains the genomic DNA sequence 112 base pairs upstream of the translation start site (-112) for Smp_106930, the schistosome HSP70 homolog; the brackets indicate the heat shock binding element (HSE), which closely resembles the HSE consensus sequence, a repeating inverted pentameric sequence (nGAAAnTTCnGAAAn) [42, 44, 56, 57]. Unlabeled oligonucleotides used for competition experiments include: oKI032 (5’-ttagaagccgccgagatct[aGAAagTTCtaGTAc]agatctagcgaagactctcct-3’) and oKI033 (reverse complement of oKI032), which match the sequence of oKI068(ds); oKI030
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(5’-ttagaagccgcaggagatct[cGAAtTTCgaCTAg]agatctacggaagactctcct-3’) and oKI031 (reverse complement of oKI030), which contain the genomic sequence 239 base pairs upstream of the translation start site of SmHSP70 (-239); oKI034 (5’-ttagaagccgcaggagatct[cGAAtTTCg]agatctacggaagactctcct-3’) and oKI035 (reverse complement of oKI034), which contain a shortened binding sequence from -239; oKI036 (5’-ttagaagccgcaggagatct[aCTTagTTCtaGTAc]agatctacggaagactctcct-3’) and oKI037 (reverse complement of oKI036), which contain three mutated nucleotides in the first pentameric repeat from -112; double-stranded oligonucleotide oAT012(ds)(5’-gctgaaggat[CTAAAAATAG]gcggatcggc-3’), which contains a DNA binding sequence for the putative schistosome myocyte enhancer factor 2 [54]; and oKI073 (5’-gatcgtcat[aGAAagTTCtaGAAc]gatc-3’) and oKI074 (reverse complement of oKI073), which contain the HSE consensus sequence [57]. To make the oligonucleotide probes double-stranded, matched single-stranded oligonucleotides were incubated at 100°C for 2 minutes, after which the temperature was reduced by 1°C each minute, ending when the temperature reached 30°C. The oligonucleotide names and sequences are summarized in S2.1 Table.

Biotin-labeled DNA was detected using the LightShift chemiluminescent EMSA kit (Thermo Scientific) according to the manufacturer’s guidelines. Briefly, 3.5 μg each of purified maltose binding protein (MBP) and MBP-SmHsf1 fusion protein was incubated with 100 fmol of the biotin-labeled oligonucleotide probes either alone or together with 25 pmol of non-labeled oligonucleotide probes in binding buffer, glycerol, MgCl2,
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator poly-dIdC (nonspecific DNA competitor), and NP-40 detergent for 30 minutes. The protein-DNA complexes were run on a 5% native polyacrylamide gel in 0.5× TBE at 200 V for 1 hour, transferred to a nylon membrane in 0.5× TBE at 350 mA for 1 hour, and crosslinked on a CL-1000 Ultraviolet Crosslinker (CVP) at an energy setting of 120 mJ/cm². After crosslinking, the membrane was blocked, incubated with a stabilized streptavidin-horseradish peroxidase conjugate, washed, incubated with luminol/enhancer and stable peroxide solution, and visualized on a CCD camera (Fotodyne, Hartland, WI).

2.4.6 Comparison of protein sequences

Hsf1 protein sequences from Schistosoma mansoni (GeneDB, Smp_068270.2), Schistosoma japonicum (GeneDB, Sjp_0064040), Caenorhabditis elegans (GenBank: AAS72410.1), Saccharomyces cerevisiae (Saccharomyces genome database, strain S288C: YGL073W), Drosophila melanogaster (NCBI RefSeq: NP_476575.1), Xenopus laevis (NCBI RefSeq: NP_001090266.1), Mus musculus (GenBank: AAH94064.1), and Homo sapiens (NCBI RefSeq: NP_005517.1), along with the protein sequence corresponding to the conserved domain of Hsf1 (NCBI conserved domains, Cdd:smart00415) [9, 10, 58], were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) [7, 8] using the default parameters. TreeViewX software was used to generate a phylogram.

2.4.7 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Primers specific to Smp_068270.2 (forward primer oKI040: 
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5'-TGGTAATGACGAGTGTGACGTA-3', reverse primer oKI042:

5'-TCAACATTAAGGCCTACAGGAAA-3') were designed using the Primer3 web applet [59, 60]. One microgram each of sporocyst, cercaria, 4-hour schistosomulum, and adult stage RNA was subjected to a reverse transcriptase reaction with oligo dT (Promega), and 50 ng of the resulting cDNA was used for a relative $\Delta\Delta C_T$ qPCR using SYBR Green PCR Master Mix (Applied Biosystems) and primers oEJ548 (5'-AGTTATGCGGTGTGGGTCAT-3') and oEJ549 (5'-TGCTCGAGTCAAAGGCCTAC-3') with cytochrome c oxidase subunit 2 (TC7399, TIGR database) as the reference gene. qRT-PCR products are intron-spanning. All experiments were done in triplicate. A two-tailed t-tested was applied to $\Delta C_T$ values as the statistical test to determine significant differences in transcript expression levels relative to the cercaria stage.

2.4.8 Recombinant protein purification

Smp_068270.2 was subcloned into pMAL-c5X (NEB) at NdeI and EcoRI by ligation using T4 DNA ligase (NEB, Ipswitch, MA) to make plasmid pKI058, and transformed into BL21(DE3) chemically competent E. coli bacterial cells (Invitrogen). BL21(DE3) cells carrying either plasmid pKI058 or empty vector pMAL-c5X were induced with isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich, Saint Louis, MO) to a concentration of 0.4 mM at 37°C for 6 hours, and cell pellets were frozen overnight. Cells were lysed by pulse sonication (Sonifier 250, Branson, Danbury, CT) in a phosphate lysis buffer (50 mM potassium phosphate at pH 8.0, 200 mM NaCl) containing
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10 mM phenylmethylsulfonyl fluoride (PMSF) and 100 μL Halt Protease Inhibitor Cocktail (Thermo Scientific). The lysate was cleared by centrifugation at 10,000 × g for 30 minutes at 4°C (Sorvall), and the cleared supernatant was incubated with amylose resin beads (NEB) at 4°C overnight with gentle rocking. Purified protein (MBP and MBP-SmHsf1, respectively, from pMAL-c5X and pKI058) was eluted from the amylose beads (50 mM potassium phosphate at pH 8.0, 200 mM NaCl, 10 mM maltose), dialyzed against 3 changes of protein storage buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol) Slide-a-lyzer, Thermo Scientific) and concentrated with 30k and 50k MWCO Amicon columns (Millipore, Billerica, MA). The proteins were quantified using the Bradford reagent (Bio-Rad, Hercules, CA) and an ND-8000 spectrophotometer (Thermo Scientific).

2.4.9 Custom antibody production

A polyclonal antibody raised in New Zealand white rabbits against the peptide with sequence Cys-KYKKEIRKQHKI from Smp_068270 (SmHsf1) was designed and purchased (Pacific Immunology, Ramona, CA). The peptide sequence used for antibody production was Blasted against the NCBI schistosome protein database to prevent production of an antibody cross-reactive with other schistosome proteins. IgG was purified from the pre-immune serum using Melon Gel IgG Spin Purification Kit (Thermo Scientific).
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2.4.10 Western blotting

To detect *Sm*Hsf1 protein, 1 µg purified MBP, 1 µg and 7 µg MBP-*Sm*Hsf1 fusion, and approximately 5 µg of cercarial protein extract were resolved on a 5% polyacrylamide gel and transferred to nitrocellulose membranes in ice-cold Towbin transfer solution (25 mM tris, 192 mM glycine, 20% methanol) at 400 mA for 2 hours. Following the transfer, the membranes were blocked in 5% milk dissolved in phosphate buffered saline, 0.1% Tween-20 (PBSTw) on an orbital shaker at room temperature for 1 hour. Purified IgG from pre-immune serum or immune serum was added to a concentration of 0.5 µg/mL, and the membranes were gently rocked at 4°C overnight. The membranes were washed in PBSTw on an orbital shaker for 5, 10, and 15 minutes, after which an HRP-linked goat anti-rabbit secondary antibody (GE Healthcare) was added at a dilution of 1:2500 in 1% milk/PBSTw, followed by orbital shaking at room temperature for 1 hour and washing in PBSTw. Amersham ECL Western blotting detection reagent (GE Healthcare) was added (2 mL per nitrocellulose membrane) and incubated at room temperature for 1 minute before the membranes were exposed to autoradiography film.

2.4.11 Immunohistochemistry

A protocol adapted from Collins *et al.* (2011) was used to prepare samples for immunohistochemistry [61]. Briefly, cercariae were fixed for 20 minutes at room temperature in a 4% paraformaldehyde/PBSTw (PBS/ 0.1% Tween-20) solution, washed in PBSTw, then dehydrated in a methanol/PBSTw series and stored in 100% methanol at -20°C until use. Prior to use, cercariae were rehydrated, digested for 10 minutes at room
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator temperature in permeabilization solution (1× PBSTw, 0.1% SDS, and proteinase K (1 µg/mL)), and washed in PBSTw (all subsequent washes were carried out with nutation at room temperature). Cercariae were re-fixed for 10 minutes at room temperature in a 4% paraformaldehyde/PBSTw solution, and washed in PBSTw. Samples were incubated with rocking in block solution (PBSTw, 5% horse serum (Jackson ImmunoResearch Laboratories, West Grove, PA), 0.05% Tween-20, and 0.3% Triton X-100) for 2hrs at RT or overnight at 4°C. Samples were incubated with a polyclonal primary rabbit anti-\textit{Sm}Hsf1 antibody (described above) in block solution at a concentration of 0.6 µg/mL or 2.5 µg/mL, overnight at 4°C and washed >2hrs at room temperature. Samples were then incubated with an Alexa 647 donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) at a concentration of 1:400 or 1:800 in block solution, overnight at 4°C. Samples were washed in PBSTw (>2hrs), at room temperature with the second wash containing DAPI (1 µg/mL). After washing, samples were mounted in Slow Fade Gold (Invitrogen, Grand Island, NY). Pre-immune serum IgG (5 µg/mL), and no primary controls were run in parallel with experimental samples. For the no primary controls, samples were incubated in block solution alone during the primary incubation step.

2.4.12 Imaging

All samples were mounted in Slow Fade Gold mounting media. Samples were imaged on a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany) (Plan-Apochromat 63×/1.2 W objective). The Alexa 647 fluorophore was excited with a 633 nm laser and
the DAPI with a 405 nm laser. Images were processed using Zeiss LSM Image Browser (Carl Zeiss) or ImageJ.

