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Abstract

Title of Dissertation:

Schistosoma mansoni c-AMP dependent protein kinase (PKA): A potential new drug target

Brett E. Swierczewski, Doctor of Philosophy, 2010

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Schistosomiasis, a disease caused by parasitic blood flukes of the genus *Schistosoma*, afflicts over 200 million people in tropical and subtropical regions of the world and is responsible for approximately 280,000 deaths in Sub-Saharan Africa alone annually. Currently, praziquantel (PZQ) is the sole drug that is used for treatment due to its ability to kill the mature adult worms of the medically important *Schistosoma* species (*S. mansoni, S. haematobium,* and *S. japonicum*). However, it is unrealistic to expect that reliance on this single drug for all treatment and control of schistosomiasis will be sustainable in the long-term and highlights the necessity for the identification of new chemotherapeutic targets in schistosomes. We therefore explored the anti-parasite potential of targeting cAMP-dependent protein kinase (PKA) enzymes in *S. mansoni*. Here

we provide biochemical evidence for the presence of a PKA signaling pathway in adult *S*. *mansoni* and show that PKA activity is required for parasite viability *in vitro*. We identified a cDNA which encodes for a PKA catalytic subunit homologue in *S. mansoni*, named SmPKA-C. RNA interference studies showed that SmPKA-C is an essential gene and is required for *S. mansoni* viability *in vitro*. SmPKA-C mRNA was shown to be differentially expressed throughout the parasite life cycle with highest levels being present in the infectious larval stage of the parasite, cercariae, and adult females by real-time PCR. PKA activity, similar to adult worms, was shown to be required for cercariae viability. Our data also suggests that PKA signaling may be required for parasite growth and development and egg production both *in vivo* and *in vitro*. PKA-C subunit orthologues were also identified in *S*. *haematobium* (ShPKA-C) and *S*. *japonicum* (SjPKA-C) and are 99 % identical to SmPKA-C at the amino acid level. Additionally, we showed that *S*. *mansoni* contains a gene for a PKA regulatory subunit homologue that was highly heterologous at the amino acid level to other regulatory subunits from other eukaryotic organisms. *S*. *mansoni* PKA activity was also susceptible to modulation by the site-selective cAMP analog, 8-Cl-cAMP, and this compound was lethal for adult schistosomes *in vitro*. Together our data show that PKA catalytic and regulatory gene products are essential for adult schistosomes and may represent attractive therapeutic targets for the treatment and control of schistosomiasis

Schistosoma mansoni cAMP-dependent protein kinase (PKA): A potential new drug target

By:

Brett E. Swierczewski

Dissertation submitted to the Faculty of the Emerging Infectious Diseases Program of the Uniformed Serviced University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2010.

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Introduction

Schistosomiasis

Historical Significance and Epidemiology

Schistosomiasis is a disease caused by parasitic trematodes of the genus *Schistosoma* [1]. Schistosomiasis is an ancient disease that can trace its origins to ancient Egypt and China [2,3]. There are a multitude of references to schistosomiasis in ancient Egyptian papyri and hieroglyphics and calcified schistosome eggs have been found in coprolites and mummies from Egypt and China, dating back to 1200 $_{B.\ C.}$ and 200 $_{B.\ C.}$ respectively [4]. Some have postulated that schistosomiasis has played a role in shaping world history. It has been implicated in the deaths of Napoleon Bonaparte, in his exile on Elba, and of King Herod the Great, referenced in the gospels of the New Testament [5,6]. Militarily, it has been theorized that schistosomiasis stopped Napoleon's invasion of Egypt and prevented the invasion of Taiwan by Mao Tse Tung's Chinese Red Army in 1949 [7]. Even though it is a disease from antiquity, schistosomiasis has re-emerged due to a number of factors, including major man-made irrigation and agricultural projects and socioeconomic migration of people to these areas [8]. Most recently, schistosomiasis has been classified along with several other diseases as a neglected tropical disease (NTD) and major efforts from a variety of agencies, including the Gates' Foundation, are attempting to control the transmission and morbidity of the disease through mass drug administration [9]. However, with only one drug available for treatment and no vaccine available, schistosomiasis remains on the forefront of scientific research.

Currently, there are over 200 million cases of schistosmiasis with over 93% of the cases occurring in sub-Saharan Africa [8]. Schistosomiaisis is responsible for 280,000 deaths annually in sub-Saharan Africa, with the majority occurring in populations living in extreme poverty [10]. Schistosomiasis is endemic in 74 countries in tropical and sub-tropical regions of the world (Fig. 1). Schistosomiasis is caused by seven species of schistosomes: *S. mansoni, S. japonicum, S. intercalatum, S. malayi, S. guineensis,* and *S. mekongi* which cause hepatosplenic/intestinal schistosomiasis and *S. haematobium* which causes urinary schistosomiasis [11]. The three major species of medical importance are *S. mansoni*, *S. japonicum*, and *S. haematobium*. *S. mansoni* and *S. haematobium* share considerable geographic overlap as they are both endemic in Africa and the Arabian peninsula [12]. However, *S. mansoni* is also endemic in regions of South America, particularly in Brazil. *S. japonicum* is endemic throughout the Pacific region, most notably in China, Indonesia, and the Philippines. *S. mekongi* and *S. intercalatum* are much more geographically restricted when compared to the other species. *S. mekongi* is localized to the Mekong River valley in parts of Laos and Thailand while *S. intercalatum* is restricted to regions of western Africa [11].

The geographic distribution of schistosomiasis is dependent on multiple factors, most importantly the availability of competent molluscan intermediate hosts and human water contact [12]. Schistosome migration coinciding with mass human migration from endemic areas has led to the dissemination of schistosomiasis to previously non-endemic areas. For example, *S. mansoni*

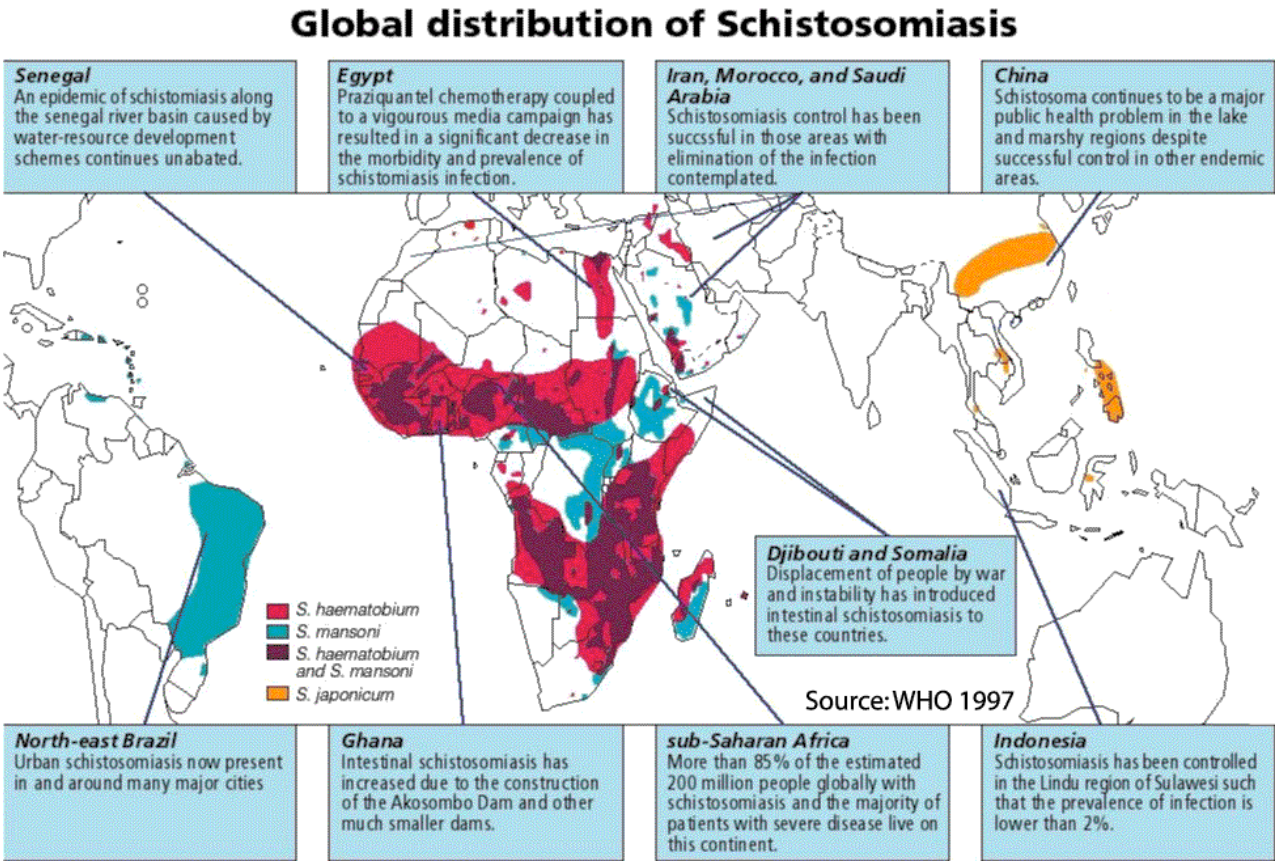
was introduced into the Americas by the African slave trade, due to the presence of a susceptible new molluscan intermediate host, *Biomphalaria glabrata,* which led to transmission and subsequent disease establishment in South America [13]. A more recent example includes establishment of schistosomiasis in a previously non-endemic focus in the Democratic Republic of Congo due to the introduction of a competent intermediate host, *Biomphalaria tenagophila*, from South America [14]. Schistosomiasis has re-emerged or has been established in some areas as a result of massive agricultural and irrigation water projects [8]. Construction projects in the Senegal River basin led to a 95% increase in schistosomiasis infections over a three year period in a community in Ndombo, Senegal [15]. In China, major surveillance programs are being established to monitor changes in the transmission of *S. japonicum* due to construction of the Three Gorges Dam on the Yangtze River [16,17]. Reports from Egypt have showed that construction of the Aswan Dam resulted in alteration of schistosomiasis patterns with an increase in *S. mansoni* prevalence and a decrease in *S. haeamtobium* [18,19]. This trend has also been observed in the southern Nile River delta, due to the changing ecological pattern of the Nile resulting in an increased distribution of new intermediate hosts [12]. These massive construction projects draw a substantial influx of people to work at these sites, resulting in recruitment of a new susceptible host population to an area of transmission.

Water contact is essential for transmission and individuals most at risk live near lakes, rivers, dams, reservoirs, and streams [20,21]. Previous control

efforts, particularly in China and Egypt, were focused on vector control of the snail intermediate host through the use of molluscicides, but these programs had only short term effects and are extremely expensive and difficult to maintain [22]. Today schistosomiasis is a man-made disease and could be decreased or potentially eliminated in numerous areas by better sanitation and the availability of a safe water supply [23].

Figure 1: Global Distribution of Schistosomiasis

Urinary schistosomiasis (red) caused by *S. haematobium* and hepatic/intestinal schistosomiasis caused by *S. mansoni* (blue) and *S. japonicum* (orange) are found in tropical and sub-tropical regions of the world and overlap particularly in sub-Saharan Africa (purple).


Global distribution of Schistosomiasis
Senegal
An epidemic of schistomiasis along the senegal river basin caused by water-resource development schemes continues unabated.
Egypt
Praziquantel chemotherapy coupled to a vigourous media campaign has resulted in a significant decrease in the morbidity and prevalence of schistomiasis infection.
Iran, Morocco, and Saudi Arabia
Schistosomiasis control has been succssful in those areas with elimination of the infection contemplated.
China
Schistosoma continues to be a major public health problem in the lake and marshy regions despite successful control in other endemic areas.
S. haematobium
S. mansoni
S. haematobium and S. mansoni
S. japonicum
Djibouti and Somalia
Displacement of people by war and instability has introduced intestinal schistosomiasis to these countries.
Source: WHO 1997
North-east Brazil
Urban schistosomiasis now present in and around many major cities
Ghana
Intestinal schistosomiasis has increased due to the construction of the Akosombo Dam and other much smaller dams.
sub-Saharan Africa
More than 85% of the estimated 200 million people globally with schistosomiasis and the majority of patients with severe disease live on this continent.
Indonesia
Schistosomiasis has been controlled in the Lindu region of Sulawesi such that the prevalence of infection is lower than 2%.

Life Cycle

In contrast to other trematodes, schistosomes have separate sexes and require constant contact with one another for growth and maturation [24]. The schistosome life cycle is complex, alternating between asexual reproduction in the snail intermediate host and sexual reproduction in the definitive vertebrate host [11].