2.5 Results

2.5.1 Schistosome Hsf1 is a transcriptional activator.

Hsf1 proteins function as activators of transcription. To test whether the Hsf1 protein from schistosomes (Smp_068270.2) is able to activate transcription, we performed a modified yeast one-hybrid analysis as previously described [54, 55]. Briefly, we made an N-terminal fusion of the DNA binding domain of the yeast Galactose 4 protein (Gal4DBD) with SmHSF1 to make the fusion protein Gal4DBD-SmHsf1. Gal4DBD can bind DNA, but it cannot activate transcription because its transactivation domain has been removed. The Gal4DBD-SmHsf1 fusion protein was expressed in a yeast strain that is auxotrophic for histidine and adenine (see Materials and Methods). Genes for alpha galactosidase (encoded by MEL1), histidine metabolism (encoded by HIS3), and adenine metabolism (encoded by ADE2), are regulated by promoter elements dependent on Gal4 binding and activation; these genes were used as reporters.

Expression of the Gal4DBD-SmHsf1 protein in yeast cells resulted in the induction of the MEL1 reporter gene, which was visualized by blue-colored yeast cells on SD-Trp/X-α-Gal plates (Figure 2.1A). To test whether SmHsf1 could also induce HIS3 and ADE2 reporter gene expression, yeast cells were selected for growth and viability by a serial dilution assay on synthetic medium lacking the respective nutritional marker.
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator (SD-His, SD-Ade). Yeast not expressing the selectable markers cannot survive. We found that yeast cells expressing Gal4DBD-SmHsf1 protein were viable and conferred histidine and adenine prototrophy to these cells (Figure 2.1B and C). Yeast cells expressing only the Gal4DBD were unable to induce activity from any reporter: they showed no blue color on SD-Trp/X-α-Gal plates and were not viable on SD-His and SD-Ade plates, while the positive control yeast cells expressing the full length Gal4 activator (Gal4Full) showed blue color on SD-Trp/X-α-Gal plates and were viable on SD-His and SD-Ade plates (Figure 2.1) These data demonstrate that SmHsf functions as a transcriptional activator.
Figure 2.1. SmHsf1 can drive transcription in a modified yeast one-hybrid system. Yeast cells expressing SmHsf1 fused to the Gal4 DNA binding domain (Gal4DBD-SmHsf1) were patched (A) or serially diluted (B and C, from 1 to $10^{-5}$) on different selective media to test the ability of SmHsf1 to activate transcription. The positive control yeast express a complete $GAL4$ gene (Gal4Full) and the negative control yeast express the $GAL4$ DNA binding domain alone (Gal4DBD). (A) Blue color on SD-Trp with X-α-Gal indicates expression of the $MEL1$ reporter gene. (B and C) Growth on the SD-His and SD-Ade plates indicates expression of the $HIS3$ and $ADE2$ reporter genes, respectively, and are essential for cell viability.
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2.5.2 SmHsf1 recognizes the heat shock DNA binding element from the schistosome HSP70 promoter.

Heat shock factors recognize promoter sequences that regulate several heat shock response genes (such as HSP70) by binding to heat shock factor DNA binding elements (HSEs) consisting of repeating inverted pentameric sequences: nGAAAnTTTCnnGAAAn [42, 56]. We tested whether SmHsf1 can bind to the HSE located 112 base pairs from the translation start site of the SmHSP70 gene using an electrophoretic mobility shift assay (EMSA) (Figure 2.2). Recombinant, purified MBP-SmHsf1 fusion protein was incubated with a double-stranded DNA (dsDNA) oligonucleotide probe, oKI068(ds), containing the HSE from the SmHSP70 promoter (Figure 2.2, lane 3). The dsDNA oligonucleotide sequence was labeled with biotin for chemiluminescent EMSA detection (see Materials and Methods). Unlabeled dsDNA oligonucleotide probes were used for competition experiments and matched the following: 1] an HSE sequence found at -239 base pairs from the SmHSP70 translation start site (DNA oligonucleotide pair oKI030/031), 2] an HSE sequence found at -112 base pairs from the SmHSP70 translation start site (DNA oligonucleotide pairs oKI032/033), 3] an HSE sequence found at -239 base pairs from the SmHSP70 translation start site lacking the third pentameric repeat (DNA oligonucleotide pair oKI034/035), 4] an HSE sequence found at -112 base pairs from the SmHSP70 translation start site with three base pairs of the first pentameric repeat mutated (DNA oligonucleotide pair oKI036/037), 5] a negative control sequence reported to bind the putative schistosome Myocyte enhancer factor 2 (dsDNA oligonucleotide oAT012(ds)) [54]; and the HSE consensus sequence (DNA oligonucleotide pair oKI073/074) (Figure
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2.2, lanes 4-9) [57]. Consistent with previous findings [49, 57], we found that MBP-\(Sm\)Hsf1 binds the HSE from the schistosome \(HSP70\) promoter. Our competition experiments show that MBP-\(Sm\)Hsf1 also recognizes the consensus HSE with great affinity (Figure 2.2, lane 9). The HSE consensus sequence is found in the promoter region of \(Drosophila\) \(HSP70\) [6, 62]. This led us to compare the protein sequence of the conserved domain (which contains the DNA binding domain) of \(Sm\)Hsf1 to that of Hsf1 from other species using ClustalW2 (Figure 2.3). Our analysis showed the expected result that Hsf1 from \(Schistosoma\) \textit{mansoni} and the related species, \(Schistosoma\) \textit{japonicum}, cluster together. However, these flatworm Hsf1 sequences appear to cluster more closely to sequences from \(Drosophila\) than to those from other organisms, including the roundworm \textit{C. elegans}.
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Figure 2.2. SmHsf1 binds the heat shock binding element from the schistosome HSP70 promoter. Recombinant SmHsf1 cloned as a fusion protein with maltose binding protein (MBP-SmHsf1), or maltose binding protein (MBP) alone, was incubated with the double-stranded biotin-labeled oligonucleotide oKl068(ds) containing the heat shock binding element sequence from the schistosome HSP70 promoter. Unlabeled competitor oligonucleotide probes were added at a 250-fold molar excess relative to oKl068(ds) in lanes 4-9. Labeled DNA was detected by chemiluminescence. Oligonucleotide sequences for the probes are shown in S2.1 Table.
Figure 2.3. Phylogram of *SmHsf1*. Protein sequences of the Hsf1 conserved domain from various species were aligned using ClustalW2, and the phylogram was generated using TreeViewX software. The following settings were used for the protein alignment: Protein Weight Matrix (Gonnet); Gap open (10); Gap extension (0.20); Gap distances (5); No end gaps allowed; Single iteration; Clustering (NJ).
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2.5.3 *SmHSF1* is expressed across schistosome developmental stages.

To determine the expression level of *SmHSF1* during schistosome development, we used quantitative reverse transcriptase PCR (qRT-PCR) to assess *SmHSF1* transcript levels from sporocyst, cercaria, 4-hour schistosomula, and adult stages. Relative to the cercaria stage, *SmHSF1* was expressed 2.3, 1.8, and 1.4-fold in sporocyst, 4-hour schistosomula, and adult stages, respectively (Figure 2.4, p < 0.05). Thus, for all schistosome developmental stages analyzed, *SmHSF1* is expressed. Values of ΔΔCT were used for a two-tailed t-test to determine significant differences in expression levels.

![Figure 2.4](image)

**Figure 2.4.** *SmHSF1* is expressed across several schistosome life-cycle stages. qRT-PCR of *SmHSF1* transcript was performed on sporocyst, cercaria, 4-hour schistosomula, and adult stages with 3 replicates. Cytochrome c oxidase was used as the reference gene and cercaria as the reference stage. The ΔΔCT method was used to analyze the qRT-PCR data.
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2.5.4 A polyclonal antibody detects the SmHsf1 protein.

A custom polyclonal antibody was designed against peptide sequence (Cys-KYKKEPIRKQHKI) that is common to the known splice variants of the SmHsf1 protein. Prior to antibody production, the peptide sequence was compared to known schistosome protein sequences by BLASTP search and no statistically significant alignment to other proteins was identified. To test whether the antibody recognizes SmHsf1, we expressed and purified SmHsf1 as a fusion protein to the maltose binding protein (MBP-SmHsf1) to increase solubility and to aid in purification. We performed a Western blot on the recombinant MBP-SmHsf1 and cercarial protein extract separated by SDS-PAGE, using both pre-immune serum and the purified polyclonal anti-SmHsf1 antibody (Figure 2.5). The antibody detected bands at approximately 130, 110, and 70 kDa for the recombinant MBP-SmHsf1 fusion protein (Figure 2.5, lanes 2 and 3), all of which were sequence confirmed to contain Hsf1 protein by LC-MS/MS. In cercarial extract, bands were observed at approximately 110, 65, and 50 kDa for the cercarial protein extract, (Figure 2.5, lane 4), and no non-specific reactivity was observed to the extract using IgG from pre-immune serum (Figure 2.5, lane 5). In the context of highly abundant background proteins in a complex lysate, it was not possible to detect Hsf1 peptides in the 110, 65, and 50 kDa bands from cercarial extract.
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**Figure 2.5.** The *SmHsf1* antibody recognizes the *SmHsf1* protein. Purified IgG from *SmHsf1*-immunized rabbit bleeds (lanes 1–4) or pre-immune serum (lane 5) were used in a Western blot to test for reactivity against bacterially expressed recombinant proteins and cercarial extract; (lane 1), 1 µg MBP negative control; (lane 2), 1 µg MBP-*SmHsf1* fusion protein; (lane 3), 7 µg MBP-*SmHsf1* fusion protein; (lanes 4 & 5), 7 µg cercarial extract.

The expected molecular weight of *SmHsf1* is approximately 73 kDa in molecular weight,
which when fused to 42 kDa MBP should result in an approximately 115 kDa MBP-SmHsf1 fusion protein. Since Hsf1 can be highly post-translationally modified [63], we assessed whether treatment with alkaline phosphatase or deglycosidase could collapse the higher molecular weight bands at 130 and 110 kDa, but observed no band shift. Analysis of MBP signal in these recombinant protein bands by Western blot (S2.2 Figure) demonstrated a similar band pattern to the anti-SmHsf1 blot. Treatment of recombinant MBP-SmHsf1 with Factor Xa protease produced the desired cleavage result by liberating the 42 kDa MBP; however, after cleavage SmHsf1 is not detected using the antibody against Hsf1, suggesting the Hsf1 protein is not very stable (data not shown).

2.5.5 Antibody raised against SmHsf1 localizes to the acetabular glands of S. mansoni cercariae.

We used indirect immunohistochemistry to determine the location of SmHsf1 expression in fixed cercariae (Figure 2.6 and Figure 2.7; S2.3-S2.7 Movie). We predicted that SmHsf1 should produce punctate staining to nuclei throughout the cercariae. We reasoned this because as a transcription factor, Hsf1 is usually localized to the nucleus to induce activation of HSPs [37, 39]. To our surprise, we observed targeted SmHsf1 localization to the cercarial acetabular glands, which run the length of, and comprise a large percentage of, the cercarial head (Figure 2.6I, Figure 2.6K, and Figure 2.7; S2.3 Movie and S2.6 Movie). Cercarial acetabular glands are composed of three pairs of postacetabular and two pairs of preacetabular glands, whose secretions are thought to be involved in host invasion [31, 64, 65]. These anucleated glands are unicellular, have large fundi located
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator.

Anterior and posterior to the acetabulum (i.e., pre and post), and have ducts composed of long cellular processes that extend anteriorly to the tip of the oral sucker [64, 66]. Both sets of glands are filled with secretory granules whose contents are thought to be involved in attachment to, and subsequent penetration of, the definitive host. The postacetabular glands contain secretory granules of mucigen, and the preacetabular gland granules contain proteinases [29, 31, 65, 67]. Labeling with the \( Sm \)Hsf1 antibody occurred along the length of the glands, rather than being restricted to the fundus as might be expected of a transcription factor (Figure 2.6I and Figure 2.7; S2.3 Movie and S2.6 Movie). Our data show that \( Sm \)Hsf1 is primarily localized to the postacetabular glands. Additionally, \( Sm \)Hsf1 localization and DAPI staining seem to be mutually exclusive (S2.5 Movie). The no primary and pre-immune controls did not show labeling (Figure 2.6, panels A and E, respectively).
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Figure 2.6. *SmHsf1* is localized to the acetabular glands in *S. mansoni* cercariae. (A-L) Single, representative confocal sections of cercariae. A custom, rabbit polyclonal primary antibody against *S. mansoni* Heat shock factor 1 protein (*SmHsf1*) and a donkey anti-rabbit Alexa 647 secondary antibody were used to detect *SmHsf1* in cercariae. (A-D) No primary negative control. The anterior region (mouth) is located near the bottom of the panels. (E-H) Pre-immune serum IgG negative control. The anterior region is located to the left. (I-L) Anti-*SmHsf1*. In panel I, *SmHsf1* is localized to the acetabular glands (red) which traverse the entire head of the cercariae from the posterior (left) to anterior (bottom right). Panels B, F, and J are stained with DAPI. Panels C, G, and K are merged Alexa 647 and DAPI images. Panels D, H, and L are Differential Interference Contrast (DIC) images for each treatment.
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Figure 2.7. Antibody raised against SmHsf1 localizes to the acetabular glands extending the entire length of the *S. mansoni* cercarial head. Immune staining, as in Figure 2.6, was used to localize anti-SmHsf1 signal (red) to acetabular glands of the *S. mansoni* cercariae. The anterior (mouth) is to the bottom left of the image. The image is a maximum confocal projection, and the magnification is with a 63×/1.2 W objective.

2.6 Discussion

Hsf1 proteins are highly conserved and well-characterized transcriptional activators. Hsf1 in schistosomes has been previously described [49, 57, 68]. We expand the initial observations on SmHsf1, and we increase our knowledge of this protein. Hsf1 functions as a transcriptional activator of HSPs in other systems. We cloned the *SmHSF1* gene and assessed whether SmHsf1 functions as an activator in a heterologous yeast reporter system. Our results confirm that SmHsf1 is a positive regulator of transcription (Figure 2.1). Using qRT-PCR, we show that *SmHSF1* transcript is expressed in all stages tested, but that the level of *SmHSF1* is relatively high in sporocysts and relatively low in cercariae. Our findings of transcript levels of *SmHSF1* support the idea that a larger pool
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator of heat shock proteins is required to maintain cell homeostasis in sporocysts because of their elevated protein levels for the mass production of cercariae [69]. Alternatively, this may reflect the general lower transcript levels observed in cercariae, or priming of cercarial transcripts by production of some cercarial transcripts in sporocysts.

The transcript levels of the Hsf1 target, \textit{SmHSP70}, do not change in response to salt or temperature increases in cercariae as expected of a stress response gene [51]. It was speculated that the lack of increase in \textit{HSP70} transcript levels in cercariae prior to the loss of the cercarial tail during transformation is due to tail-dependent inhibitory signals that terminate the transcription of \textit{HSP70} [51]. In support of these observations, we found that the HSP activator, \textit{SmHsf1} protein, is primarily localized to the cercarial acetabular glands, which are unicellular and lack nuclei [64, 66]. We also found that the \textit{SmHsf1} staining does co-localize with the DAPI nuclear stain elsewhere in the cercariae. Thus, it appears that in the absence of nuclear localization, cercarial \textit{SmHsf1} would be unable to induce the transcription of new HSPs, including \textit{HSP70}, despite expectations for this transformation to be a high stress condition. Furthermore, localization of anti-\textit{SmHsf1} signal to cercarial acetabular glands is suggestive of alternative function for this transcription factor. This motivates our further research into the specificity of this novel immunolocalization.

The schistosome acetabular glands are long, unicellular structures that produce, store, and release a variety of substances such as mucins and elastases/proteinases to assist in
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator adhesion and invasion of human skin [64]. Curiously, SmHsf1 has not been detected in cercarial secretions [20, 21], suggesting that SmHsf1 protein may be bound directly or indirectly to the acetabular cell membrane. At the time of host invasion, the acetabular glands are no longer nucleated [64, 66], raising the possibility of an alternative function for SmHsf1 beyond a transcriptional role in cercariae and newly transformed schistosomula. SmHsf1 may be required for the production of chaperone proteins during the development of the glands in early embryonic cercariae in the molluscan host. After fragmentation of the gland nucleus, SmHsf1 could be released into the cytoplasm, where it can interact with membrane-associated proteins (SmHsf1 has no known transmembrane domains), facilitating SmHsf1 to remain bound to the glands during the secretion of other gland contents. A non-nuclear Hsf1 is also observed in non-small cell lung cancer line cells, in which Hsf1 associates with and disables the anti-apoptotic membrane-bound Ralbp1 protein [47, 48]. One scenario is that SmHsf1 functions to block a related anti-apoptotic factor, allowing apoptosis of the glands to occur. Indeed, in 5-day old schistosomula, the acetabular glands are disintegrated [34], and our immunohistochemical analysis using the anti-SmHsf1 antibody shows no acetabular SmHsf1 localization in 5-day old schistosomula (data not shown). SmHsf1 could be involved in the degradation of acetabular glands in schistosomula, allowing space for the development of organs such as the gut. Additional investigation is required to reveal the mechanism by which SmHsf1 remains in the glands, and whether it contributes to the controlled disintegration of the glands, will be of significant interest. In addition, our data suggest that the anti-SmHsf1 antibody is primarily localized to the postacetabular glands.
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator in cercariae, although we do not exclude the preacetabular glands. It is not clear why there appears to be a preference for the postacetabular glands, responsible for the deposition of cercarial mucins and for providing an adhesive substrate for the parasite to remain attached to host skin [31, 64].

Hsf1 and HSPs could play even broader roles in schistosome developmental regulation. Hsp70 was identified as being responsible for mediating the association and dissociation of the 26S proteasome in mouse embryonic fibroblasts [16]. The 26S proteasome is a conserved set of proteins that function in protein turnover and protein recycling, cell cycle progression through degradation of cyclins, and modulation of cell death [12-15, 17]. A major component of the 26S proteasome, the 20S proteasome is reported to bind tightly to Hsp90 in schistosomes, and it has different forms of reactive subunits in cercariae relative to newly transformed schistosomula [18], again connecting the heat shock pathway to cellular development. Examination of the role of SmHsf1 and HSPs during and after the invasion of human skin not only represents the study of a potentially novel developmental regulatory mechanism, but it can also help to identify key proteins necessary for parasite invasion and development that can be used as therapeutic targets to decrease schistosomula viability in the host. Alternatively, inhibiting the heat shock pathway in cercariae may have unpredictable effects on the ability of schistosomes to infect their host. The heat shock system used by schistosome cercariae during host invasion may also apply to other parasites that undergo environmental transitions, such as *Ancylostoma duodenale* (hookworm), which transition from soil to host, or *Toxoplasma*
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator

_{gondii}, which transition from extracellular to intracellular during muscle and brain invasion.

Our data demonstrate that the antibody raised against a peptide in _SmHsf1_ can recognize _SmHsf1_, but we cannot rule out the possibility that it does not interact with another cercarial protein, and that another protein is responsible for this unique localization to the acetabular glands. Given that our data support previous evidence that the _SmHsf1_ protein is extremely insoluble and unstable [49], we were unable to statistically identify the protein in cercariae using mass spectrometry. However, if _SmHsf1_ is not responsible for this novel acetabular localization and it is another protein, this observation provides a cellular marker and an excellent impetus to begin to explore molecular factors in acetabular regulatory mechanisms as well as an opportunity to further explore host-parasite interactions and parasite development. Understanding the mechanisms for schistosome invasion and development can promote the discovery of novel treatments to combat parasitic infections.

### 2.7 Acknowledgements

Infected snails and adult RNA were provided to E. Jolly by the BRI via the NIAID Schistosomiasis resource center under NIH-NIAID contract No. HHSN2722010000051: _Schistosoma mansoni_, Strain NMRI-exposed _Biomphalaria glabrata_, Strain NMRI, NR-21962. We also thank Ronald Blanton and Blanton Tolbert for helpful discussions, and Anida Karahodza and Shuang Liang for critical review of the manuscript.
2.8 Supporting information

**S2.1 Table. Names and sequences of oligonucleotides used for EMSA.**
doi:10.1371/journal.pntd.0003051.s007 (Table S1 in published version).

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Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator

S2.2 Figure. MBP antibody recognizes the MBP-SmHsf1 fusion protein. An antibody against MBP-fused with HRP (Abcam, ab49923) was used in a Western blot to probe for MBP in recombinant proteins prepared from E. coli. (lane 1) 5 µg MBP positive control (lane 2) 5 µg intact MBP-SmHsf1 fusion protein (lane 3) 5 µg recombinant MBP-SmHsf1 cleaved with Factor Xa. Signal was detected by chemiluminescence (Pierce ECL western blotting substrate, 32209). doi:10.1371/journal.pntd.0003051.s001 (Figure S1 in published version).

S2.3 Movie. Anti-SmHsf1 antibody is localized to the cercarial head. Z-stack images of an S. mansoni cercarial head showing anti-SmHsf1 localization. The anterior end the cercarial head is toward the bottom right. doi:10.1371/journal.pntd.0003051.s002 (Movie S1 in published version).

S2.4 Movie. DAPI staining of the cercarial head. Z-stack images of an S. mansoni cercarial head stained with DAPI. The anterior end of the cercarial head is toward the bottom right. doi:10.1371/journal.pntd.0003051.s003 (Movie S2 in published version).

S2.5 Movie. Anti-SmHsf1 and DAPI staining in the cercarial head. Merged z-stack images of an S. mansoni cercarial head showing anti-SmHsf1 localization and DAPI staining. The anterior end of the cercarial head is toward the bottom right. doi:10.1371/journal.pntd.0003051.s004 (Movie S3 in published version).
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S2.6 Movie. Rotational view of the cercarial head showing anti-SmHsf1 localization. Maximum projection images are shown. The anterior end of the cercarial head is toward the bottom. doi:10.1371/journal.pntd.0003051.s005 (Movie S4 in published version).

S2.7 Movie. Rotational view of the cercarial head stained with DAPI. Maximum projection images are shown. The anterior end of the cercarial head is toward the bottom. doi:10.1371/journal.pntd.0003051.s006 (Movie S5 in published version).