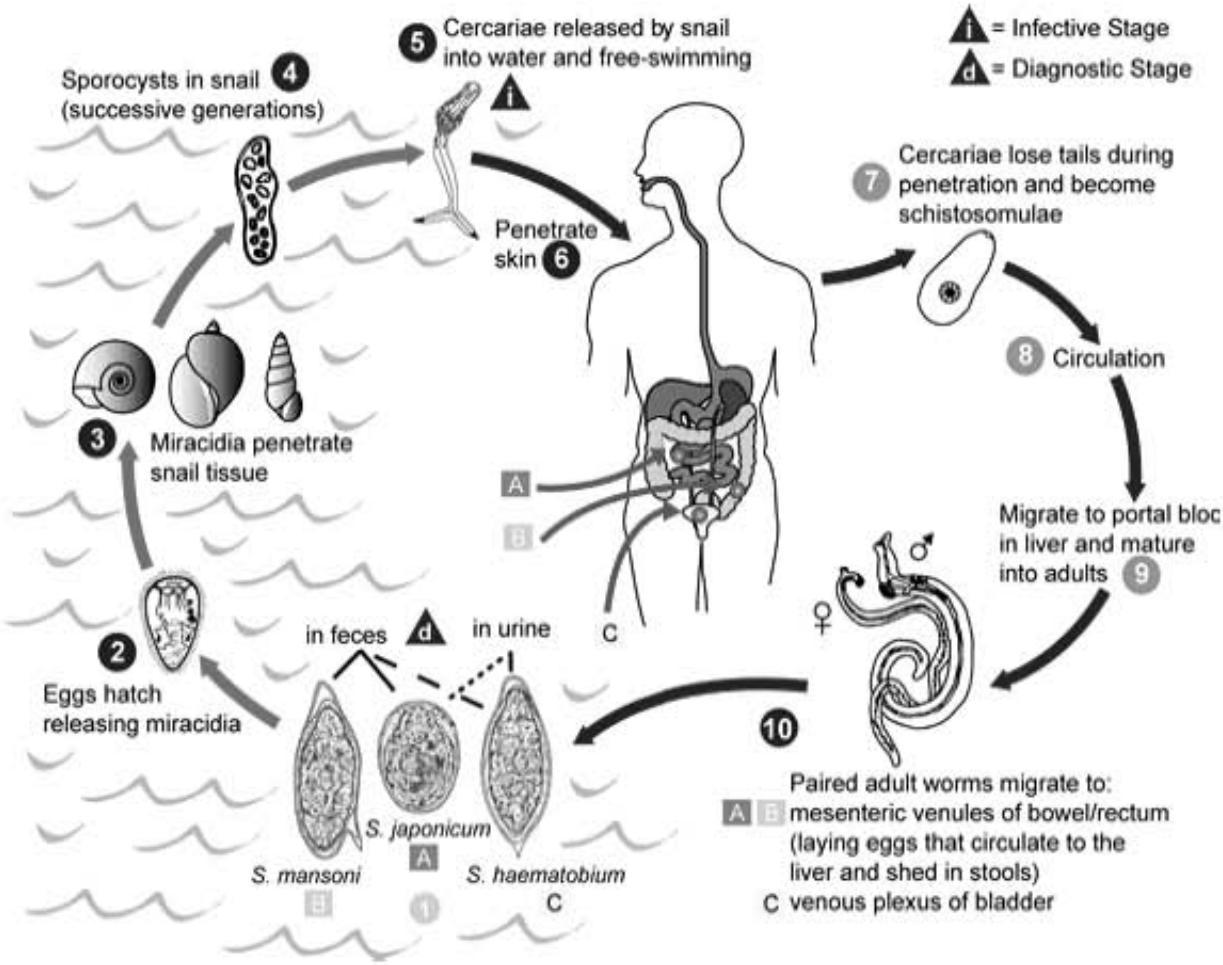
Female worms reside in the gynecophoral canal of the male worm, allowing the adult worms to remain paired within the definitive host. *S. mansoni* and *S. japonicum* adult pairs lie in the mesenteric venules draining the large intestine and small intestine respectively, while *S. haematobium* pairs live in the urinary bladder plexus (Fig 2) [11]. Eggs are excreted in the feces in *S. mansoni* and *S. japonicum*, while eggs are typically expelled in the urine in *S. haematobium*. Once the eggs reach fresh water, a miracidium will emerge from the egg and seek out the appropriate snail intermediate host. Each schistosome species is restricted in its usage of intermediate host. *Oncomelania, Bulinus*, and *Biomphalaria* are the snail intermediate hosts for *S. japonicum*, *S. haematobium*, and *S. mansoni* respectively. After infecting the snail, the miracidium will undergo a rapid transformation into a mother sprorocyst and will undergo several rounds of asexual reproduction, ultimately ending with production of the infective stage of the parasite, cercaria, approximately 4 - 6 weeks after the initial infection of the snail.

Cercariae are released from the snail into the water, in response to external stimuli such as light. Immediately on penetrating the skin of the

vertebrate host, cercariae shed their bifurcated tails, transforming into schistosomula. Schistosomula enter the host's peripheral circulatory system and travel through the pulmonary capillaries. Approximately eight days after infection, the schistosomula reach the hepatic portal vein where they remain for approximately three weeks, undergoing further growth and development. Adult worms will pair at approximately four weeks post-infection and will migrate to the superior and inferior mesenteric venules (*S. mansoni* and *S. japonicum* respectively) or the venous plexus of the bladder (*S. haematobium*). Female worms can lay approximately 200 (*S. haematobium*), 400 (*S. mansoni*) or 3000 (*S. japonicum*) eggs per day [25]. Eggs reaching fresh water inhabited by the appropriate snail intermediate host will maintain the life cycle. The average life span of schistosomes is 3 – 5 years, but there are some reports of schistosomes living 20 – 30 years [26].

Figure 2: Schistosome Life Cycle

Eggs are passed in the feces (*S. mansoni* and *S. japonicum*) or urine (*S. haematobium*). After reaching fresh water, the eggs hatch releasing a miracidium. Miracidia seek out and infect the appropriate snail intermediate host (*S. mansoni*: *Biomphalria, S. japonicum*: *Oncomelnia*, *S. haematobium*: *Bulinus*). After infection of the snail, miracidia will undergo rapid transformation to the sporocyst stage. Sporocysts undergo several rounds of asexual reproduction ultimately producing the infective stage of the parasite, cercariae, for the definitive host. Cercariae are released from the snail and penetrate the skin of the definitive host. Cercariae transform into schistosomula and enter the circulation and migrate to the hepatic portal system. After a time period of growth and development, adult male and female schistosomes pair and migrate to the mesenteric (*S. mansoni* and *S. japonicum*) or ureter venules (*S. haematobium*). Females can begin laying eggs six weeks post-infection. Eggs traverse and pass through the intestinal or ureteral wall and are passed in the feces or urine.



(http://www.dpd.cdc.gov/dpdx/HTML/Schistosomiasis.htm)

Pathology/Disease

Schistosomiasis is a unique disease in that the eggs are the cause of pathology and not the adult worms. Schistosomiasis can be divided into acute and chronic illness. Acute schistosomiasis or Katayama Fever occurs approximately 4 – 10 weeks after a heavy initial infection and is characterized as a systemic hypersensitivity reaction to migrating schistosomula [27]. Symptoms of Katayama Fever include fever, chills, malaise, headache, non-productive cough and general gastrointestinal discomfort, and patients typically recover spontaneously [28]. The immune response consists of a marked eosinophilia and production of several inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1 [29]. Katayama fever associated with *S. mansoni* is seen mainly in naïve travelers, military personnel, and tourists traveling to endemic areas, while Katayama Fever associated with *S. japonicum* affects people living in endemic areas with previous exposure to *S. japonicum* [30,31].

The chronic form of disease can be classified into urinary (*S. haematobium*), intestinal (*S. mansoni*, *S. japonicum*, *S. mekongi* and *S. intercalatum)* and hepatic/hepatosplenic (*S. mansoni*, *S. japonicum* and *S. mekongi*) schistosomiasis. Eggs which are not excreted in the urine or feces are transported back into the circulation and can become embedded in other tissues. A local immune response to egg antigens results in the formation of granulomas around these trapped eggs [29].

Intestinal schistosomiasis occurs as a result of eggs becoming trapped in the intestinal wall, which provokes a local granulomatous response around the trapped eggs. Symptoms include abdominal pain, loss of appetite, and diarrhea that may contain blood [11]. Hepatic schistosomiasis occurs when eggs do not cross the intestinal lumen and are transported and become lodged in the liver. The resulting granuloma consisting of CD4+ T cells, eosinophils, and macrophages results in the production of cytokines (IL-13, IL-21) which over time can induce the development of fibrotic lesions in the liver and the gut [32]. Constant re-infection and the development of more severe hepatic fibrosis can eventually impede portal blood flow and result in portal hypertension, porto-systemic shunting and the formation of gastro-intestinal varices [29,33]. Mortality due to *S. mansoni* and *S. japonicum* is usually caused by bleeding and massive hemorrhage from gastro-intestinal varices [11]. Additionally, eggs can become lodged in other organs such as the spleen, central nervous system and lungs.

Urinary schistosomiasis is caused by local granulomatous inflammation around eggs of *S. haematobium* trapped in the ureteral walls of the bladder [11]. Urinary schistosomiasis affects mostly children and symptoms include dysuria and haematuria. *S. haematobium* is categorized as carcinogen by the World Health Organization as chronic urinary schistosomiasis is associated with the development of bladder cancer in Egypt and other parts of Africa [34,35]. Chronic urinary schistosomiasis can eventually lead to renal failure as well. Genital schistosomiasis can occur when eggs provoke a local granulomatous response in the genitals or cervix [29].

As mentioned earlier, there are an estimated 200 million cases of schistosomiasis worldwide with the majority of cases in sub-Saharan Africa [8]. Of those 200 million, 120 million are symptomatic with 20 million having severe symptoms [36]. It is estimated that 1.7 – 4.5 million Disability Adjusted Life Years (DALYs) are lost annually due to schistosomiasis [9] . Of the 280,000 approximate deaths per year caused by schistosomiasis, 130,000 are attributed to portal hypertension caused by *S. mansoni* and 150,000 are attributed to kidney failure caused by *S. haematobium* [37].

Treatment/Current Research Efforts

Current chemotherapy for treatment of all forms of schistosomiasis is dependent solely on the anthelmintic, praziquantel (PZQ) [38]. PZQ is cheap (0.06 – 0.19 U. S. dollars), safe, and effective against the adults of all the medically important schistosomes. A single dose of 40 – 60 mg/kg is usually sufficient to produce cure rates of 60 – 90% [39]. Some alternative drugs are used to treat schistosomiasis, but their use is restricted due to higher costs of production and limited efficacy. For example, oxamniquine is used on a limited basis in Brazil, but has largely been replaced by PZQ as it is only effective against the adult worms of *S. mansoni* [40].

Though PZQ has been used in the field for over 30 years, its exact mechanism of action remains unknown. PZQ has been shown to cause rapid calcium intake, tegument disruption, and musculature contraction in treated worms, suggesting that calcium ion channels may be potential molecular targets

of PZQ [41,42]. Additionally, indirect evidence suggested that actin could possibly be a molecular receptor for PZQ [43]. Further studies on these potential targets have yielded inconclusive results, and thus the exact action of PZQ still remains elusive [44,45]. Since no vaccine exists for schistosomiasis, PZQ is also used for prophylactic control of morbidity and transmission control in schistosomiasis endemic areas [46]. Population-based chemotherapy has become the hallmark of schistosomiasis control efforts today, resulting in PZQ distribution on a mass scale to several African nations through the Schistosomiasis Control Initiative, which has focused increased attention on the limitations of the drug [47].

The most significant limitation of PZQ is that it is not efficacious against juvenile worms approximately 7 – 28 days old [48,49]. Subsequently, a second dose is typically administered 6 -8 weeks after the initial dose to kill those worms which have matured during the period after the first dose. Sole reliance and increased dissemination of PZQ for schistosomiasis highlights the real possibility for PZQ resistance to develop in the field. PZQ-tolerant strains of schistosomes can be reared in the laboratory by exposing worms to sub-curative doses of the drug [50,51]. To date, there has been no definitive evidence of clinically relevant PZQ resistance in the field, but decreased PZQ sensitivity has been demonstrated. Resistance to PZQ in a field setting was first suspected in a *S. mansoni* outbreak in Senegal, where low cure rates were observed after one dose of PZQ [15]. Further analysis revealed that this was a hyperendemic area of transmission and that most patients also harbored immature parasites at the

time of treatment, which are significantly less susceptible to PZQ [52]. Additionally, patients at this site treated multiple times with PZQ still continued to shed viable eggs. In another study, *S. mansoni* isolates obtained from patients in Egypt who had been unsuccessfully treated multiple times with PZQ showed an effective dose 50 (ED_{50}) three fold higher when compared to previously unexposed strains [53]. A follow-up study conducted several years later showed no increased resistance to PZQ in the same area [54]. Most recently, *S. mansoni* isolates obtained from car wash workers who had been treated with PZQ for the last 15 years showed decreased susceptibility to PZQ with several of the workers still shedding eggs after more than 20 treatments [55]. Resistance to PZQ may be a matter of time and major efforts are focused on studying the efficacy of alternative drugs in treatment of schistosomiasis

Artemether and artesunate, cornerstones of malaria treatment, have been found to be effective against juvenile schistosomes of *S. mansoni, S. japonicum*, and *S. haematobium* and combination therapy with PZQ is currently being investigated [56,57,58]. Another malaria drug, mefloquine, has shown promising results in treatment of *S. mansoni* and *S. japonicum* infections *in vivo* and is lethal for schistosomes *in vitro* [59,60,61]. K11777, a drug in late-stage pre-clinical testing for treatment of Chagas' disease, has been found to be effective in significantly reducing parasite burden and egg production in a murine model of schistosomiasis [62,63].

In addition to using existing drugs, other efforts have focused on identifying novel chemotherapeutic targets in schistosomes. Thioredoxin

glutathione reductase, an essential enzyme in the *S. mansoni* detoxification of reactive oxygen species, is currently being validated as a novel drug target in schistosomes [64,65].

For several years, researchers have used detailed Expressed Sequence Tag (EST) databases for both *S. mansoni* and *S. japonicum* to identify gene products that could potentially represent novel drug targets. Most recently, the genomes of both of these parasites have been fully sequenced, adding a tremendous molecular tool for identification of novel schistosome targets [66,67]. One protein family whose role in schistosome biology and potential for chemotherapeutic targeting has recently been explored is the protein kinase family.

Protein Kinases

General Characteristics

Protein kinases (PKs), through the reversible phosphorylation of specific amino acid residues on protein substrates, are the major mediators of signal transduction pathways in eukaryotic organisms [68]. In the human genome, there are over 500 genes which encode for PKs and PKs participate in a wide variety of developmental and differentiation processes in cells, including gene expression, metabolism, apoptosis, tissue differentiation and cellular proliferation [69,70]. Phosphorylation by PKs occurs by the transfer of the terminal phosphoryl group of ATP, in complex with a divalent cation (usually Mg^{2+}), to

specific tyrosine (Tyr) or serine/threonine (Ser/Thr) residues on protein substrates. Thus, PKs can be broadly characterized as Ser/Thr kinases (STKs) or Tyr kinases (PTKs) [71]. PTKs and STKs can be classified into two distinct catergories: receptor or non-receptor (cytoplasmic) [72,73]. Examples of each class of PTKs are the epidermal growth factor receptor (EGF-R) and Src kinases respectively [74,75]. Examples of Ser/Thr receptor kinases (RSTK) are members of the transforming growth factor β (TGF-β) receptor family, while c-AMP dependent PK (PKA) is the classic example of a cytoplasmic STK [76,77]. There are four general groups of PKs: 1) The AGC group, which includes the cyclic nucleotide dependent PKs (PKA and PKG), and the protein kinase C family (PKC); 2) The CMGC group, which includes the cyclin-dependent kinases and mitogen-activated kinases (MAPK); 3) the CaMK group, which includes the calcium/calmodulin dependent kinases group; and 4) the PTKs [68].

The key distinguishing features that all PKs have in common are a catalytic domain, which binds and phosphorylates the target protein, and a regulatory region, which interacts with other proteins to control activation of the catalytic domain [78,79]. All PKs contain a highly conserved catalytic domain consisting of approximately 250 amino acid residues. The catalytic domain can be further divided into 12 distinct sub-domains each with a defined role in kinase function [68]. For example, sub-domain I contains the consensus motif, GTGSFGRV, which is responsible for ATP binding. Since protein phosphorylation is involved in a myriad of cellular and regulatory processes, subtle alterations in PK activity can have pathogenic effects in eukaryotes.

The unregulated signaling of specific PKs has been shown to cause several diseases in humans such as cancer, diabetes, central nervous system disorders, and inflammatory disorders [80,81,82,83]. For example, overexpression of EGFR and PKA has been shown to cause bladder, ovarian, cervical, and several other types of cancer [81,84,85]. Hence, the development of specific PK inhibitors as anti-cancer therapeutics is a major area of study in both academia and industry. There are several PK inhibitors which have been approved for the treatment of multiple types of cancer. These inhibitors typically act by blocking the binding of ATP, thus locking the kinase in its inactive confirmation [86]. One of the most successful of these inhibitors, imatinib (Gleevec®, Novartis), is used for the treatment of chronic myeloid leukemia (CML) [87,88]. CML is typically caused by the fusion of the *abl* and *bcr* genes, resulting in a constitutively expressed PK named BCR-Abl [89]. Imatinib binds with high affinity to the ATP-binding site of BCR-Abl, keeping the kinase in its inactive confirmation [90].

Extensive research and development of PK inhibitors for cancer treatment has encouraged investigators to explore the potential for targeting these enzymes in other eukaryotic pathogens, particularly protozoan parasites [91]. The sequencing of a number of protozoan genomes to include *Plasmodium falciparum* (causative agent of malaria), *Leishmania major* (causative agent for cutaneous leishmaniasis), and *Trypansoma brucei* and *T. cruzi* (causative agents of African and American Trypanosomiasis respectively) have identified numerous PKs to which there are inhibitors available [92,93]. Though these are unicellular

organisms, there is a high degree of homology between PKs from these organisms and those of mammalian species. In particular, the kinome of *P. falciparum* has been a major focus of research in recent years in trying to elucidate the role of multiple PKs in the parasite's life cycle and the effect of their subsequent inhibition. It was shown that treating *P. falciparum* with the cyclin-dependent kinase inhibitor, Puravalanol B, prevented infection of human erythrocytes by inhibiting the activity of the *P. falciparum* PK, CK1 [94]. Studies on targeting PKs in protozoan parasites are ongoing, supporting the rationale that PKs should be explored as targets in other parasites such as schistosomes.

Protein Kinases in S. mansoni

The identification of PKs and their roles in the life cycles of schistosomes has been studied extensively for the last several decades. With the recent publishing of the *S. mansoni* genome, it has been shown that *S. mansoni* contains genes which encode for 249 PKs corresponding to approximately 2% of the entire proteome [66]. Studies aimed at understanding signal transduction pathways in *S. mansoni* have identified a number of PK homologues. These include homologues to PTKs such as the insulin receptor (IR) and EGF-R [95,96] and STK homologues such as PKC and TGF-β receptor kinases [97,98,99]. There is considerable evidence that schistosomes require signals from the host for normal development and reproduction [100,101,102]. For example, it has been shown that CD4+ T cells are required for normal schistosome development

and reproduction to occur [103]. There is also evidence that host TNF-α is required for normal production and excretion of eggs in *S. mansoni* [100]. Thus, it is no surprise that schistosomes may possess PK homologues to bind and interact with these host factors. As these studies have suggested that the parasite requires host signals for growth, there is also considerable evidence for communication between adult male and female schistosomes via cell signaling pathways.

Female schistosomes require a constant male stimulus for female-specific gene expression, reproductive development, and egg production, but the molecular identity of this male stimulus remains unknown [24,104,105]. Since eggs are the cause of morbidity and transmission of schistosomiasis, elucidating the signaling pathways between adult schistosomes could possibly lead to novel drug targets within these pathways. Numerous studies have demonstrated that signaling and crosstalk between TGF-β pathways and several PTKs of the Src kinase and MAPK families regulate adult pairing, female-specific gene transcription and egg production in *S. mansoni* [106,107,108]. One study in particular showed that treating adult *S. mansoni* with the broad spectrum PTK inhibitor, Herbimycin A, reduced egg production *in vitro* and also decreased transcription of a specific eggshell protein [109]. Continued characterization of schistosome PKs has prompted discussion regarding whether schistosome PKs should be considered as potential new drug targets [110].

The study by Knobloch et al., in which *S. mansoni* were treated with Herbimycin A, is one of but a handful of studies that have actually treated intact

worms or other life cycle stages with PK inhibitors. Unpublished studies have shown that inhibiting the insulin receptor of *S. mansoni*, with the tyrphostins AG1024 and AG538, blocked parasite glucose uptake, which is required for parasite survival [110]. Inhibitors and agonists of cAMP and PKA were used in several studies to examine the role of cAMP in miracidial locomotion and sprorocyst transformation respectively [111,112,113]. cAMP and PKA were found to be essential for miracidial locomotion, while subsequent downregulation was required for normal transformation to the sporocyst stage. Although PKA has been well characterized in multiple organisms, these were the first studies to show that *S. mansoni* had an intact cAMP signaling pathway, albeit in the larval stages of the parasite. As PKA overexpression can lead to the development of several cancers and other autoimmune diseases [81], researchers have spent the better part of three decades developing inhibitors to this important protein.

PKA

General characteristics

PKA was first described in 1968 and was the first PK to be cloned, sequenced and to have its crystal structure elucidated [114,115]. PKA is highly conserved in eukaryotic organisms from *Saccharomyces cerevisae* to *Homo sapiens.* PKA is the major mediator of cAMP signaling in eukaryotic cells and is involved in a variety of biological processes such as cell proliferation, gene transcription, apoptosis, and metabolism [116,117,118]. In the absence of

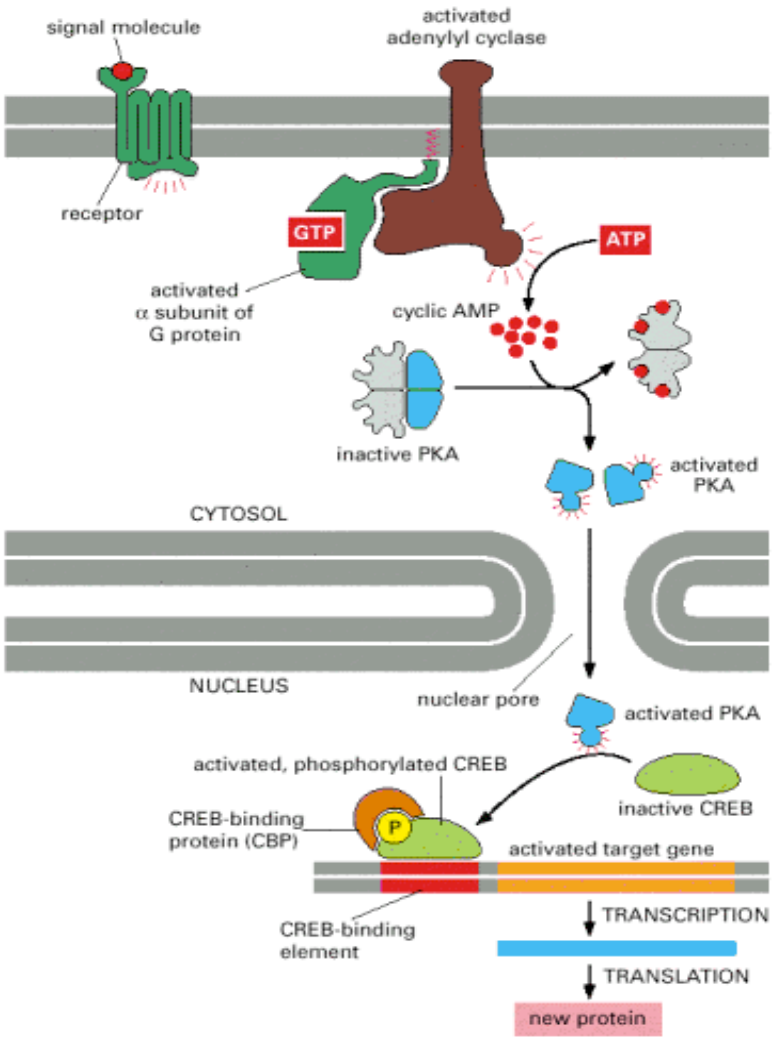
cAMP, PKA is a holoenzyme containing two regulatory (PKA-R) and two catalytic (PKA-C) subunits. There are two families of PKAs, RI and RII, based on the R subunit types involved in formation of the holoenzyme [77]. RI is involved in cell proliferation processes and RII is involved in tissue differentiaton and growth inhibition processes [77]. Four isoforms of PKA-R subunits, RIα, RIβ, RIIα, and RIIβ have been identified in mammalian tissues [119]. PKA-R subunits exhibit differential expression patterns, with RIα and RIIα expressed ubiquitously and RIβ and RIIβ restricted to neural and endocrine tissues respectively [120]. Three isoforms of the PKA-C subunit, Cα, Cβ, and Cγ, have been identified with Cα being ubiquitously expressed and Cβ and Cγ being expressed in brain and adipose tissue and the male testis respectively [121]. The diversity in PKA-R and C subunit genes allows for a variety of holoenzymes to be formed, each with different functions and specifications.

PKA activation is initiated by ligands such as extracellular hormones that bind to G-protein coupled receptors on the cell surface (Fig. 3). Upon binding of the ligand, a conformational change is induced in the G-protein, allowing for its disassociation and the release of the stimulatory subunit (Gsα). Gsα binds and activates membrane bound adenylyl cyclase, which catalyzes the conversion of ATP to cAMP. There are two cAMP binding sites on PKA-R subunits (Sites A and B). cAMP binding to Site B induces a conformational change in the PKA-R subunit, allowing for further binding of cAMP to site A. Once both cAMP sites are occupied, the holoenzyme disassociates and the PKA-C subunits are released and translocated to the nucleus from the cytosol as they contain a nuclear

translocation site. Active PKA-C subunits then phosphorylate Ser/Thr residues on protein substrates that contain the peptide recognition sequence Arg/Lys – Arg/Lys – X – Ser/Thr [116]. Major targets of PKA in the nucleus are cAMP response binding-element (CREB), activating transcription factor 1 (ATF1), and the cAMP response element modulator (CREM) [122].

Figure 3: PKA Activation Process

A G-protein coupled receptor is stimulated after binding of an extracellular signal. Membrane-bound adenylyl cyclase is activated after binding the GSα subunit and induces conversion of ATP to cAMP. cAMP will bind to two sites on the R subunits causing the C subunits to be released. The C subunits translocate from the cytosol to the nucleus and will phosphorylate specific Ser/Thr residues on protein substrates (CREB).



(*Molecular Biology of the Cell, 4th edition*, 2002, Garland Science)

PKA and Cancer

The RI/RII ratio is tightly regulated in cells and increases in this ratio have been implicated in a number of human diseases. There is direct evidence that the RIα subunit is preferentially expressed in proliferating and transformed cell lines (i. e. HL-60 leukemia cell line, LS-174T colon carcinoma cells, e.g.), while increased expression of the RIIβ is associated with non-proliferating and differentiated cell types [81,123]. Overexpression of the RIα subunit at the mRNA and protein level has also been shown to be associated with carcinogenesis and neoplastic transformation in several types of human cancers such as breast, colon, ovarian, and lung cancer [81,124]. Additionally, up-regulation of the RIα subunit is associated with poor prognosis and increased malignancy in cancer patients. Overexpression of the RIα subunit results in the constitutive expression and the uncontrolled activity of PKA. Subsequent studies have shown that up-regulation of only the RIIβ subunit induces a phenotype reversion and growth inhibition in various human cancer cell lines [125,126]. These studies have identified PKA as a potential cancer therapeutic target and considerable effort has therefore been invested in the development of inhibitors of the RIα subunit, with a moderate degree of success.