2.9 References


Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator


Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator


Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator


Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator


50. Neumann S, Ziv E, Lantner F, Schechter I. Cloning and sequencing of an hsp70
Chapter 2: Immunolocalization of anti-Hsf1 to the acetabular glands of infectious schistosomes suggests a non-transcriptional function for this transcriptional activator


60. Koressaar T, Remm M. Enhancements and modifications of primer design


Chapter 3: Hsp70 may be a molecular regulator of schistosome host invasion

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The numbering for the supporting information here differs from that in the published version. Digital object identifier (DOI) references are provided in section 3.8.
Chapter 3: Hsp70 may be a molecular regulator of schistosome host invasion

3.1 Abstract

Schistosomiasis is a debilitating disease that affects over 240 million people worldwide and is considered the most important neglected tropical disease following malaria. Free-swimming freshwater cercariae, one of the six morphologically distinct schistosome life stages, infect humans by directly penetrating through the skin. Cercariae identify and seek the host by sensing chemicals released from human skin. When they reach the host, they burrow into the skin with the help of proteases and other contents released from their acetabular glands and transform into schistosomula, the subsequent larval worm stage upon skin penetration. Relative to host invasion, studies have primarily focused on the nature of the acetabular gland secretions, immune response of the host upon exposure to cercariae, and cercaria-schistosomulum transformation methods. However, the molecular signaling pathways involved from host-seeking through the decision to penetrate skin are not well understood. We recently observed that heat shock factor 1 (Hsf1) is localized to the acetabular glands of infectious schistosome cercariae, prompting us to investigate a potential role for heat shock proteins (HSPs) in cercarial invasion. In this study, we report that cercarial invasion behavior, similar to the behavior of cercariae exposed to human skin lipid, is regulated through an Hsp70-dependent process, which we show by using chemical agents that target Hsp70. The observation that biologically active protein activity modulators can elicit a direct and clear behavioral change in parasitic schistosome larvae is itself interesting and has not been previously observed. This finding suggests a novel role for Hsp70 to act as a switch in the cercaria-schistosomulum transformation, and it allows us to begin elucidating the pathways associated with
Chapter 3: Hsp70 may be a molecular regulator of schistosome host invasion

cercarial host invasion. In addition, because the Hsp70 protein and its structure/function is highly conserved, the model that Hsp70 acts as a behavior transitional switch could be relevant to other parasites that also undergo an invasion process and can apply more broadly to other organisms during morphological transitions. Finally, it points to a new function for HSPs in parasite/host interactions.

3.2 Author summary

Parasitic schistosome worms cause morbid disease in over 240 million individuals worldwide. Acute infections with these worms can lead to Katayama fever, while chronic infections can lead to portal hypertension, enlarged abdomen, and liver damage. The infective larval stage, called cercariae, are free-swimming and can detect, seek, and penetrate human skin to enter the human host circulatory system, eventually developing into egg-laying adult worms that cause schistosomiasis. Molecular pathways associated with the initial cercarial invasion of the host, however, are largely unknown, especially with respect to the parasite-specific signals involved in host detection and subsequent decision to invade. Here, we describe a role for Hsp70 in cercarial invasion behavior. To date, only generic stimulation with skin lipid, linoleic acid or L-arginine are known to induce cercarial invasion behavior; thus, we can begin an initial investigation of molecular requirements for host invasion and environment transition for schistosomes and possibly other parasitic organisms.
3.3 Introduction

Schistosome parasites have six different morphological stages during their life cycle, which requires an intermediate molluscan and a definitive mammalian host that the parasite must correctly identify and invade. Free-swimming, freshwater cercariae (singular: cercaria) are released from infected molluscs and invade mammals and humans for further development into larval worms called schistosomula (singular: schistosomulum or schistosomule). Schistosomula adapt to survival in the host blood environment, evade the immune system, develop a gut to begin digesting red blood cells, elongate and traverse the human circulatory system, and eventually develop into egg-laying adult worms [1].

Cercariae are highly adapted for swimming and invading their mammalian hosts. Transcriptional studies show that cercariae have elevated expression of genes associated with metabolism and motility when compared with other stages [2, 3]. Free-swimming cercariae have a limited energy supply and a limited duration during which they can infect their host [4]. Thus, they must correctly identify and quickly respond to an appropriate host (or source of chemoattractant), swim toward it, and begin the host penetration process. For the purposes of this report, we call this behavior cercarial honing or simply, honing. Swimming cercariae respond to changes in light levels, to thermal gradients, and to chemicals such as linoleic acid and L-arginine released from human skin [5-9]. After reaching the skin, the cercariae crawl along the skin surface until they identify a suitable location to penetrate. Parasite invasion through the skin involves the
physical motion of swimming into the skin, in coordination with release of their acetabular gland contents, which include mucins to enhance the attachment to skin and proteases to degrade skin molecules [10-12].

While the ultrastructure of cercariae has been described before and after entry into the host [13-15], protein regulators of cercarial honing and invasion have not been studied, with the exception of two reports [16, 17]. In 1991, Matsumura and others proposed that protein kinase C and calcium metabolism are involved in proteolytic enzyme release from cercariae acetabular glands [16]. Almost 25 years later, Ressurreição followed up on the work by Matsumura and recently reported that PKC, ERK, and p38 MAPK phosphorylation is involved in release of proteolytic enzymes from cercarial acetabular glands following the observation that inhibition of PKC, ERK, and p38 MAPK activities blocked linoleic acid-induced release of acetabular gland contents [17]. The current report further explores the molecular requirements for cercarial host invasion. We identify heat shock protein 70 (Hsp70) as a potential molecular component involved in cercarial honing and show that inhibition of Hsp70 can bypass the requirement for linoleic acid, L-arginine, or any host-derived signal to induce cercarial host targeting behavior. Interestingly, numerous reports corroborate regulatory interplay between Hsp70, PKC, ERK, and p38 MAPK activities [18-21].

Several studies led us to investigate the potential role for a heat shock pathway during cercarial honing and invasion. First, the heat shock response has traditionally been
associated with cellular stress [22-24], and cercariae are no exception to this, since they must transition from a cooler, low-saline and freshwater environment to the warmer, saline environment of a human host. Second, we recently observed an unexpected localization of heat shock factor 1 (Hsf1), the major transcriptional activator responsible for transcribing heat shock genes (such as HSP70 and HSP90), to the acetabular glands of cercariae [25]. This observation helps corroborate the findings of another study that showed the presence of Hsp70 in released acetabular gland contents [26]. Third, the heat shock response may play a role in other stages of schistosome infection as well. In particular, an induced heat shock response in the schistosome intermediate host Biomphalaria glabrata renders them susceptible to schistosome infection, while absence of a strong heat shock response leads to resistance [27]. Together, these studies suggest an important role for a heat shock pathway in parasitic schistosomes.

Hsp70, a member of the heat shock protein (HSP) superfamily, is structurally and evolutionarily conserved from prokaryotes to eukaryotes and generally functions as a chaperone protein that aids in (re)folding nascent and denatured proteins through interactions with its substrate domain and ATP hydrolysis (for review, [28]). However, additional roles for Hsp70 outside of its well-established chaperone functions have also been described. Together with various co-chaperones, Hsp70 can also direct signaling pathways that control cell death, differentiation, homeostasis, and proliferation by modulating the function of key regulatory proteins (client proteins) [29]. This is observed in the regulation of tumor necrosis factor receptor 1 (TNFR1) signaling [30]. Aggregation
of TNFR1 leads to cell death; however, TNFR1 aggregation is inhibited when TNFR1 interacts with silencer of death domain (SODD). Hsp70 is thought to bind to SODD, modifying it to induce SODD/TNFR1 interaction, thereby inhibiting TNFR1-dependent cell death [30]. Hsp70 also plays a role in modulating Smad-mediated transcription [31]. Smad proteins are essential transducers of the transforming growth factor superfamily. Smad-mediated transcription is enhanced by the activity of the melanocyte specific gene (Msg1) protein, a transcriptional activator that cannot independently bind DNA but does so indirectly through interaction with p300/CBP. Hsp70 forms a complex with Msg1, suppressing its interaction with p300/CBP, and consequently blocks Msg1 enhancement of Smad-mediated transcription [31]. As another example, in clathrin-mediated endocytosis, Hsp70 binds and holds clathrin triskelia, preventing their aggregation during the uncoating of clathrin-coated vesicles; in the other half of the clathrin cycle, Hsp70 releases the triskelia to allow the coating of new vesicles upon activation by some unknown signal(s) [32]. The role of Hsp70 in clathrin-mediated endocytosis resembles that which we propose here for cercarial honing, especially with respect to the sequestering of important cellular components until Hsp70 receives an activating signal to release its client protein. While identification of the mechanism for Hsp70 mediated regulation for clathrin-mediated endocytosis is a topic of much interest [33-35], a similar mechanism and question just as interesting may apply to the cercarial honing and invasion process.

In this study, we treated cercariae with modulators of Hsp70 protein that inhibit or
activate Hsp70 via different mechanisms to explore whether Hsp70 functions in cercarial host invasion. Of interest, we found that 2-phenylethanesulfonamide (PES), also known as pifithrin-µ, initiated the process of cercarial honing and invasion in the absence of any host-specific stimulants such as skin lipids or linoleic acid, and it did so with 100% effectivity, which is greater than that observed with either skin lipids or linoleic acid, albeit at a slower rate. PES specifically binds to Hsp70 (Kd ~ 2.9 µM), and its derivatives do not interact with Grp75 or Grp78, organelle-specific members of the Hsp70 family [36, 37]. X-ray crystallographic analysis shows that PES interacts with residues L394, P398, L401, G484, N505, and D506 in human Hsp70. We propose a model that Hsp70 is involved in a signaling pathway that causes cercariae to begin host invasion maneuvers and that inhibition of Hsp70 bypasses the need for upstream host signals that normally initiate this process.

We have recorded and observed over 200 videos of cercarial mobility in response to small molecule modulators that target Hsp70, heat shock protein 90 (Hsp90), or apoptosis. To our knowledge, this is the first investigation of a molecular signaling pathway in cercariae that points to a role for Hsp70 as a regulatory factor for the transition between parasite development stages. In addition to providing a potential pathway to which we can direct drug development against schistosomes, these data could apply more broadly to other parasites and to other organisms during transitions or periods of rapid development [38]. Finally, we add to the current model in describing cercarial host invasion.
Chapter 3: Hsp70 may be a molecular regulator of schistosome host invasion

3.4 Methods

3.4.1 Phylogenetic analysis of Hsp70

Protein sequences of Hsp70 from various species most closely related to that of *Schistosoma mansoni* (NCBI accession numbers: CCD76164 (Smp_106930) and CCD76236 (Smp_049550) were identified by the NCBI BLASTp function [39] and aligned using ClustalW2 using its default parameters [40]. A phylogenetic tree was generated using the output of the ClustalW2 alignment and TreeView X software.