The site-selective cAMP analog, 8-Cl-cAMP, has been shown to be a potent growth inhibitor both *in vitro* and *in vivo* in a wide spectrum of human cancer cell lines and animal models [123,127,128]. While 8-Cl-cAMP has completed two Phase I clinical trials and has recently begun Phase II clinical trials [129,130], to date, the mechanism of action of 8-Cl-cAMP is not completely

understood. The majority of studies have shown that 8-Cl-cAMP activates and induces downregulation and possible truncation of RI_{α} subunits, facilitating the upregulation of RII_{β} subunits and leading to an increased RII/RI ratio in transformed cells [123]. 8-Cl-cAMP preferentially binds with high affinity to both sites B and A on RIα subunits, while only binding with high affinity to Site B on the RIIβ subunit [131,132]. However, 8-Cl-cAMP has also been shown to induce apoptosis in human cancer lines, in addition to its down-regulation of RI_{α} subunits, suggesting two modes of action for the drug [133,134]. Furthermore, other studies have suggested that the metabolic by-product of 8-Cl-cAMP, 8-Cl-adenosine (8-Cl-ADO), is responsible for the anti-proliferative effects observed with 8-Cl-cAMP, independent of RI/RII isotype switching [135,136].

Using an antisense strategy, a synthetic RIα antisense oligodeoxynucleotide (ODN) corresponding to the first 21 bases of the RIα N-terminus has produced similar results to those observed with 8-Cl-cAMP in a number of human transformed cell lines and an animal tumor models, with no signs of cytotoxicity [123,127]. Treatment with this ODN resulted in increased RIIβ mRNA and protein expression, as well as a marked reduction in RIα expression, thus increasing the RII/RI ratio and resulting in inhibition of cell growth [137,138]. As a consequence of these promising results, a second generation RIα antisense ODN, GEM®231 (HYB165, Hybridon, Inc.) with a mixed RNA-DNA backbone was developed. GEM®231 has been shown to have a longer half-life, increased biological activity, and synergistic effects with other cancer drugs when compared to the original RIα ODN [139,140]. GEM®231 has

recently completed Phase I clinical studies and is under Phase II review [141,142].

PKA in Protozoan Parasites

Due to the high degree of PKA conservation in eukaryotic organisms and its role in cancer development, PKA has been studied in eukaryotic parasites to determine the role and potential suitability of PKA inhibitors as anti-parasitic therapeutic agents. In particular, the role of PKA in a number of protozoan parasites has been examined in recent years. A PKA-C subunit (gPKA) was cloned and sequenced in the intestinal parasite, *Giardia lamblia* [143]. Treating the parasite with the PKA-C inhibitor, PKI 14-22 amide, showed an inhibition in the transition from the dormant cyst stage to the active trophozoite, a process known as excystation. gPKA was also found to be localized to the flagella of the parasite and PKA-C inhibition resulted in a significant decrease in motility in a dose-dependent manner. PKI 14-22 amide is a synthetic peptide that contains a pseudosubstrate site which PKA-C subunits bind to with high affinity [144].

Studies have also shown that PKA plays an essential role in *P. falciparum*. A study by Syin et al showed that PKA-C inhibition by treatment with the ATP-competitive inhibitor, H-89, blocked erythrocyte invasion and the activation of gametocytogenesis [145,146]. Recently, a PKA-C subunit has been characterized in *P. falciparum* (PfPKA). Subsequent studies have shown that recombinant PfPKA activity is significantly decreased when treated with the PKA-C inhibitors, PKI 14-22 amide and H-89, confirming the results obtained in the

study by Syin et al. [147]. Due to the high degree of drug resistance in *P. falciparum*, studies to validate PKA as a novel drug target are ongoing.

PKA in S. mansoni

As mentioned earlier, the role of cAMP and PKA has been examined only in the larval stages of *S. mansoni*. In a study by Matsuyama et al., treatment of miracidia with the adenylyl cyclase antagonist, SQ22536, and the PKA-C inhibitors, H-89 and PKI 14-22 amide, significantly decreased miracidial motility in a dose-dependent manner [113]. Conversely, treatment of miracidia with the cAMP agonists, forskolin and serotonin, inhibited miracidial transformation to the sporocyst stage within the snail intermediate host [112]. These studies suggest that the intramolluscan stages of the parasite contain a functional cAMP signaling pathway. Currently, there is very little data on this vital enzyme and the role it plays in the intramammalian stages of the parasite. As PKA has been shown to be an essential protein in several other parasitic organisms, we hypothesized that PKA plays an essential role in adult *S. mansoni* and that targeting PKA may represent a novel anti-schistosome therapeutic target

Hypotheses and Specific Aims

Based on the evidence that PKA is conservated in eukaryotic organisms and is essential in a wide array of cellular processes, we hypothesized that *S. mansoni* possess genes for PKAs and express them in the intramammalian stages of the life cycle. Furthermore, we hypothesized that PKA plays an important role in schistosome cell biology and that targeting PKA would represent a novel approach to eliminating adult *S. mansoni* from infected hosts.

Specific Aim 1: To identify and characterize *S. mansoni* PKA.

Specific Aim 2: To elucidate the biological function of *S. mansoni* PKA in the intramammalian stages of the life cycle.

Specific Aim 3: To test whether *S. mansoni* is a potential therapeutic target.

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Chapter 2

A SCHISTOSOME cAMP-DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT IS ESSENTIAL FOR PARASITE VIABILITY

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Abstract

As eukaryotes, protozoan and helminth parasites make extensive use of protein kinases to control cellular functions, suggesting that protein kinases may represent novel targets for the development of anti-parasitic drugs. Because of their central role in intracellular signaling pathways, cyclic nucleotide-dependent kinases such as cAMP-dependent protein kinase (PKA) represent promising new targets for the treatment of parasitic infections and neoplastic disorders. However, the role of these kinases in schistosome biology has not been characterized and the genes encoding for schistosome PKAs have not been identified. Here we provide biochemical evidence for the presence of a PKA signaling pathway in adult *Schistosoma mansoni* and show that PKA activity is required for parasite viability *in vitro*. We also provide the first full description of a gene that encodes for a PKA catalytic subunit in *S. mansoni*, named SmPKA-C. Finally we demonstrate, through RNA interference, that SmPKA-C contributes to the PKA activity we detected biochemically and that inhibition of SmPKA-C expression in adult schistosomes results in parasite death. Together our data show that SmPKA-C is a critically important gene product and may represent an attractive therapeutic target for the treatment and control of schistosomiasis.

Keywords: *Schistosoma*, cAMP-dependent protein kinase, kinase inhibitor, adenylyl cyclase, RNA interference

Author Summary

Schistosomes are parasitic flatworms that inhabit the circulatory system and are the cause of a debilitating and insidious disease for millions of people worldwide. Like other complex organisms, schistosomes and other parasitic worms regulate their cell biology through extensive use of enzymes called protein kinases that phosphorylate other proteins to alter their function. One such protein kinase, cAMP-dependent protein kinase (PKA), has been proposed as a therapeutic target for the treatment of parasitic infections and cancer. Here we use biochemical techniques to show that schistosome worms possess a functional PKA pathway that is required for survival of the parasites. We also identify a parasite gene that encodes for a functional PKA enzyme and show that silencing this gene results in both significant loss of PKA activity in schistosome worms and parasite death. These findings suggest that the gene we have identified is critically important to schistosomes and that it's protein product may represent a target for the development of much-needed new drugs to treat schistosome infections.

Introduction

Schistosomiasis, a disease caused by trematodes of the genus *Schistosoma*, afflicts approximately 200 million people in tropical and subtropical regions of the world and is responsible for approximately 280,000 deaths annually in Sub-Saharan Africa alone [1]. The schistosome life cycle is remarkably complex, involving multiple life cycle stages that are morphologically and physiologically adapted for survival within and transmission between the molluscan and vertebrate hosts these parasites require for life cycle completion. Within each host, evasion of host defenses is balanced with requirements for host resources and signals that are necessary for schistosome growth and development. While specific examples have not been characterized at the molecular level, there is considerable evidence for interactions between schistosomes and host factors, such as hormones and growth factors, which influence aspects of parasite biology such as development and reproduction [2-5]. This intimate relationship, where schistosomes exploit host factors to facilitate establishment of infection while simultaneously evading host defenses, is presumably a reflection of extensive host-parasite co-evolution that has occurred since the emergence of the genus *Schistosoma* some 12-19 million years ago [6].

Currently the anthelmintic praziquantel is the sole drug used for treatment of schistosomiasis, due to its ability to kill the adult worms of all medically important *Schistosoma* species [7]. However, there are reasons to suspect that reliance on this single drug for all treatment and control of schistosomiasis will

not be sustainable in the long term. First, praziquantel-tolerant strains of *S. mansoni* can be derived in the laboratory by exposure to sub-curative doses of praziquantel [8,9]. Second, evidence for decreased sensitivity to praziquantel has been found following mass drug treatment efforts [10]. Thus the potential for praziquantel resistance is real and the increasingly wide scale use of praziquantel, through programs such as the Schistosomiasis Control Initiative, highlight the necessity for the identification of new chemotherapeutic targets in schistosomes [11].

Protein kinases represent a potentially new class of therapeutic targets for the treatment of parasitic diseases [12]. Through the phosphorylation of substrate proteins, protein kinases play a central role in the cellular signaling pathways of eukaryotic organisms and are involved in biological processes as diverse as gene expression, metabolism, apoptosis, and cellular proliferation [13]. The unregulated activity of protein kinases has been implicated in the pathogenesis of several human diseases, including cancer, autoimmune diseases, and inflammation [14,15]. Consequently, the development of protein kinase inhibitors as therapeutics for cancer and other diseases has been actively pursued [16]. As eukaryotes, protozoan and helminth parasites presumably also make extensive use of protein kinases to control cellular functions, suggesting that protein kinases may represent novel targets for the development of anti-parasitic drugs [17,18]. Examples of promising protein kinase targets in parasites include the cyclic guanosine monophosphate- (cGMP-) dependent protein kinases (PKGs) of *Toxoplasma* [19], *Eimeria* [20] and *Plasmodium* [21],

and a *Plasmodium* cyclic adenosine monophosphate- (cAMP-) dependent protein kinase (PKA) [22], as inhibition of these kinases resulted in significant anti-parasitic effects *in vivo* or *in vitro* [20,23]. Regulated by cyclic nucleotide second messengers produced by purine nucleotide cyclases, cyclic nucleotide-dependent protein kinases represent particularly attractive drug targets as, in addition to targeting the kinase domain directly, their activity can also be manipulated by targeting the regulatory cyclic nucleotide binding (CNB) domains with cyclic nucleotide analogs [24]. Indeed, an experimental therapy for some cancers in which PKA is implicated utilizes this latter approach and is now in clinical trials [25]. A consistent difference between PKA and PKG across highly divergent taxa is that in PKA, the regulatory and catalytic activities are contained within separate gene products known as PKA-R and PKA-C respectively, whereas in PKG the CNB sites and catalytic domain are usually contained within the same polypeptide. Thus the inactive conformation of PKA is a heterotetramer of two PKA-R and two PKA-C subunits, while PKG exists as a homodimer. Segregation of the catalytic and regulatory functions of PKA into separate proteins provides an opportunity for diversification in the function of PKA, as different PKA-C and PKA-R isoforms can combine to produce holoenzymes with different functions [26]. Mammalian genomes contain as many as three *pka-c* genes and four *pka-r* genes, allowing for a variety of different holoenzymes to be formed [27].

While cyclic nucleotide-dependent kinases have been extensively characterized in a variety of eukaryotic organisms, including several parasites,

there is relatively little data available on the role of these kinases in the biology of schistosomes. A study by Matsuyama et al. showed that treatment of schistosome miracidia with cAMP analogs and PKA antagonists completely inhibited miracidial locomotion in a dose-dependent manner, suggesting a role for PKA in miracidial swimming [28]. In contrast, Kawamoto et al. found that treatment of miracidia with adenylyl cyclase agonists inhibited miracidium to mother sporocyst transformation, while drugs that decreased cAMP levels triggered transformation [29]. These studies suggest that cAMP and PKA play important roles in the larval stages of the schistosome life cycle. However, no studies have examined the role of PKA in adult schistosome biology and full-length nucleotide sequences encoding schistosome PKAs have not been identified. We hypothesized that PKA plays a vital role in adult worms and that targeting PKA may represent a novel approach to eliminating adult schistosomes from infected mammalian hosts. In this report, we provide a biochemical characterization and molecular identification of a *S. mansoni* PKA (SmPKA). Furthermore, we show that the schistosome PKA is an essential gene product for adult worms and as such represents an attractive therapeutic target for the treatment and control of schistosomiasis.

Materials and Methods

Ethics statement

All experiments involving mice were performed in accordance with protocols approved by the USUHS Institutional Animal Care and Use Committee.

Parasite materials

Biomphalaria glabrata snails infected with NMRI/Puerto Rican strain of *S. mansoni* were supplied by Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD). Cercariae were obtained by exposing infected snails to light for 2 h in 50 mL of filtered water. Schistosomula were prepared by mechanical transformation of cercariae according to published protocols [30]. Adult *S. mansoni* were obtained from 6 week-infected C57BL/6 mice that were infected with 150 cercariae using the tail immersion method [30].

Western blotting

Freshly isolated adult worms were homogenized in cell extraction buffer (100 mM NaCl, 25 mM Tris pH 7.5) containing a protease inhibitor cocktail (Sigma; 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; 1 mM), aprotinin (0.8 μM), leupeptin (20 μM), bestatin (40 μM), pepstatin A (15 μM), and E-64 (14 μM). The resulting homogenate was incubated on ice for 30 min and centrifuged at 13,000 rpm for 20 min at 4 °C to remove insoluble material. The protein concentration of the resulting supernatant (Sm lysate) was determined using the Quick Start™ Bradford Protein Assay. Western blots were performed

using the WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit (Invitrogen). Briefly, 7 µg of total protein from adult worms, HT1080, and 293FT cells were used per sample. Reduced samples were separated by SDS-PAGE on 12% Bis-Tris gels and transferred onto polyvinyl difluoride (PVDF) membranes. After an initial blocking step in 5 % non-fat dried milk, 20 mM Tris pH 7.5, membranes were incubated for 12 h with polyclonal anti-PKA C-α antibody (Cell Signaling Technology®) diluted 1:1000 and then with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Invitrogen) for 2 h. Bound antibody was detected according to manufacturer's instructions using Kodak BioMax Light Film.

Detection of PKA enzymatic activity

PKA activity was measured using a fluorescent peptide substrate-based assay (Omnia® Lysate Assay for PKA kit, Biosource). Freshly isolated adult worms were used to prepare Sm lysate, in the presence of protease and phosphatase inhibitor cocktails (Phosphatase Inhibitor Cocktail 1(Sigma), containing cantharidin, bromotetramisole and microcystin LR, diluted 1:100) as described above. The protein concentration was determined using the Quick Start™ Bradford Protein Assay and adjusted to a final concentration of 0.2 µg/µL with additional extraction buffer. Kinase reactions containing 1 µg total protein (equivalent to 5 µL Sm lysate), 10 mM ATP, 15 µM PKA peptide substrate, 10 mM DTT, and a non-PKA inhibitor cocktail (64 µM PKC inhibitor peptide, 10 µM GF109203X, 20 µM calmidazolium) in kinase reaction buffer were assembled in

opaque 96-well assay plates, according to the manufacturer's recommendations. Accumulation of phosphorylated substrate was monitored during a 1 h incubation at 30 °C by recording fluorescent emissions at a wavelength of 485 nm upon excitation at 360 nm, using a Spectramax M2 microplate fluorometer (Molecular Devices). Fluorescence measurements were recorded every 30 s in relative fluorescence units (RFUs). Kinase reactions containing 2 ng recombinant human PKA-Cα catalytic subunit (Invitrogen) were used as positive controls. PKA activity was plotted using GraphPad Prism software version 4 (Graphpad Software, Inc.). Symbols at each time point represent the means of three biological replicates and experiments were performed at least twice.

PKA inhibitor assays

H-89 and protein kinase A inhibitor fragment 14-22 (PKI 14-22 amide) were purchased from Invitrogen. H-89 was dissolved in dimethyl sulfoxide (DMSO) and PKI 14-22 amide was dissolved in water. Kinase reactions were performed as described above in the presence of H-89 and PKI 14-22 amide or appropriate vehicle control at the following concentrations: 500, 100, and 10 μM. The maximum concentration of DMSO in any reaction was less than 5 % and no differences in kinase activity were observed between controls treated with water or DMSO. PKA activity in presence and absence of inhibitor was determined as described in the previous section.

Adenylyl cyclase agonist and inhibitor assays

Forskolin and SQ22536 were purchased from Sigma and stock solutions prepared in DMSO. To examine the effects of forskolin and SQ22536 on PKA activity, triplicate groups of freshly isolated adult worm pairs (10 pairs per group) were incubated for 2 h at 37 ºC in 24 well tissue culture plates containing Dulbecco's modified Eagle's medium (DMEM) in the presence of 100 or 50 μM of forskolin, SQ22536 or DMSO alone. Then Sm lysate was prepared from the treated worms and PKA activity was measured as described above.

In vitro treatment of adult worms with PKA inhibitors

Effects of SmPKA inhibition in adult worms were assessed using H-89 and PKI 14-22 at the following concentrations: 500, 250, 100, 50, 25, 10, and 1 μM. Individual adult worm pairs (6 pairs per concentration) were placed in the wells of 24 well tissue culture plates containing 1 mL total of DMEM (with 10% fetal bovine serum and 5% penicillin/streptomycin) and appropriate concentration of inhibitor. Equal amounts of appropriate vehicle alone were added to the wells containing control worms. Medium containing inhibitor or vehicle was replaced daily. Worms were incubated at 37 °C in 5 % CO_2 and observed every 24 h for a period of 7 d. Worms were considered to be dead when all evidence of motility, including gut peristalsis, had ceased. Kaplan-Meier survival curves were generated using GraphPad Prism software. Photomicrographs were obtained using a Zeiss CL1500ECO dissecting microscope.

SmPKA-C cDNA cloning and sequence analysis

Total RNA was extracted from adult worms using the RNAzol B Method (IsoTex Diagnostics, Inc.). 1 µg RNA was used to synthesize cDNA using the iScript™ Select cDNA Synthesis Kit and an oligo $(dT)_{20}$ primer (Bio-Rad). The full-length cDNA sequence of SmPKA-C was obtained using the RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RACE) kit (Invitrogen) and internal gene specific primers designed from the *S. mansoni* EST Sm11052. RACE products of interest were purified using the QIAquick® Gel Extraction Kit (Qiagen), cloned into the pCR2.1-TOPO vector (Invitrogen), and sequenced using the Big-Dye Terminator cycle sequencing kit (Applied Biosystems). The SmPKA-C cDNA sequence was compared to genomic sequences available at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the *S. mansoni* Genome Project website (http://www.genedb.org/genedb/smansoni/blast.jsp) blast servers. Vector NTI software (Invitrogen) was used to align the nucleotide and amino acid sequences of SmPKA-C with PKA-C subunit sequences from other eukaryotic organisms. Phylip (Phylogeny Inference Package) software [31] was used to construct the phylogenetic tree using the protein parsimony method (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=protpars).

Detection of SmPKA-C gene expression in S. mansoni life cycle stages

Total RNA was extracted and cDNA was synthesized from male and female adult worms, schistosomula, and cercariae as described above. Egg, miracidium, and sporocyst cDNA were kindly provided by Dr. Conor Caffrey (Sandler Center for Basic Research in Parasitic Diseases, San Francisco, CA). To detect SmPKA-C expression, the following primers were used to amplify a 900 bp fragment of the SmPKA-C cDNA (nucleotide positions 50 – 952): forward 5'–GGTAATGCACAAGCTGCTAAA–3' and reverse 5'-CCAATCGGTTGTTGCAAACC-3'. The *S. mansoni* alpha tubulin cDNA (GenBank Accession No. S79195) was used as a positive control and a 100 bp fragment (nucleotide positions 1711 – 1824) was amplified by PCR using the following primers: forward 5'-GGTTGACAACGAGGCCATTTATG-3' and reverse 5'-TGTGTAGGTTGGACGCTCTATATCT-3'. Amplicons were visualized by agarose gel electrophoresis.

SmPKA-C gene silencing using RNAi

A 900 bp SmPKA-C cDNA fragment was generated by PCR using the primers and cycling parameters described above and the resulting amplicon was cloned into pCRII-TOPO vector (Invitrogen). Plasmid DNA was linearized using *Sac*I or *Xho*I and used as template to transcribe ssRNA using T7 and Sp6 RNA polymerases and the MEGAscript RNA transcription kit (Ambion) [32]. SmPKA-C dsRNA was generated and purified using the MEGAscript RNAi kit (Ambion) according to the manufacturer's instructions. A non-schistosome control dsRNA

was generated from the pCR-II TOPO vector as described above. Integrity of the final dsRNA products was assessed by agarose gel electrophoresis. 10 or 30 µg of SmPKA-C or control dsRNA, diluted in 100 µl of electroporation buffer (Ambion), were delivered to groups of 12 mixed-sex adult worms via electroporation as described previously [33]. Adult worms, placed in 4 mm cuvettes, were pulsed at room temperature with a single 20 ms square wave pulse at 125 V using the GenePulser Xcell Electroporation system (Bio-Rad). Adult worms were immediately transferred to pre-warmed DMEM (10% FBS and 5% penicillin/streptomycin) and maintained at 37 ºC. To assess the efficacy of SmPKA-C RNA knockdown, RNA was extracted after 3 d and transcript levels assessed by PCR as described above, except that primers which hybridize outside the targeted region were used (forward 5'-CGCGTAATATCACTTGAGAGTCAAAATAG-3' and reverse 5'-AAATTCACTAAATTCTTTTGCACATTTCTCTGTTGTAGCAATACG-3', to amplify a fragment corresponding to nucleotide positions 18-1096), and the accumulation of PCR product was monitored in real time by detection of SYBR Green fluorescence, using a M.J. Research Chromo4 PCR cycler (Bio-Rad). Relative SmPKA-C RNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method [34] and *S. mansoni* alpha tubulin RNA as the control transcript. Data are representative of two independent experiments.

Statistical analyses

The statistical significance of differences between treated and control groups in activity assays was calculated using one-way ANOVA of repeated measures. The statistical significance of differences between Kaplan-Meier survival curves was calculated using the logrank test. *P* values ≤ 0.05 were considered statistically significant. Student's T test with Welch's correction was used to test the significance of differences in expression levels detected by real-time PCR. GraphPad Prism software was used for all statistical analyses.

Results

Detection of PKA protein expression and PKA enzymatic activity in adult S. mansoni

To detect putative PKA-C subunit homologues in adult *S. mansoni* protein lysate (Sm lysate), western blot analysis using a polyclonal antibody generated against a conserved epitope of the C-terminus of human PKA-Cα subunit was conducted (GenBank Accession No. P17612). A band of 40 kDa was detected in Sm lysate, which was similar in molecular weight to the human PKA-Cα detected in the two human cell lines (Fig. 4A).

We next sought to determine if protein extracts from adult *S. mansoni* had measurable PKA activity. Using a fluorescent peptide substrate-based assay, a putative PKA activity was detected in adult Sm lysate, as determined by the accumulation of a fluorescent, phosphorylated peptide reaction product (Fig. 4B). While reaction product accumulated more slowly in the Sm lysate reaction than in a positive control reaction containing recombinant human PKA-Cα (hereafter

referred to as control PKA), similar total amounts of product accumulated in both reactions by the end of the assay.

While the peptide substrate used in the kinase activity contains a specific PKA target sequence, other protein kinases such as protein kinase C (PKC) and calmodulin-dependent protein kinases (CDPKs) share similar substrate specificity to PKA [35]. For this reason, a kinase inhibitor cocktail that inhibits PKC and CDPK was included in all assays to eliminate non-PKA mediated phosphorylation. To further confirm that the kinase activity detected in Fig. 4B was attributable to a PKA enzyme, adult Sm lysate was treated with inhibitors that target PKA-C subunits, H-89 and PKI 14-22 amide, and the resulting activity was compared to PKA activity in untreated control lysate. The PKA activity of adult Sm lysate and control PKA was completely inhibited by 10 μM H-89 ($P < 0.0001$) (Figs 4C and 4D), an ATP-competitive inhibitor that is a potent inhibitor of PKA both *in vitro* and *in vivo* [36]. Identical results were obtained with 100 and 500 μM H-89 (data not shown). Similar to treatment with H-89, 10 μM PKI 14-22 amide also significantly inhibited the PKA activity of Sm lysate and the control PKA ($P < 0.0001$) (Figs 4E and 4F). PKI 14-22 amide is a highly specific inhibitor of PKA as it contains a pseudosubstrate site which facilitates high affinity binding to the substrate binding site of PKA-C subunits, preventing docking of the substrate [37,38].

Modulation of parasite PKA activity through manipulation of cAMP

Since cAMP binding to PKA-R subunits is required for PKA-C release and activation, we hypothesized that if the kinase activity we detected in Sm lysate was attributable to a PKA, its activity would be sensitive to alterations in the availability of cAMP for R subunit binding. To test this hypothesis, we tested whether the schistosome PKA activity was sensitive to manipulation of endogenous adenylyl cyclase activity [39]. The adenylyl cyclase inhibitor, SQ22536 [40], and adenylyl cyclase agonist, forskolin [41], were each used to either inhibit or activate endogenous adenylyl cyclase activity, respectively. In order to maintain the integrity of intracellular signaling pathways in these experiments, intact adult worms were treated with inhibitor or agonist rather than Sm lysate, as cellular structure is lost during preparation of the parasite lysate. 100 μM SQ22536 significantly decreased PKA activity in treated adult *S. mansoni* worms when compared to the untreated controls ($P < 0.0001$) (Fig. 5A), presumably by inhibiting cAMP production and preventing the disassociation of PKA-C subunits from the holoenzyme. In contrast to SQ22536, 100 μM forskolin significantly increased PKA activity in treated adult worms as compared to the untreated controls ($P < 0.0001$) (Fig. 5C). Similar activation and inhibition were seen with 50 μM of forskolin and SQ22536, respectively (data not shown). As expected, the activity of the recombinant control PKA preparation was not affected by SQ22536 or forskolin, as this preparation does not contain adenylyl cyclase or PKA-R subunits (Figs 5B and 5D).

Taken together, these data further support the conclusion that the kinase activity we detected in adult *S. mansoni* extracts is attributable to a PKA enzyme, as this activity can be inhibited and activated using inhibitors and agonists of adenylyl cyclase. Furthermore, our data suggest that *S. mansoni* possesses a functional cAMP signaling pathway, containing adenylyl cyclase and both regulatory and catalytic PKA subunits.