3.4.2 Animals and parasites

*Biomphalaria glabrata* snails infected with *S. mansoni* (NMRI strain) were obtained from Biomedical Research Institute (BRI; Rockville, MD). Cercariae were collected from infected snails by light-induced shedding: the snails were kept in the dark overnight and then placed under bright light for 2 hours [41].

3.4.3 Parasite observation and treatments

Cercariae were observed in 12-well or 24-well culture plates (respectively about 1,000 or 500 cercariae per well) using an inverted (VanGuard 1493INi) and upright stereo (Olympus SZ30) microscope fitted with a camera (Canon T5i). Videos were captured with the focus on the bottom of the wells at 40× and 10× magnification and a camera setting of 1280 by 720 at 60 fps. Images shown in figures are frames extracted from the videos.
Treatments of cercariae included the addition of the following substances; the treatment concentrations were chosen based on those used in the studies indicated (typically increased several-fold over those used in cell-based studies): human skin lipid (finger swipe), linoleic acid (Sigma L1012) [42], Hsp70 modulators 2-phenylethynesulfonamide (PES; Sigma P0122) [36], MKT-077 (Sigma M5449) [43], 115-7c (Stressmarq SIH-123) [44], and VER-155008 (Sigma SML0271) [45], Hsp90 inhibitors geldanamycin and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; these Hsp90 inhibitors were a kind gift of Giselle Knudsen and Jonathan Choy from the Small Molecule Discovery Center at UCSF) [46], pan-caspase inhibitor Z-VAD-FMK (Santa Cruz Biotechnologies sc-3067) [47], anthelmintic praziquantel (Sigma P4668) [48], and adenosine phosphates ATP (Sigma A1852), AMP-PNP (non-hydrolyzable ATP analog; Sigma A2647), and ADP (Sigma A2754) [49, 50]. These substances were either vortexed with a volume of water before treatment or added directly to water containing cercariae. Cercariae were treated within 3 hours of collection, and the time points expressed in this report refer to the time elapsed after the administration of a given treatment.

3.5 Results

3.5.1 Schistosome Hsp70 is highly conserved

We obtained a 637 amino acid protein sequence for *S. mansoni* Hsp70 from NCBI (CCD76164) and used this sequence as a query (NCBI BLASTp) to identify homologous Hsp70 proteins from different organisms. Using the available sequences (incomplete sequences were omitted), we performed an alignment using ClustalW2 to determine the
phylogenetic relationship among these proteins (S3.1 Figure). As expected, we found that
*Sm*Hsp70 proteins are highly conserved across organisms with greater than 50% identity
and that they cluster into different Hsp70 classes [51]. *Sm*Hsp70 (NCBI accession
CCD76164, 637 amino acids (aa)) clustered with the human Hsp70 (NCBI accession
NP_006588, 646 aa), which is constitutively expressed and recognized as the heat shock
protein 70 cognate (Hsc70) protein. The second *Sm*Hsp70 protein (NCBI accession
CCD76236, 648 aa) represents a non-constitutive heat-inducible form of Hsp70, and it clustered with *Hs*Hsp70 (NCBI accession AA112964, 655 aa), also called heat shock
protein 70 family A (Hsp70) member 5, which is localized to the lumen of the
endoplasmic reticulum (ER) where it is thought to mediate protein trafficking of
ER-derived proteins, thereby regulating protein signaling [52].

### 3.5.2 Establishment of cercarial swimming in culture

Previously, we published the observation that *Sm*Hsf protein is localized to the acetabular
glands of schistosome cercariae [25]. *Sm*Hsf is a transcriptional activator of HSPs. While
we do not think that Hsf1 can directly regulate the actions of its transcriptional targets in
acetabular glands, we became interested in the idea that Hsf1 or HSPs may be involved in
the transition between cercariae and schistosomula, either for cercarial invasion or for
newly transformed schistosomula.

We began by experimentally repeating observations of cercarial responses to human skin
lipid that have been well established since the 1970s [26, 53]. Our descriptions of
cercariae are based on observations from inverted and upright microscopes. However, because cercariae continuously moved vertically in our 1 mL water samples, a consistent location to image between samples was not possible. Thus, images described here focus on the bottom of the culture wells, with approximately 1,000 cercariae per well for a 12-well culture plate or 500 cercariae per well for a 24-well culture plate. When observing cercariae by microscopy in a culture well, the relatively large depth of the water column and the nature of standard microscopes precludes a meaningful side-view visualization. Swimming cercariae, in wait of a host, are distributed vertically in a water column with few touching the bottom surface of a culture well. Thus, most cercariae will not be seen at the bottom of a culture well from this viewpoint. In contrast, when the cercariae have settled in response to a stimulus, many more cercariae can be observed at the bottom of a culture well (Figure 3.1). The apparent lack of cercariae in some of the images described later is not caused by a discrepancy in the number of cercariae added, but rather by their specific distribution (vertical and horizontal) in the water column.
Chapter 3: Hsp70 may be a molecular regulator of schistosome host invasion

Figure 3.1. Visual illustration of cercariae swimming. During active swimming, most cercariae are found in the water column, while almost no cercariae can be seen at the bottom of the culture well. As cercariae hone, more of them can be seen at the bottom of the well when observed at higher magnification.

Since many drugs are often diluted or dissolved in DMSO, we established a baseline for cercarial DMSO tolerance, relative to what we observed in water. We compared cercariae treated with filtered water, 0.5% DMSO, and 1% DMSO. Cercariae treated with water and 0.5% DMSO were distributed in a similar manner and exhibited a similar swim (up)-sink-swim behavior at both 10 minutes and 2 hours (S3.2 Video).

3.5.3 Treatment of cercariae with human skin lipid, linoleic acid, and PES

Given the potential connection for a heat shock response during the cercaria-schistosomulum transformation and that Hsp70 is widely conserved, we compared the effect of treating cercariae with human skin lipid, linoleic acid, and PES (Figure 3.2; S3.3 Video). PES has been shown to prevent Hsp70 from interacting with several Hsp70 client proteins [36]. Experimentally, cercariae respond to a skin lipid smear on the bottom of a petri dish by settling to the bottom of the petri dish and
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beginning the penetration process [26]. Our observations confirmed this. However, only cercariae located in close proximity to the skin lipid smear seemed to gather at the site where skin lipid was placed; the majority of the cercariae settled to the bottom of the well without regard for the location of the lipid smear. It should be noted that when cercariae were exposed to human skin lipid or linoleic acid (mixed into water and added to cercariae), the cercarial honing response occurred within minutes. We also note that not all cercariae in our 1 mL sample responded to the skin lipid stimulus, as some cercariae could be seen swimming higher in the water column, out of the focal plane (S3.3 Video); this may correlate with the 60-70% cercarial response previously described in response to human lipids or L-arginine [9].

Figure 3.2. Cercariae treated with PES hone and transform more completely than those treated with skin lipid. Cercariae were treated with filtered water (A-C), 0.5% DMSO (D-F), human skin lipid (G-I), or 250 µM PES (J-L), and observed at various time points. The treatments and observation timings are as follows: (A) water, 8 minutes; (B) water, 56 minutes; (C) water, 1 hour 45 minutes; (D) DMSO, 9 minutes; (E) DMSO, 57 minutes; (F) DMSO, 1 hour 46 minutes; (G) lipid, 0 minutes; (H) lipid, 9 minutes; (I) lipid, 1 hour; (J) PES, 3 minutes; (K) PES, 51 minutes; (L) PES, 1 hour 42 minutes. Each treatment used about 1,000 cercariae in a volume of 1 mL in a 12-well plate well (40× view).
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We next tested the effect of PES, a selective inhibitor of Hsp70. When cercariae were exposed to PES, they initially behaved similarly to the 0.5% DMSO control treatment, whereas the cercariae exposed to skin lipid responded immediately and started settling to the bottom of the well and swimming into or crawling along the surface (Figure 3.2D, G, J; S3.3 Video). However, we were surprised by the result just minutes later. After 5-10 minutes, PES (250 µM)-treated cercariae began to swim to the bottom of the culture plate well, eventually losing their tails to transform into schistosomula (Figure 3.2J, K, L). We observed the same effect with a lower treatment concentration (50 µM) of PES but at a later time point (S3.5 Video). While the majority of the cercariae treated with human skin lipid or linoleic acid honed downward, we observed that 100% of the PES-treated cercariae settled to the bottom of the well and began the penetration behavior (S3.3 Video). When we co-treated cercariae with PES and skin lipid, the cercariae responded with an effect similar to that of PES alone: all of the cercariae were present at the bottom of the well (S3.4 Video).

We observed that after exposure to skin lipid (9 minutes) or PES (51 minutes), the cercariae formed clusters (Figure 3.2H, K); this effect was not seen in the 0.5% DMSO control treatment (57 minutes, Figure 3.2E; S3.3 Video). Cercariae under PES treatment had not yet formed these clusters at 20 minutes (S3.5 Video). A majority of the cercariae lost their tails by 1-3 hours in the PES treatment and 1 hour in the skin lipid treatment (Figure 3.2L, I; S3.3, S3.5 Video); again, this effect was not seen in the 0.5% DMSO control treatment (1 hour 56 minutes, Figure 3.2F; S3.5 Video). Within 3 hours, both PES
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and skin lipid-treated cercariae transformed into schistosomula. For PES-treated cercariae, it should be noted that this honing and transformation occurred in the absence of any host signaling molecules.

Transformation involves several events, notably the loss of tails and loss of water tolerance. The flat appearance of the heads of the cercariae in the skin lipid- and linoleic acid-treated sample at 2 hours indicates the loss of water tolerance and lysis, and further progression in the transformation to the schistosomulum stage, as compared with the corresponding PES-treated sample, in which the heads have a round appearance and are motile (S3.3 Video). We should also note that the timing for all events seemed to vary somewhat, albeit consistently between cercarial sheds. For example, in one cercarial shed, honing with skin lipids may begin within a minute, in another 3 minutes.

3.5.4 Treatment of cercariae with other Hsp70 modulators

To further determine whether the effect of PES is specific to Hsp70, we treated cercariae with several different Hsp70 modulators, including MKT-077, 115-7c, and VER-155008. MKT-077 functions as an allosteric inhibitor of Hsp70, binding within the nucleotide binding domain of Hsp70 next to its ATP/ADP binding pocket and inhibiting ATP turnover rate. MKT-077 is a rhodacyanine dye originally identified as an anti-tumor agent, and it has been shown to bind mortalin, an Hsp70 family member, and disrupt its interaction with p53 [43]. However, we found no obvious change in behavior of the cercariae in our MKT-077 treatments at 100, 250, or 500 μM concentrations (Figure
3.3D), with the exception of increased death at 22 hours (S3.6 Video). Note that the mechanism of action of MKT-077 differs from that of PES, which binds to the Hsp70 substrate binding domain and competitively blocks protein-protein interactions of Hsp70 and its client proteins.