PKA activity is required for the viability of adult S. mansoni worms in vitro

To test whether the schistosome PKA activity plays a significant role in parasite biology, we next analyzed the effect of the inhibitors H-89 and PKI 14-22 amide on adult worms *in vitro*. Treating worms with H-89 at concentrations of 50 – 500 μM resulted in 100 % mortality within 24 h (Fig. 6A). Incubation of worms with H-89 at 25 μM resulted in 75 % mortality by Day 3 and 100 % by Day 4 (Fig. 6A). Treatment with H-89 at 10 μM resulted in 100% mortality by Day 5 (Fig. 6A). Prior to parasite death, exposure to H-89 caused a cessation in egg production and resulted in dissociation of males and females (Fig. 6B), effects that were evident at H-89 concentrations as low as 1 μM concentration (data not shown), despite the lack of a killing effect (Fig. 6A). Similarly, incubation with 500 and 250 μM of PKI 14-22 amide resulted in 100 % worm mortality within 24 h of exposure (Fig. 6C), while at 100 μM some worms survived until Day 4 (Fig. 6C). Incubating worms with 1 - 50 μM PKI 14-22 amide resulted in 100 % survival (Fig. 6C). As with H-89, PKI 14-22 amide caused cessation of egg production and unpairing prior to parasite death (Fig. 6D). These data show that loss of

PKA activity by inhibition of PKA-C subunits is lethal for adult *S. mansoni in vitro* and suggest that the schistosome PKA is essential for maintaining parasite viability.

Identification of a SmPKA-C cDNA and its expression in S. mansoni life cycle stages

To identify cDNA sequences that might encode for PKA-C subunits in *S. mansoni*, BLASTX searches of the *S. mansoni* genome database (http://www.genedb.org/genedb/smansoni/blast.jsp) were performed using PKA-C protein sequences from other organisms. A 622 bp EST (Sm11052) with significant similarity to other PKA-C subunits was identified. Sm11052 contained an incomplete open reading frame (ORF) that encoded for the N-terminal portion of a protein kinase domain, as determined by the presence of a complete ATP-binding site (corresponding to the consensus motif Gly – x – Gly –x – x - Gly – x – Val) and a serine/threonine kinase active site containing the motif Arg - Asp – Asp – Leu – Lys – x – x – Asn [13]. The complete sequence of the cDNA was obtained by 5' and 3' RACE using gene-specific primers that annealed within the Sm11052 sequence and the entire cDNA was then amplified from adult *S. mansoni* cDNA. The full-length cDNA is 1899 bp long and contains a complete ORF of 1053 bp, encoding for a protein of 350 amino acids in length and with a predicted molecular mass of 40.4 kDa (GenBank Accession No. GQ168377). The ORF encoded for a putative protein kinase, with intact N and C-termini and a C-terminal kinase domain that contained all 12 conserved subdomains

characteristic of protein kinase domains [13]. BLAST comparison of the amino acid sequence with the non-redundant protein sequence database at NCBI showed that the putative *S. mansoni* PKA-C (SmPKA-C) protein shared 70% similarity with PKA-C subunits from other eukaryotic organisms (*Caenorhabditis elegans, Drosophila melanogaster, Mus musculus*, and *Homo sapiens*) (Fig. 7A) and was most similar to the PKA-C subunit from *Aplysia californica* (Fig. 7B). The estimated molecular mass of SmPKA-C protein was also similar to that of other PKA-C proteins and approximately matched the apparent mass of the band detected in *S. mansoni* extracts by western blot using anti-human PKA-C antibodies in Fig. 1A. Qualitative analysis of SmPKA-C expression in various life cycle stages by reverse transcriptase PCR revealed that SmPKA-C transcript was detectable in all *S. mansoni* life cycle stages tested (egg, miracidium, sporocyst, cercaria, schistosomulum, adult male and female; Fig. 7C).

The ORF of SmPKA-C was then compared using BLAST analysis to the *S. mansoni* genome database to identify other putative PKA-C subunit sequences encoded by the *S. mansoni* genome. One sequence, Smp_152330, was identified that was 95% identical to the SmPKA-C nucleotide sequence. PCR analysis showed that the 3'end of the predicted database sequence was incorrect and, using 3' SmPKA-C gene-specific primers, we were able to amplify the correct cDNA sequence of Smp_152330 from adult *S. mansoni* cDNA. Translation of the Smp_152330 nucleotide sequence showed it contained 18 more amino acids at the N-terminus than the SmPKA-C protein, but the remainder of both the nucleotide and amino acid sequences were identical (Fig.

7A and data not shown), suggesting that the corrected Smp_152330 sequence represents an alternatively spliced form of SmPKA-C.

Silencing of SmPKA-C expression by RNAi

To determine the effect of silencing SmPKA-C expression in *S. mansoni*, adult worms were treated via electroporation with 30 µg of SmPKA-C dsRNA or control dsRNA. We observed 75% mortality of adult worms treated with 30 µg of SmPKA-C dsRNA by Day 3, while all control dsRNA-treated parasites survived (Fig. 8A). To reduce parasite mortality and provide more surviving worms for subsequent analysis, the amounts of dsRNA were reduced in subsequent experiments. Treatment with 10 µg of SmPKA-C dsRNA resulted in 92 % survival of worms, while treatment with 10 µg of control dsRNA again resulted in 100 % survival to Day 3 (data not shown). Real-time PCR analysis of SmPKA-C transcript levels in surviving worms on day 3 post-electroporation revealed a significant reduction of SmPKA-C mRNA in SmPKA-C dsRNA-treated worms to approximately 1 % of the levels detected in control dsRNA-treated worms (P = 0.0034) (Fig. 8B).

To determine whether SmPKA-C contributes to the PKA activity detected in Sm lysate, PKA activity was measured in lysates prepared from worms that were treated with 10 µg SmPKA dsRNA or control dsRNA 7 days previously. Worms were treated with 100 µM forskolin for 2 hours prior to lysate preparation in order to reactivate PKA activity. SmPKA activity was significantly reduced in

SmPKA-C dsRNA treated worms as compared to the control dsRNA treated worms ($P < 0.05$) (Fig. 8C).

Discussion

In this study, we provide the first direct evidence for the expression of an active PKA in adult *S. mansoni* worms. First, antibodies to a highly conserved motif from the PKA-C kinase domain of other organisms reacted with a protein of the expected size for a PKA-C subunit in protein extracts from adult worms. Second, a protein kinase assay utilizing a peptide substrate that is preferentially phosphorylated by PKAs demonstrated significant PKA kinase activity in adult worm lysates. Third, the activity of the putative PKA was sensitive to known inhibitors of PKA-C kinase activity, providing further evidence that a PKA-C protein was responsible for the activity we detected in parasite extracts. Finally, exposure of intact parasites to an adenylyl cyclase inhibitor and agonist either decreased or increased, respectively, the PKA activity, demonstrating that the putative parasite PKA exhibits the expected sensitivity to modulation of cAMP levels. Taken together, these data support the conclusion that an active PKA is expressed in adult *S. mansoni*, and that adult schistosomes also express regulatory proteins that control PKA activation by cAMP, such as adenylyl cyclase and PKA regulatory subunits.

We are aware of one other report which demonstrated that targeting protein kinases in adult schistosomes can be detrimental to the parasites. In this report, the authors demonstrated that, while exposure of adult *S. mansoni* worms

to the broad spectrum tyrosine kinase inhibitor herbimycin A *in vitro* was not lethal to schistosome worms, parasite egg production was significantly inhibited in a dose-dependent manner [42]. Here we demonstrate that exposure of intact adult worms to inhibitors of PKA kinase activity is lethal to the parasites, suggesting that, in contrast to herbimycin-sensitive tyrosine kinases, the schistosome PKA is critically important for viability. H-89 in particular killed schistosomes rapidly at low concentrations *in vitro*. However, while H-89 is considered a specific inhibitor of PKA and its IC_{50} for PKA is in the low nanomolar range, it has also been shown to inhibit other protein kinases. For example, previous studies showed that H-89 also inhibited ribosomal protein S6 kinase 1 (S6K1) and mitogen-and stress-activated protein kinase (MSK1) at lower concentrations than PKA [36,43]. Since these two kinases play a major role in eukaryotic cell biology, the lethality observed on treating adult worms with H-89 may not be due to PKA inhibition alone, but rather the result of inhibition of several other kinases in addition to PKA. However PKI 14-22 amide, a highly specific inhibitor of PKA-C subunits that does not affect other protein kinases [37], also caused parasite death, albeit at higher concentrations than H-89, supporting the conclusion that PKA activity is important for maintaining parasite viability. A possible explanation for the difference in parasite killing we observed with these two inhibitors is that PKI 14-22 amide is a peptide, which would not be expected to penetrate the treated parasites as well as H-89. Alternatively, inhibition of additional kinases by H-89 may enhance its toxicity for schistosome worms.

Partial coding sequences for putative PKA-C genes have been generated by the *S. mansoni* genome project, but no full-length sequences for a schistosome PKA-C have been identified. Here we report the isolation of a full-length cDNA encoding for a *S. mansoni* PKA-C subunit we named SmPKA-C. Expression of this transcript was detected in all life cycle stages we examined, including adults, suggesting this cDNA may encode the PKA activity we detected in adult worm extracts. Subsequent targeting of the SmPKA-C transcript in adult schistosomes using RNAi was lethal for the parasites, demonstrating that this gene is essential for parasite viability, at least *in vitro*, and providing further support for the conclusion that the parasite death we observed with the inhibitors H-89 and PKI 14-22 amide were the result of PKA inhibition. Consistent with this conclusion, RNAi inhibition of SmPKA expression resulted in significant loss of PKA activity in parasite lysates, confirming that at least a portion of the PKA activity in adult worms is encoded by this transcript. Interestingly, not all PKA activity was ablated by RNAi of SmPKA, despite significant knock-down of transcript levels to approximately 1 % of the levels observed in control worms, raising the possibility that other PKA isoforms are expressed by adult worms. Alternative splicing in the N-termini of PKA-C subunits has been observed in mammalian species and in invertebrates, such as *C. elegans* [44,45], and our RACE experiments suggest that alternative splicing results in the expression of at least two SmPKA-C isoforms in adult schistosomes. However, the remaining activity cannot be attributed to either of these splice variants, as the two transcripts share the sequence targeted by the dsRNA fragment we used for

RNAi. These observations suggest that *S. mansoni* expresses other PKA isoforms, perhaps encoded by additional PKA genes or generated by additional, less conservative alternative splicing, and argue that a search for additional PKA-encoding sequences in *S. mansoni* mRNA and genomic DNA is warranted. Alternatively, the remaining PKA activity detected in RNAi-treated worms may be due to residual SmPKA-C expression, as ablation of SmPKA-C transcript was not absolute. Interestingly we were unable, using RACE, to isolate full-length cDNAs that correspond to other putative PKA genes that have been predicted from sequences of *S. mansoni* genomic DNA (Smp_080770, Smp_047150.2/1) suggesting that the predicted coding regions are incorrect. Thus the extent to which adult schistosomes express multiple PKA isoforms remains unclear. However, as RNAi knockdown of SmPKA-C resulted in parasite death, we conclude that SmPKA-C is a critically important protein and propose that SmPKA may be an attractive target for the development new schistosomicidal therapeutics.

Tight regulation of PKA activity by PKA-R subunits provides additional opportunities for pharmacological manipulation of PKA, beyond targeting the PKA-C subunit directly with kinase inhibitors. To date, there are no published reports that identify or characterize PKA-R subunits in schistosomes, but the cDNA sequence of a putative PKA-R subunit from *S. mansoni* was recently released by the *S. mansoni* genome project (GenBank accession no. CAY17201) and others may await identification. In other organisms, variation in CBD sequences amongst PKA-R isoforms results in differential affinity for cAMP and

for cAMP analogs that either induce or inhibit holoenzyme dissociation and activation of the PKA-C subunits [46,47]. Thus there is considerable potential for pharmacological manipulation of PKA activity using cAMP analogs. One such analog, 8-Cl-cAMP, has been shown to be a potent growth inhibitor in numerous human cancer cell lines and has completed phase I clinical trials for the treatment of some cancers [25]. Identification and analysis of schistosome PKA-R subunits may identify opportunities for the pharmacological targeting of parasite PKA in a similar manner to that now in development for cancer treatment. These observations highlight the obvious parallels between the treatment of cancer and parasitic infections, which both involve the targeting of eukaryotic cells, and suggest that novel approaches to cancer chemotherapy may provide new leads for the development of much needed anti-parasitic drugs.

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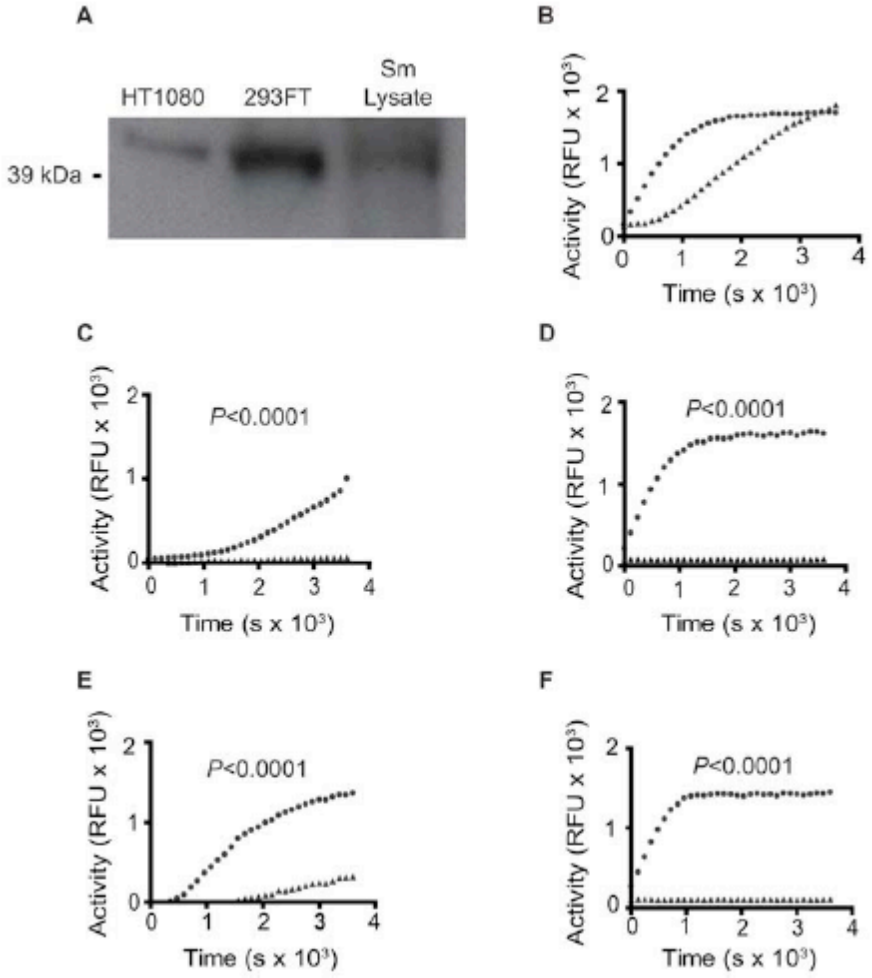
Figure 4: Detection of a putative schistosome PKA in parasite lysates.

A, Western blotting of lysates from adult *S. mansoni* (Sm lysate) and two human cell lines (293FT, HT1080) using a polyclonal antibody specific for the human PKA-Cα subunit revealed the presence of a putative PKA-C subunit of the expected molecular weight of 40 kDa in the schistosome lysate. B, Putative PKA activity is detectable in lysate of adult *S. mansoni* worms using a fluorescence-based kinase assay. ▲, Sm lysate, ● recombinant human PKA-Cα. C-F, Kinase activity was measured in kinase reactions containing Sm lysate (C and E) and recombinant human PKA-Cα (D and F) and the PKA inhibitors H-89 (C and D) and PKI 14-22 (E and F). ▲, inhibitor-treated; ●, no inhibitor. B-F, each time point represents the mean of three biological replicates.


A
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E
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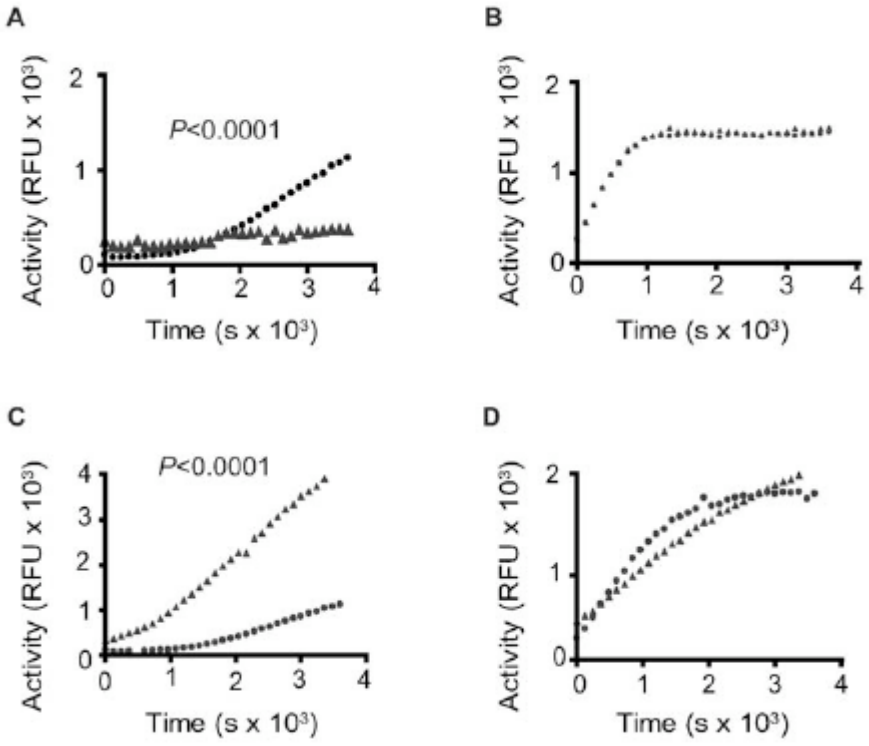
Time (s x 10³)
F
P<0.0001
Activity (RFU x 10³)
Time (s x 10³)

Figure 5: Schistosome PKA activity is sensitive to adenylyl cyclase modulation.

Kinase activity was measured in kinase reactions containing Sm lysate from adultworm pairs (A and C) that were previously treated with SQ22536 (A) or forskolin (C). Treatment groups contained 10 worm pairs (20 worms total) each and were performed in triplicate. Kinase reactions containing recombinant human PKA-Cα (B and D) were treated directly with SQ22536 (B) or forskolin (D). ▲, inhibitor-treated; ●, no inhibitor. Data are representative of two independent experiments.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Activity (RFU x 10³)
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B
Activity (RFU x 10³)
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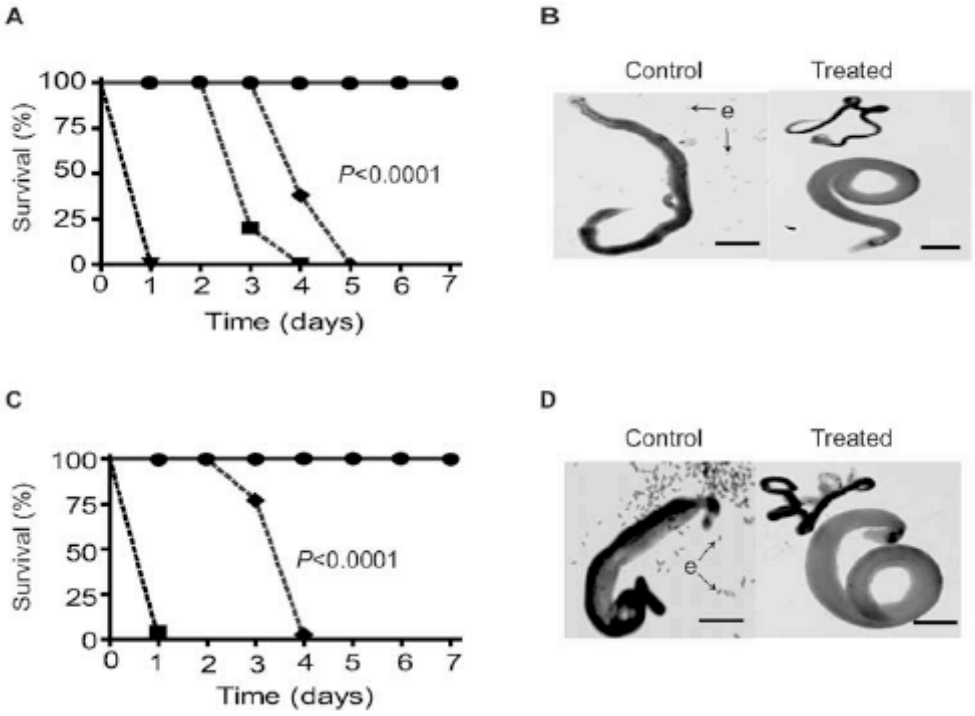
C
Activity (RFU x 10³)
P<0.0001
Time (s x 10³)
D
Activity (RFU x 10³)
Time (s x 10³)

Figure 6: PKA activity is essential for adult *S. mansoni* viability *in vitro*. Adult *S. mansoni* worm pairs were maintained in medium containing varying concentrations of H-89 (A and B) or PKI 14-22 amide (C and D). Survival in the presence of each inhibitor was plotted against time (A and C). For H-89, concentrations of inhibitor are as follows: 0 and 1 μM (●); 10 μM (♦); 25 μM (■); 50, 100, 250, and 500 μM (▼). For PKI 14-22 amide concentrations of inhibitor are as follows: 0, 1, 10, 25, and 50 μM (●); 100 μM (♦); 250 and 500 μM (■). Treatment groups containing 6 worm pairs each (12 worms total) were used for each concentration. B and D, micrographs of representative worm pairs incubated in the presence of 100 μM H-89 (B) or 100 μM PKI 14-22 amide (D). Scale bars = 1 mm. Data are representative of three independent experiments.


A
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Time (days)
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Survival (%)
P<0.0001
0 1 2 3 4 5 6 7
Time (days)
D
Control
Treated
e

Figure 7: Identification of SmPKA-C as a PKA-C subunit from *S. mansoni*. A, Amino acid alignment of both SmPKA-C and putative splice variant peptide sequences with PKA-C sequences from *Caenorhabditis elegans* (Cekin-1; NP_493605)*, Drosophila melanogaster* (DmPKA; NP_476977)*, Mus musculus* (MmPKAa; P05132) and *Homo sapiens* (HsPKAa; P17612). B, Phylogenetic analysis comparing both SmPKA-C amino acid sequences to the following PKA-C sequences from other organisms: *Homo sapiens* PKA-Cα (HsPKAa; P17612), *H. sapiens* PKA-Cβ (HsPKAb; P22694), *H. sapiens* PKA-Cγ (HsPKAg; P22612), *Mus musculus* PKA-Cα (MmPKAa; P05132), *M. musculus* PKA-Cβ (MmPKAb; P68181), *Rattus norvegicus* PKA-Cα (RnPKAa; P27791), *R. norvegicus* PKA-Cβ (RnPKAb; P68182), *D. melanogaster* (DmPKA; NP_476977), *Aplysia californica* (AcPKA; CAA45014), and *Acyrthosiphon pisum* (ApPKA; XP_001946114). C, SmPKA-C transcript is expressed in cDNA of all the life cycle stages of *S. mansoni*. 1, egg, 2, miracidium, 3, sporocyst, 4, cercaria, 5, schistosomulum, 6, adult female, 7, adult male.

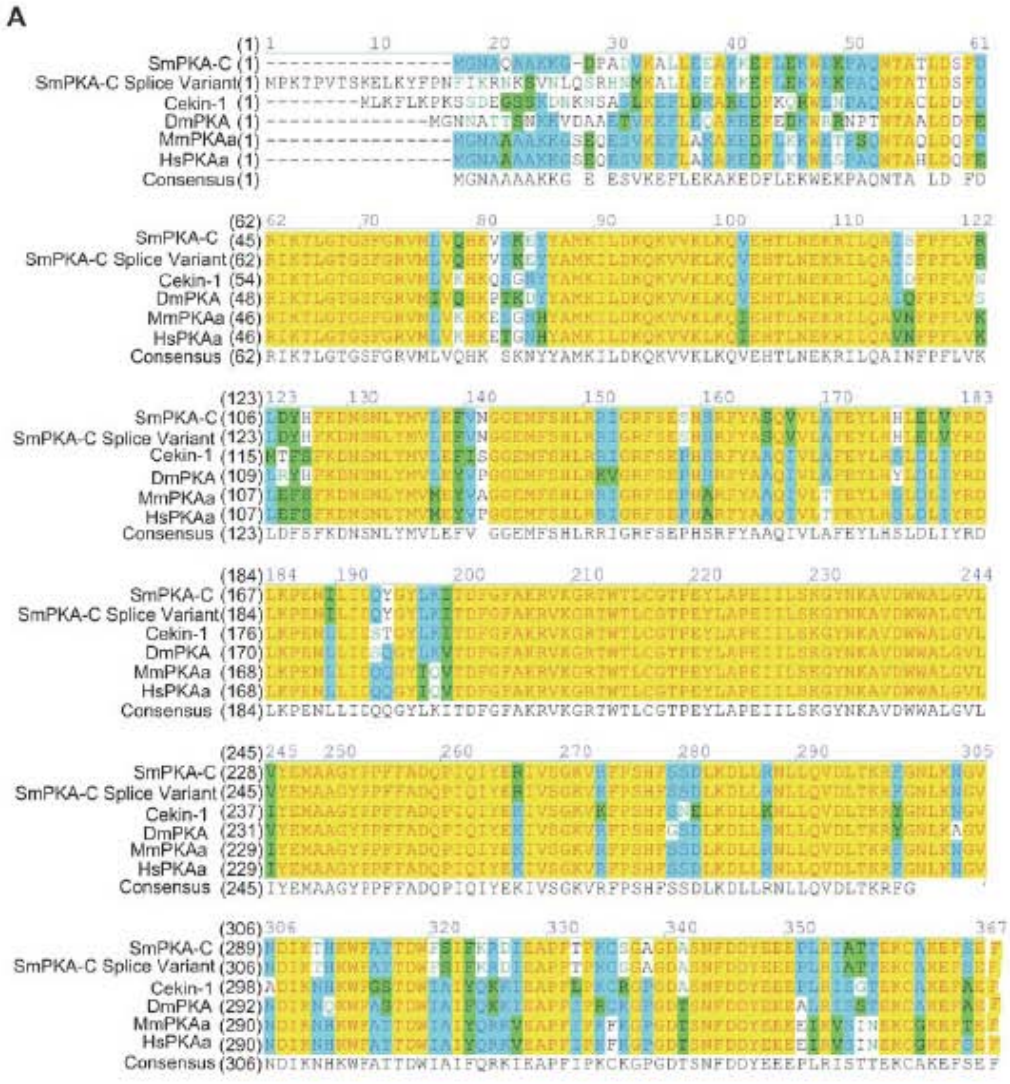
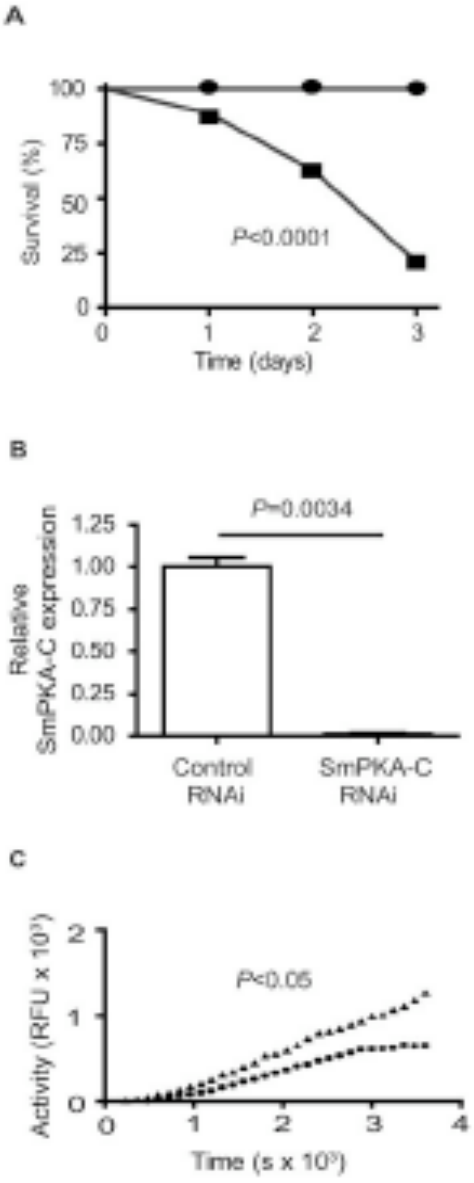