![Figure 3.3. Cercariae treated with other Hsp70 inhibitors do not hone.](image)

Cercariae were treated with filtered water (A), 1% DMSO (B), 250 µM PES (C), 500 µM MKT-077 (D), 400 µM 115-7c (E), or 100 µM VER-155008 (F); observed at 2 hours. Each treatment used about 1,000 cercariae in a volume of 1 mL in a 12-well plate well (10× view).

While most pharmacological agents target and inhibit the function of proteins, 115-7c has the unusual property of acting as an activator of Hsp70 protein folding function, leading to an enhanced rate of substrate refolding [44]. It binds to Hsp70 and promotes complex formation between Hsp70 and Hsp40. In our treatments of cercariae with 115-7c, we observed the induction of honing behavior by 2 hours, especially in the 400 µM treatment (Figure 3.3E); by 22 hours, a majority of the cercariae had lost their tails (S3.7 Video).

VER-155008 at the concentration used (100 µM) is insoluble in water, and it did not change the behavior of the cercariae (Figure 3.3F; S3.8 Video). While there are numerous
inhibitors of Hsp70, most utilize a similar mechanism of action. For example, all of the following Hsp70 modulators inhibit Hsp70 nucleotide binding activity or ATPase activity: apoptozole, JG-98, methylene blue, MKT-077, VER-155008, YM-01, and YM-08 (stressmarq.com).

3.5.5 Treatment of cercariae with Hsp90 inhibitors

Since Hsp70 can work with other HSPs as a major effector of the heat shock response pathway, we asked whether another highly conserved HSP, Hsp90, could be involved. We treated cercariae with the Hsp90 inhibitors geldanamycin and 17-DMAG, a water-soluble derivative of geldanamycin. However, treatment with these compounds did not produce a change in cercarial behavior; the cercariae resembled those treated with 1% DMSO (Figure 3.4; S3.9 Video).
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Figure 3.4. Cercariae treated with Hsp90 inhibitors do not hone. Cercariae were treated with 1% DMSO (A), 250 µM PES (B), 100 µM geldanamycin (C), or 50 µM 17-DMAG (D); observed at 2 hours. Each treatment used about 1,000 cercariae in a volume of 1 mL in a 12-well plate well (10× view).

3.5.6 Treatment of cercariae with other compounds

Although PES is a potent inhibitor of Hsp70, it was initially described in a screen to identify molecules that block p53-dependent transcriptional activation and apoptosis [54, 55]. PES can also block cisplatin-induced p53 interaction with mitochondrial Bak, a pro-apoptotic molecule responsible for the permeabilization of the mitochondrial membrane, and which thereby blocks p53-dependent activation of apoptosis-associated caspases 8 and 3 [56]. However, it is thought that PES inhibition of p53 acts by inhibition of Hsp70, as PES does not directly interact with p53, BAK, BCL-xL, Grp78, Hsc70, or Hsp90 [36]. The molecular targets or mechanism for p53 regulation of apoptosis is unclear. To determine whether the apoptosis pathway is involved in the honing behavior of cercariae, we blocked caspase activity by treating cercariae with a pan-caspase
inhibitor, Z-VAD-FMK. When cercariae were treated with Z-VAD-FMK, we found no change in cercarial honing behavior. Co-treatment with Z-VAD-FMK and PES resulted in a honing behavior similar to that of PES treatment alone (S3.10 Video).

As an additional treatment, we included praziquantel, the long-standing drug treatment for human schistosome infection. The efficacy of praziquantel treatment depends on the parasite stage for schistosomes; notably, while it can kill cercaria and adult stage schistosomes, it cannot kill the intermediate schistosomulum stage schistosomes [48, 57]. Our treatment of cercariae with 300 nM praziquantel resulted in settling, similar to honing behavior; however, at 24 hours, we observed that while most of the cercariae had died, very few had lost their tails, in contrast to the PES treatment, which resulted in tail loss (in addition to death) for nearly all of the cercariae (S3.11 Video).

Functional roles for Hsp70 in the regulation of signal transduction through the binding of client proteins have been recently described and correlate with its intrinsic ATPase activity [29]. When Hsp70 is in an ADP bound state (Hsp70*ADP), Hsp70 interacts with its client protein stably and the Hsp70 lid is in a “closed” state, preventing release of the client protein. When in the ATP bound state (Hsp70*ATP), the Hsp70 lid is opened, allowing the release of the client protein and increasing the on/off rate at the substrate interaction domain [58]. We propose in the regulation of cercarial honing that Hsp70 binds a client protein and functionally inhibits the client protein’s ability to initiate cercarial honing (Figure 3.5). In accordance with this, if Hsp70 is critical to honing, then
we predict that increasing the ATP concentration should cause the Hsp70 lid to open, leading to the release of the client protein and consequentially result in cercarial honing (Figure 3.5). To test this, we treated cercariae with ATP, AMP-PNP (a non-hydrolyzable ATP analog), and ADP, each at a concentration of 5 mM (intracellular ATP concentration in mammalian cells has been suspected to occur in the millimolar range [59]). While ATP and ADP treatments at this concentration did not show any difference compared to the water alone control treatment, AMP-PNP induced honing behavior within 2 hours 30 minutes (Figure 3.6; S3.12 Video).
Figure 3.5. Predictive model of a role for Hsp70 in cercarial honing. (A) In the absence of strong host signals, Hsp70 binds tightly to its client protein, HHF, inhibiting its activity. (B) Host signals are transmitted through a cercarial signal transduction pathway, releasing Hsp70 inhibition of HHF, which functions in cercarial honing. (C) The inhibitor PES blocks Hsp70 activity by binding to the Hsp70 substrate binding domain and releasing Hsp70 inhibition of HHF, resulting in cercarial honing. (D) Addition of 10 mM ATP leads to release of HHF, possibly by binding to the Hsp70 ATPase domain and reducing its affinity for HHF, resulting in cercarial honing. (E) Addition of a non-hydrolyzable form of ATP leads to release of HHF, possibly by preventing ATP hydrolysis and maintaining the weak affinity state of Hsp70 for binding client proteins, resulting in cercarial honing.
Figure 3.6. Cercariae treated with ATP, AMP-PNP, and ADP. Cercariae were treated with filtered water (A), 5 mM ATP (B), 5 mM AMP-PNP (C), or 5 mM ADP (D), and observed 2 hours 30 minutes after treatment. Each treatment used about 500 cercariae in a volume of 0.5 mL in a 24-well plate well (40× view).

3.6 Discussion

Understanding the requirements for schistosome infection at the parasite-host interface can expedite the identification of novel targets for prevention of infection or the elimination of newly established infections. This has been observed using a topical skin treatment with inhibitors of the schistosome proteases used by the larval cercarial form, during invasion [60, 61]. The process by which cercariae invade a mammalian host has been well described, but molecular requirements regulating this process are unknown. We present evidence that Hsp70 is involved in the process of cercarial honing and plays a role in a signal transduction pathway to regulate cercarial invasion behavior. Cercariae are released from their molluscan host and have less than 24 hours to find a mammalian
host before depletion of their glycogen stores in their tail and body prevents their ability to penetrate host skin [4]. In search of a host, cercariae are distributed in the water column with minimal up and down motion, presumably lying in wait in what might be described as a “still hunting” mode. Cercariae swim randomly in response to water turbulence, light and shadows, and it is thought that they swim toward their host through gradients of body heat and skin chemicals, including linoleic acid, human skin lipid, and L-arginine, with the latter two being the most directionally significant [8, 12, 62]. This chemotactic process is modeled by placing cercariae in water and exposing them to a surface streaked with skin lipid as stimulus. We observed limited chemotaxis in our treatments of cercariae with skin lipid, such that only the cercariae in close proximity to the site where the skin lipid was placed made contact with the lipid. This suggests that cercariae do not swim toward the host over long distances, but lie in wait for a host that comes into close proximity and swim more actively to increase the chance of making contact with the host.

Pharmacological targeting is one way to dissect the molecular pathways that may be involved in cercarial honing. Here, we used a selection of chemical compounds to query a role for Hsp70, Hsp90, and apoptosis in this honing behavior. Based on our observations, we propose that a heat shock pathway is specifically involved in cercarial honing for host invasion. HSPs have been identified in cercarial gland secretions [26, 63] and are among the highest abundance transcripts identified in newly transformed schistosomula [3]. In fact, HSPs have been correlated with cercarial transformation since the late 1980s [64].
However, the role of HSPs has traditionally been connected with the stress response (for review, [22-24]), correlating with the transition from cercaria to schistosomulum, which involves a temperature change from that of ambient water to 37ºC host body temperature. Recent evidence in other systems has suggested that HSPs have more diverse functions outside of stress response, including roles in oogenesis and development, lifespan extension, regulation of cancer, fertility and viability [65-71]. In the schistosome molluscan host *B. glabrata*, the snail heat shock response is necessary for snail susceptibility to infection, such that a reduced heat shock response in the snail results in resistance to schistosome infection [27]; this suggests an important function in host HSP level for schistosome host invasion.

Our observation that cercariae treated with PES undergo a behavioral change is novel, and it allows for the initial identification of molecular components involved in cercarial honing. Honing occurs in response to skin lipid [72]; however, since cercariae treated with the Hsp70 inhibitor PES show a similar behavior to cercariae treated with skin lipid, we predicted that Hsp70 plays a regulatory role in the signaling required for the honing behavior. Honing induced by PES is concentration-dependent with time, such that lower concentrations require more time for induction to occur (S3.5 Video). Our treatment of cercariae with two other Hsp70 modulators, MKT-077 and 115-7c, resulted in different behaviors. MKT-077 treatment resulted in a lack of honing, similar to the water control treatment, while treatment with the Hsp70 activator 115-7c resulted in honing behavior, similar to the PES treatment. We propose that while all three Hsp70 modulators tested in
this study bind to Hsp70, only PES and 115-7c actively promote the release of its client protein, which can then function to initiate cercarial honing.

Treatment with ATP and its non-hydrolyzable analog, AMP-PNP, could also cause Hsp70 to release its client protein by skewing the Hsp70*ATP/ADP binding state distribution toward Hsp70*ATP (the state at which Hsp70 has low affinity for client proteins).

Specifically, we model that in the uninduced honing state (absence of lipid stimulus), Hsp70 interacts with and negatively regulates or inhibits the function of an Hsp70 client protein, which we call Hsp70 Honing Factor (HHF). Upon upstream signal activation by skin lipid, Hsp70 releases HHF, which allows the activation of further signaling to trigger the honing behavior (Figure 3.5). This model is not in disagreement with current models describing a function for Hsp70 signaling [29].

A signaling pathway required to induce cercarial honing implies that many potential signaling factors could be involved, beginning from the receptor(s) that senses skin lipid, potential kinases or phosphatases, through Hsp70, HHF and its targets. We reasoned that other HSPs, such as Hsp90, could be involved, as Hsp90 is reported to interact with client proteins in signaling as well [73]. However, in our treatment of cercariae with Hsp90 inhibitors geldanamycin and 17-DMAG, a geldanamycin derivative, we did not observe any obvious change in the behavior of the cercariae. Next, we considered the potential involvement of apoptosis in honing induction. PES can block cisplatin-induced p53 activation of apoptosis [36]. Our treatment with the apoptosis inhibitor Z-VAD-FMK also
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did not result in any obvious change in the behavior of the cercariae. Interestingly, praziquantel treatment led to honing behavior similar to that resulting from PES treatment; however, at 24 hours, most of the cercariae had not lost their tails, indicating that transformation did not occur. In contrast, tail loss occurred for the skin lipid, linoleic acid, PES, and 115-7c treatments by 24 hours (S3.3 Video). This observation leads us to speculate that cercarial honing involves specific signaling to cause the loss of tails (transformation) in addition to a change in the swimming pattern (settling).

Further effort will be necessary to identify the signaling components involved in cercarial honing, including the proposed Hsp70 client protein, HHF, and to better understand the relatively recently described role of Hsp70 in signaling [29]. Under ideal circumstances, genetics approaches such as gene knock-downs and knock-outs would be appropriate to identify honing components. However, these tools have not been thoroughly developed for use in developing or mature cercariae. Our group and others are working on developing methods to overcome these technical challenges [74-79]. Analysis in cercariae is challenging, as cercariae are short lived, transient, and the necessary proteins for swimming and host invasion have already been produced prior to exit from the snail host. Genetic manipulations of early developing cercariae within sporocysts may be possible, but in the case of Hsp70 and potentially other proteins, knock-down or knock-out could result in the loss of viability or production, not because of protein targeting problems, but because of the multipurpose nature of this particular protein. HSPs are the most abundant proteins expressed in the schistosome egg and miracidium.
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[80]. However, a reduction of the heat shock response in the intermediate snail host, *B. glabrata*, makes the snail resistant to schistosome infection [27], suggesting a critical role for the heat shock pathway for intermediate host susceptibility. Consequently, it would not be a far stretch to speculate whether inhibition of miracidial HSPs could affect invasion of the snail host.

In this study, we have just pierced the surface and glimpsed at molecular components that contribute to cercarial honing. We have found no similar observation where Hsp70 signaling affects a whole organism and its behavior directly, leading to stimulating questions such as: how does signaling quickly and directly regulate cercarial behavior, and are there other organisms that are similarly regulated? Additionally, schistosomiasis affects nearly 240 million people globally. Understanding the molecular requirements for cercarial honing and invasion, as well as those for early schistosomulum survival, could identify new potential drug targets and transition schistosome control from treatment to prevention.

3.7 Acknowledgments

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3.8 Supporting information

S3.1 Figure. Phylogenetic tree of Hsp70. Peptide sequences of Hsp70 closely homologous to those of S. mansoni (NCBI accession CCD76164, labeled S. mansoni 637 aa; and CCD76236, labeled S. mansoni 648 aa) were chosen from several species (A. thaliana 651 aa, NP_195870; C. elegans 640 aa, NP_503068; D. melanogaster 641 aa, NP_524063; D. rerio 643 aa, AAH56709; D. rerio 650 aa, AAH63946; E. coli 638 aa, WP_000516131; H. sapiens 646 aa, NP_006588; H. sapiens 655 aa, AAI12964; M. musculus 646 aa, BAE30272; M. musculus 655 aa, AAH50927; S. cerevisiae 649 aa, NP_009478; S. haematobium 648 aa, KGB42118; S. japonicum 648 aa, AAC00519; X. laevis 650 aa, NP_001080068; X. laevis 655 aa, NP_001080064) and aligned using ClustalW2. The phylogenetic output was used to generate the tree using TreeView X software. doi:10.1371/journal.pntd.0004986.s001 (S1 Figure in published version).
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S3.2 Video. Cercariae treated with filtered water or DMSO (0.5, 1%) at approximately 10 minutes and 2 hours (40× view). doi:10.1371/journal.pntd.0004986.s002 (S2 Video in published version).

S3.3 Video. Cercariae treated with 0.5% DMSO, human skin lipid, 0.1% linoleic acid, or 250 µM PES at approximately 10 minutes and 1 hour (40× view). doi:10.1371/journal.pntd.0004986.s003 (S3 Video in published version).

S3.4 Video. Cercariae treated with 0.5% DMSO, human skin lipid, skin lipid / 250 µM PES, or 250 µM PES at approximately 10 minutes and 1 hour (40× view). doi:10.1371/journal.pntd.0004986.s004 (S4 Video in published version).

S3.5 Video. Cercariae treated with 0.5% DMSO or PES (50, 150, 250 µM) at approximately 2 minutes, 10 minutes, 20 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 22 hours (10× view). doi:10.1371/journal.pntd.0004986.s005 (S5 Video in published version).

S3.6 Video. Cercariae treated with filtered water or MKT-077 (50, 250, 500 µM) at approximately 2 minutes, 10 minutes, 20 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 22 hours (10× view). doi:10.1371/journal.pntd.0004986.s006 (S6 Video in published version).

S3.7 Video. Cercariae treated with 1% DMSO or 115-7c (100, 200, 400 µM) at approximately 2 minutes, 10 minutes, 20 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 22 hours (10× view). doi:10.1371/journal.pntd.0004986.s007 (S7 Video in published version).

S3.8 Video. Cercariae treated with filtered water, 0.1% DMSO, or 100 µM VER-155008 at approximately 10 minutes, 30 minutes, and 1 hour (10× view). doi:10.1371/journal.pntd.0004986.s008 (S8 Video in published version).

S3.9 Video. Cercariae treated with filtered water, 1% DMSO, human skin lipid, 0.1% linoleic acid, 100 µM geldanamycin, 50 µM 17-DMAG, 250 µM PES, 500 µM MKT-077, or 400 µM 115-7c at approximately 20 minutes, 40 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 24 hours (10× view). doi:10.1371/journal.pntd.0004986.s009 (S9 Video in published version).

S3.10 Video. Cercariae treated with 1% DMSO, 25 µM Z- VAD-FMK, 25 µM Z-VAD-FMK / 250 µM PES, or 250 µM PES at approximately 10 minutes, 30 minutes, and 2 hours (40× view). doi:10.1371/journal.pntd.0004986.s010 (S10 Video in published version).

S3.11 Video. Cercariae treated with filtered water, 0.1% ethanol, 300 nM praziquantel, or 250 µM PES at approximately 10 minutes, 4 hours, and 24 hours (10× view). doi:10.1371/journal.pntd.0004986.s011 (S11 Video in published version).
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S3.12 Video. Cercariae treated with filtered water, 5 mM ATP, 5 mM AMP-PNP, or 5 mM ADP at approximately 6 minutes, 32 minutes, 1 hour 2 minutes, and 2 hours 32 minutes (40× view).
doi:10.1371/journal.pntd.0004986.s012 (S12 Video in published version).

3.9 References

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Chapter 4: Discussion

There is little known about the molecular and signaling components important for schistosome cercariae to sense and invade their human host. Our understanding of the components required for the processes after reaching the host, notably penetration of the host skin, tail loss, and transformation into schistosomula, is likewise limited. While investigation of the mechanism involved in these processes increases our potential to identify targets for novel drugs for schistosomiasis, it will also increase our understanding of schistosome biology and provides an opportunity to explore general mechanisms for helminth host invasion. Given the conserved cytoprotective function of the heat shock response and associated proteins from prokaryotes to eukaryotes, and because cercariae undergo a change in environment from swimming in freshwater to invading a warm-blooded host, we hypothesized that heat shock response proteins such as heat shock transcription factor and heat shock effector proteins may play an important role during this transition.

Our initial studies focused on the further characterization of the *S. mansoni* heat shock transcription factor (*SmHSF1*), which followed a series of studies by the Schechter group [1–6]. As expected for the conserved gene, we found that *SmHSF1* has the ability to function as a transcriptional activator and can also bind specific heat shock-related DNA sequences. Further, we produced a custom antibody against a peptide with a sequence corresponding to that of a C-terminal region of *SmHsf1* and tested whether it could detect purified recombinant maltose binding protein (MBP)-*SmHsf1* protein. SDS gel
electrophoresis separation and Western blotting of both purified recombinant full-length MBP-\(Sm\)Hsf1 protein and cercaria protein extract in our study resulted in a larger apparent molecular weight than expected (Figure 2.5; \(~140\) kDa instead of \(~110\) kDa for the recombinant and \(~110\) kDa instead of \(~70\) kDa for the extract). We speculated that post-translational modifications and/or a limitation of the SDS separation method could cause such a discrepancy. Interestingly, the same phenomenon has been observed with \textit{Drosophila} [7] and yeast Hsf proteins [8], suggesting that conserved modifications or structures in Hsf are responsible for this shift in apparent size. This strengthens the assertion that \(Sm\)Hsf1 protein contains the same conserved structural characteristics, and will likely function in a similar way, as Hsf proteins from other species.

Following the positive result from the Western blot experiment, we asked whether \(Sm\)Hsf1 protein has a specific localization in cercaria stage schistosomes. We expected and observed general punctate staining corresponding with nuclei because of its function as a transcription factor. However, immunostaining of cercariae using the anti-Hsf antibody gave the striking observation that Hsf is also localized to the cercarial acetabular glands, which play an important role as storage and release vessels for proteins required during host invasion. This localization is especially surprising because in mature cercariae, these glands no longer contain nuclei [9] and are instead full of vesicles that carry proteins such as mucins [10] and elastases [11] that aid in host skin penetration. A closer examination of the staining revealed that Hsf seems to be localized to the membrane of the glands, instead of throughout the lumen. Such membrane localization for Hsf has not been well described in the literature, with the exception of one study. In
non-small cell lung cancer line cells, Hsf has been shown to interact with Ralbp1, a membrane-bound transport protein [12,13]. While a homolog of Ralbp1 does not exist in schistosomes, a similar mechanism could explain this localization. In the future, the use of higher resolution microscopy could help to better define the localization of Hsf within the glands. For example, it could determine whether the membrane localization exists throughout the entirety of the glands, from the most anterior end of the cercaria, to the fundus, or base of the glands.

Hsf localization to the acetabular glands of cercariae provokes further thought into the potential role that Hsf plays in the life of these glands, from the development of the glands while cercariae are produced in the sporocyst, to their disintegration after the transition to the schistosomulum stage. As cercariae develop within the sporocyst, the concurrently developing glands within the cercariae must produce all the proteins necessary for host skin invasion. This high protein production in the glands implies a need for increased surveillance of these proteins by chaperones such as Hsp70 so that these proteins do not aggregate. In support of this idea, mass spectrometry analysis of the gland secretions has indicated an abundance of Hsp70, a downstream product of Hsf [14]. Hsf could therefore play an important role in the development of these glands as an indirect protein homeostasis factor via Hsp70. Hsf may also function as a protein homeostasis factor in sporocysts, considering that they 1) each produce hundreds of cercariae every day [15], requiring high protein production, and 2) show the highest SmHSF1 transcript levels among sporocyst, cercaria, schistosomulum, and adult stages according to our transcript expression analysis in the first study presented in this thesis.
Further experiments of Hsf localization in sporocysts and intermediate cercariae, while technically difficult, may give more insight into Hsf function in the developing glands. Similarly, immunohistochemical staining of Hsf, paired with apoptosis assays, in newly transformed schistosomula at several time points up to 24 hours, could track the change in gland morphology as the glands degrade.

If we could disable the ability of Hsf to interact with other proteins through treatment with a small molecule inhibitor, we could determine whether Hsf contributes to the apoptosis of the glands. Existing Hsf inhibitors block its ability to bind DNA, which, in the anucleated cercarial glands, may not exhibit a significant effect. Fortunately, there exist inhibitors against other heat shock response proteins, such as the chaperone proteins Hsp70 and Hsp90, which may play an important role during cercarial host invasion. While we have argued above that high levels of Hsp70 exist in the glands to support the production of gland-related proteins, Hsp70 may also function more directly in host invasion, given the abrupt change in environment. To begin to address this question, we asked whether inhibiting Hsp70 function would affect cercarial behavior during host invasion. This became the focus of the second major study in this thesis.

The second study we present here shows that treatment of cercariae with an inhibitor of Hsp70, 2-phenylethynesulfonamide (PES), recapitulates the response of cercariae to human skin lipid. Cercariae execute a host invasion process, which begins with the detection of human skin lipid molecules, followed by the reduction of upward swimming motion (cercarial honing), an increase of acetabular gland secretions, and an increase of
penetrative swimming against the bottom of the vessel/culture well [14].

In the study of compounds that induce cercarial honing, chemotaxis of cercariae toward host molecules has been a topic of interest. A notable study by the Haas group demonstrated a chemotactic preference of cercariae for L-arginine [16]. This study further showed that human skin lipid extract (which contains L-arginine) elicited chemotaxis, while the L-arginase-treated extract did not [16]. However, we must carefully consider that many of the experiments that identified substances to which cercariae respond were performed in small volume vessels (with diameters on the order of one to tens of millimeters), which may not accurately recapitulate the larger physical dimensions (on the order of tens to hundreds of centimeters) in the field. In the L-arginine study, the authors observed chemotactic preference using a W-shaped vessel embedded within a cube measuring 5 mm per side in which the cercariae from the middle arm swam to other arms containing either empty agar or agar mixed with an experimental compound [16]. While this study shows statistical significance for cercarial swimming preference toward L-arginine at this close distance, we can only guess at whether cercariae would show the same chemotactic preference over a distance of tens to hundreds of centimeters.

Our study of cercarial honing presented in this thesis involves treatments of cercariae with chemical compounds using slightly larger vessels (12 or 24-well plate vessels, respectively about 22 or 15 mm in diameter and 2 or 1 mL in volume). When we treated cercariae with skin lipid, we observed limited chemotaxis. This means that either the skin lipid concentration in our treatment had saturated the putative chemotactic receptors in
the cercariae or that cercariae respond in an all-or-nothing fashion: once they sense skin lipid, they change their swimming behavior. Considering the physical problem that cercariae probably cannot swim fast enough to catch up with a moving host, the second explanation seems more likely. Future quantitative experiments could examine the amount of cercarial honing behavior-inducing substance necessary to activate such behavior to infer the distance at which cercariae can sense host molecules.

Cercariae activated by skin lipid that reach a surface eventually lose their tails and transform into schistosomula. Treatment with PES resulted in the honing behavior described here, with an inverse time-concentration correlation, such that higher treatment concentrations induced this change sooner compared to lower treatment concentrations. To verify our observation with PES, we used another Hsp70 inhibitor, MKT-077, and an Hsp70 activator, 115-7c. Treatment with MKT-077 resulted in no honing, while treatment with 115-7c resulted in honing. To understand these contradictory results, we must first discuss the chaperoning function of Hsp70.

Hsp70 undergoes a client protein chaperoning cycle, in which its association with either ATP or ADP (ATP/ADP state) and other proteins such as Hsp40 and nucleotide exchange factor (NEF) control Hsp70-client protein binding. In this model, Hsp70 acts as a chaperone by interacting with nascent or unfolded client proteins (often abbreviated as “S” for Hsp70 Substrate) to prevent undesirable protein aggregation [17]. Closer examination of the mechanism of action of the treatment compounds reveals that both PES and 115-7c facilitate the release of Hsp70 client proteins, while MKT-077 inhibits
Hsp70 without releasing the client proteins. This brings us to the model in which Hsp70 could be interacting with and sequestering a putative honing factor, which we termed Hsp70 honing factor (HHF), in unexposed cercariae. Upon reception of host molecules or facilitated release of client proteins by the appropriate chemical treatment, Hsp70 would release HHF, allowing it to activate downstream signaling that leads to cercarial honing.

While the Hsp70-HHF model asserts that Hsp70 holds a specific protein inactive, the client protein in the Hsp70 chaperone model can represent any nascent or unfolded protein. Another model that describes a specific client protein controlled by Hsp70 is the model for the role of Hsp70 in clathrin-mediated endocytosis. In clathrin-mediated endocytosis, a cell creates a vesicle coated with a network of clathrin proteins as it prepares to take up extracellular cargo. The clathrin coating needs to break down (uncoat) to allow the underlying vesicle to fuse with the appropriate downstream endosome. During the uncoating process, the constitutive version of Hsp70, Hsc70, is known to specifically interact with clathrin to prevent its aggregation [18]. After the reception of an unknown signal, Hsc70 releases clathrin to allow for the coating cycle to proceed. The ability for Hsc70 to interact with clathrin and act as a regulatory switch opens the possibility that Hsc70/Hsp70 can also regulate other processes through interactions with specific client proteins, such as in the Hsp70-HHF model.

According to the Hsp70-HHF model, any process or manipulation that causes Hsp70 to release its client protein would result in HHF release and cercarial honing. Accordingly, treatment of cercariae with the Hsp70 activator, 115-7c, resulted in honing. Treatment
with AMP-PNP, a non-hydrolyzable ATP analog, which forces Hsp70 into a low-affinity state for interaction with a client protein, leading to a decreased chance of client protein retention, also resulted in honing. Interestingly, elevation of the water temperature to 42°C caused the cercariae to stop swimming and settle to the bottom of the culture well; after the water returned to room temperature, the cercariae returned to normal swimming behavior (unpublished observations). This suggests that if Hsp70 released HHF, HHF could not activate downstream signaling because of the elevated temperature. Alternatively, HHF could have become unfolded at the elevated temperature, and the majority remained bound with Hsp70, resulting in continued sequestration of HHF after the temperature returned to normal.

We do note, however, that, while similar, the cercarial responses to PES, 115-7c, or adenosine phosphates do not perfectly replicate the response to skin lipid, most noticeably in terms of time until response. Treatment with skin lipid results in a honing response within seconds, while the other substances take more time to elicit a response. We can speculate that cercariae respond more rapidly to skin lipid owing to specific receptors that can quickly activate signaling pathways, although such receptors for skin lipid molecules have not yet been described. In contrast, the other compounds may require time to reach their intracellular targets to exert their effects. Behaviorally, while these treatments ultimately result in reduced upward swimming and increased overall swimming activity at the bottom of the vessel, the skin lipid treatment also induced a crawling behavior that is less prominent in the other treatments. This may suggest that different mechanisms or signaling pathways may control the change in swimming
behavior and crawling behavior: skin lipid would activate both, while Hsp70 inhibition would mainly affect swimming behavior. Further studies to identify schistosome receptors for skin lipid components, as well as investigation of putative HHF and its upstream and downstream signaling components, will help answer this question.

In the future, we would aim to identify proteins involved in the cercarial host sensing and honing activation signaling pathway, beginning with putative HHF. An affinity purification/mass spectrometry (AP/MS) approach using an Hsp70 antibody, comparing PES treatment against DMSO control treatment, could yield a list of possible HHF candidates. However, because Hsp70 also interacts with other proteins and PES does not discriminate between HHF and other client proteins, the AP/MS approach may only give broad possibilities for the identity of HHF. Genetic approaches, such as gene knockdown, have not been established in cercariae, mostly owing to the difficulty of introducing genetic material into developing cercariae while inside the snail host. Moreover, until transformation into schistosomula, cercariae have been demonstrated to undergo little transcription of \textit{HSP70} [19], and little transcription in general, likely resulting from histone methylation and histone position relative to transcriptional start sites [20]. This means that any transfected genetic material requiring transcription has little chance of affecting cercariae.

Manipulation of mature cercariae is limited to treatment with pharmacological agents or other compounds because all of the proteins necessary for host invasion have already been produced. Combining this idea with the observation that cercariae begin honing
within seconds of skin lipid exposure leads to the question of how HHF could rapidly activate signaling. We propose that HHF is a kinase or other post-translational protein modifying agent, considering three lines of evidence. First, phosphorylation has been observed to increase seven-fold between cercaria and 3-hour schistosomulum stages [21]. Second, treatment of cercariae with phorbol esters, which induce protein kinase C (PKC) activity, has been shown to result in penetration behavior and the release of host invasion-associated proteolytic enzymes [22]. Third, PKC has been shown to interact with Hsp70 [23], and it, along with extracellular signal-regulated kinase (ERK) and p38 mitogen-activated kinase (p38 MAPK), responds to temperature elevation, light intensity modulation, and linoleic acid treatment [24]. An initial experiment could probe PKC activity upon exposure to PES, followed by the same experiment but with the addition of a PKC inhibitor. Additionally, we could evaluate cercarial behavior after exposure to PES or skin lipids in the presence of a PKC inhibitor to determine whether the activation of cercarial honing requires PKC activity. The AP/MS approach using a PKC-specific antibody, or alternatively, a complex-immunoprecipitation/Western blot (CoIP/WB) approach could determine whether PKC and Hsp70 interact in cercariae and whether PES or skin lipids affects this interaction.

The two independent, but related, studies in this thesis provide novel observations and insights into the function of heat shock response proteins in schistosomes, particularly with regard to the development and behavior of the infective cercaria stage. Our experiments led us to the intriguing topic of protein signaling during cercarial host invasion, with a unique model that Hsp70 regulates a behavioral switch between naïve
swimming and honing. Together with the ideas that PKC, ERK, p38 MAPK transduce potential host signals and that histone methylation/positioning status causes transcriptional stalling in cercariae, we have taken a step closer to understanding the important molecular events that occur in cercariae, beginning from host detection to transformation into schistosomula.

We have yet to explore the role of heat shock response proteins in other schistosome stages. Of interest may be the question of whether the Hsp70-HHF model applies to the other host transition stage in which the schistosome miracidia infect the snail host. Ittiprasert and Knight have reported that elevated heat treatment in the snail host leads to increased susceptibility to infection [25], suggesting that Hsp70 and putative HHF could play a role in this transition. Like schistosomes, other parasites also undergo significant changes in living conditions between stages (e.g., insect to human host), during which heat shock genes have been suspected to play an important role [26–28], and further investigation could determine whether the Hsp70-HHF model applies more generally across different parasites. The ideas and results presented here contribute to a better understanding of schistosome biology and help inspire further investigation of the molecular mechanisms that support parasite survival.

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Chapter 4: Discussion


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