A
SmPKA-C
SmPKA-C Splice Variant
Cekin-1
DmPKA
MmPKAa
HsPKAa
Consensus
B
HsPKAa
HsPKAg
MmPKAa
AcPKA
SmPKA-C Splice
SmPKA-C
RnPKAa
DmPKA
HsPKAb
MmPKAb
RnPKAb
ApPKA
C
1 2 3 4 5 6 7
SmPKA-C
Tubulin

Figure 8: SmPKA-C encodes for an essential kinase activity.

A, Electroporation of adult worms with 30 µg of SmPKA-C dsRNA (■) resulted in 75% mortality while worms treated with control dsRNA (●) exhibited 100 % survival. B, Three days after electroporation, SmPKA-C transcript levels were decreased in worms electroporated with 10 µg of SmPKA-C dsRNA, while transcript levels were unaffected in control dsRNA-treated worms, as determined by real-time PCR. C, 7 days after electroporation, SmPKA-C dsRNA-treated (●) and control dsRNA-treated worms (▲) were incubated for 2 h in 100 µM forskolin. PKA activity was significantly decreased after electroporation with 10 µg SmPKA-C dsRNA compared to control dsRNA-treated worms.


A
100
75
50
25
0
Survival (%)
P<0.0001
0
1
2
3
Time (days)
B
P=0.0034
1.25
1.00
0.75
0.50
0.25
0.00
Relative SmPKA-C expression
Control RNAi
SmPKA-C RNAi
C
2
1
0
Activity (RFU x 10³)
P<0.05
0
1
2
3
4
Time (s x 10³)

Chapter 3

DEVELOPMENTAL REGULATION OF PROTEIN KINASE A EXPRESSION AND ACTIVITY IN *SCHISTOSOMA MANSONI*

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Abstract

c-AMP-dependent protein kinases (PKAs) are the main transducers of cAMP signaling in eukaryotic cells. Recently we reported the identification and characterization of a PKA catalytic subunit (SmPKA-C) in *Schistosoma mansoni* that is required for adult schistosome viability *in vitro*. To gain further insights into the role of SmPKA-C in biological processes during infection of the mammalian host, we undertook a quantitative analysis of SmPKA-C mRNA expression in different schistosome life cycle stages. Our data show that SmPKA-C mRNA expression is developmentally regulated, with the highest levels of expression in cercariae and adult female worms. To evaluate the biological role of SmPKA-C in these developmental stages, cercariae and adult worms were treated with various concentrations of PKA inhibitors. Treatment of cercariae with H-89 and PKI 14-22 amide resulted in loss of viability, suggesting that, as in adults, PKA is an essential enzyme activity during larval stages of development. In adult worms, *in vitro* exposure to sub-lethal concentrations of H-89 and PKI 14-22 amide resulted in inhibition of egg production in a dose-dependent manner. Furthermore, using a murine model of schistosome infection where *S. mansoni* fecundity is impaired, we show that reduced rates of egg production *in vivo* correlate with significant reductions in SmPKA-C mRNA expression and PKA activity. Finally, rescue of the defective parasite phenotype *in vivo* resulted in restoration of SmPKA-C mRNA expression, PKA activity and egg production. Taken together, our data suggest that PKA is likely required at

all developmental stages of the schistosome life cycle and may play a specific role in the reproductive activity of adult worms.

Introduction

Schistosomiasis, a disease caused by parasitic blood flukes of the genus *Schistosoma*, affects over 200 million people in tropical and sub-tropical regions and ranks second only to malaria as a parasitic cause of morbidity and mortality worldwide [1]. Schistosome developmental biology is complex, involving distinct life cycle stages that are adapted for survival within and transmission between the intermediate molluscan and definitive mammalian hosts [2]. Chemotherapy depends solely on the anthelmintic praziquantel (PZQ), which is effective against the adult worms of all the medically important schistosome species [3]. However, it is unrealistic to expect that reliance on PZQ for all treatment and control of schistosomiasis will be sustainable in the long-term. PZQ-tolerant strains of schistosomes have been reared in the laboratory and there is evidence of decreased PZQ sensitivity in patients following short- and long-term PZQ treatment regimens [4,5,6]. Thus the potential for the development of PZQ resistance is real and major research efforts now need to be focused on identifying new chemotherapeutic targets in schistosomes [7].

Protein kinases are signal-transducing enzymes that could represent novel chemotherapeutic targets in schistosomes and other eukaryotic pathogens [8,9]. In particular, cAMP-dependent protein kinases (PKAs) are the major mediators of cAMP signaling in eukaryotes and play a central role in biological

processes as diverse as gene expression, apoptosis, tissue differentiation, and cellular proliferation [10], through the phosphorylation of protein substrates at serine/threonine residues [11]. In its inactive state, PKA exits as an inactive tetramer consisting of two identical regulatory (PKA-R) subunits bound to two identical catalytic (PKA-C) subunits. Mammalian genomes contain as many as three *pka-c* genes and four *pka-r* genes, the products of which can combine in different combinations to produced holoenzymes that serve different functions [12]. Because the unregulated activity of PKA in mammalian cells has been implicated in the pathogenesis of several types of cancer [13], the development of PKA inhibitors has been pursued as a potential treatment for these diseases [14,15]. Furthermore PKA has been shown, through chemical inhibition, to be an essential signaling component in the life cycles of a number of eukaryotic pathogens, including *Plasmodium falciparum* [16], *Leishmania major* [17] and *Giardia lambia* [18], suggesting that PKA inhibitors may also have anti-parasitic applications.

Although PKAs represent potentially attractive new targets for the treatment of parasitic diseases, relatively little is known about the role and significance of these kinases in the biology of schistosomes. Previous studies on the role of PKA in *S. mansoni* demonstrated an important role for cAMP and PKA in miracidial locomotion and transformation to the mother sporocyst [19,20]. Furthermore, we recently showed that a PKA-C subunit (SmPKA-C; GenBank Accession No. GQ168377) is expressed in active form in adult *S. mansoni* and is required for parasite viability, at least *in vitro* [21]. However, the role of SmPKA-

C during infection of and development within the mammalian host has not been examined. To gain further insights into the role of SmPKA-C in mammalian parasitism, we first undertook a quantitative analysis of SmPKA-C mRNA expression in the different schistosome life cycle stages. We then examined the effect of PKA inhibitors on the stages in which SmPKA-C mRNA is most abundant, the cercaria and adult female. In the case of adult schistosomes, we also examined SmPKA mRNA levels and PKA activity under *in vivo* conditions where adult reproductive fitness is compromised. Our data suggest that SmPKA-C is required for invasion of the mammalian host and for the subsequent reproductive activity of adult worms.

Materials and Methods

Parasite materials

Biomphalaria glabrata snails infected with the NMRI/Puerto Rican strain of *S. mansoni* were obtained from Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD). Cercariae were collected by exposing infected snails to light for 2 h in 50 mL of ultra-filtered water. Schistosomula were prepared by the mechanical transformation method according to published protocols [22]. Adult *S. mansoni* were collected by portal perfusion from 6 week-infected wild type and recombination activating gene-1 deficient (RAG-$1^{-/-}$) C57BL/6 mice that were infected with 150 cercariae using the tail immersion method [22]. *S. mansoni* miracidia and sporocysts were kindly provided by Dr. Nithya Raghavan (Biomedical Research Institute, Rockville, MD). *S. mansoni* egg cDNA was

kindly provided by Dr. Conor R. Caffrey (Sandler Center for Basic Research in Parasitic Diseases, San Francisco, CA).

Quantitative RT- PCR of SmPKA-C transcript (qPCR)

Total RNA was extracted from miracidia, sporocysts, cercariae, schistosomula and adult female and male worms using the RNAzol B Method (IsoTex Diagnostics, Inc.). 1 µg RNA from each life cycle stage was used to synthesize cDNA using the iScript™ Select cDNA Synthesis Kit and an oligo $(dT)_{20}$ primer (Bio-Rad). cDNA was used as templates for PCR amplification using the SYBR Green fluorescence master mix and the M.J. Research Chromo4 PCR cycler (Bio-Rad). The $2^{-\Delta\Delta Ct}$ method [23] was used to quantify relative SmPKA-C expression (GenBank Accession No. GQ168377), using the *S. mansoni* alpha tubulin (GenBank Accession No. S79195) cDNA as an internal control. Primers used to amplify a ~100 bp fragment (nucleotide positions 4 - 110) of the SmPKA-C cDNA were: forward 5'–GGTAATGCACAAGCTGCTAAA–3' and reverse 5'- GTGTTCTGAGCAGGCTTCTCCC -3'. Primers used to amplify a ~100 bp fragment of the alpha tubulin (GenBank Accession No. S79195) cDNA (nucleotide positions 1711 - 1824) were: forward 5' - GGTTGACAACGAGGCCATTTATG-3' and reverse 5'- TGTGTAGGTTGGACGCTCTATATCT-3'. SmPKA-C expression levels in the various life cycle stages were then expressed as fold change relative to the expression level in adult male worms. All assays were performed in triplicate and data are representative of three independent experiments.

Detection of PKA enzymatic activity in cercariae and adult worms.

Freshly isolated adult worms from 6 week-infected C57BL/6 mice were isolated and transferred immediately to Petri dishes containing Dulbecco's modified Eagle's medium (DMEM). Adult worms were kept in DMEM at room temperature for 2 h in order for pairs to separate. Cercariae were collected in ultra-filtered water and placed on ice for 1 h, spun at 600 rpm for 10 min and the resulting pellet was washed with cold PBS three times. Adult male, female and cercarial protein lysates were prepared by homogenization in cell extraction buffer containing phosphatase and protease inhibitor cocktails (Sigma) and insoluble material removed by centrifugation, as described previously [21]. Protein concentrations were determined using the Quick Start™ Bradford Protein Assay and adjusted to a final concentration of 0.2 μg/μL with additional extraction buffer. In some experiments, triplicate groups of freshly isolated adult worm pairs (six pairs per group) were incubated for 2 h at 37 ºC in 24 well tissue culture plates containing DMEM and 100 μM forskolin or DMSO alone, prior to preparation of protein lysates. PKA activity was measured using the Omnia® Lysate Assay for PKA kit (Biosource) on a Spectramax M2 microplate fluorometer (Molecular Devices) as described previously [21]. Briefly, a master mix containing ATP, a specific PKA peptide substrate, DTT, ultra-pure water and a non-PKA inhibitor cocktail in kinase reaction buffer was prepared according to the manufacturer's instructions. 1 μg total protein (equivalent to 5 μL protein lysate) was added to 45 μL of the master mix in opaque 96-well assay plates.

Kinase reactions containing 2 ng recombinant human PKA-Cα catalytic subunit (GenBank Accession No. NP_002721.1) (Invitrogen) were used as positive controls. Accumulation of phosphorylated substrate was monitored during a 1 h incubation at 30 °C by recording fluorescent emissions every 30 s in relative fluorescent units (RFUs) at a wavelength of 485 nm upon excitation at 360 nm. PKA activity was plotted using GraphPad Prism software version 4 (Graphpad Software, Inc.). Symbols at each time point represent the means of three biological replicates and all experiments were performed twice.

PKA-C inhibitor assays

Newly released cercariae were collected in ultra-filtered water and counted. Effects of PKA inhibition on cercariae were assessed using the PKA-C inhibitors H-89 and PKI 14-22 at the following concentrations: 100, 50, 10, and 1 μM. H-89 and PKI 14-22 amide were purchased from Invitrogen and both were dissolved in water. Approximately 20 cercariae (2 biological replicates of 20 cercariae for each concentration) were placed in 500 μL of ultra-filtered water and appropriate concentration of inhibitor in the wells of 24 well tissue culture plates and incubated at room temperature and observed every hour for a period of 3 h. Cercariae were considered to be dead when all evidence of motility had ceased. Data are representative of two independent experiments. Kaplan-Meier survival curves were generated using GraphPad Prism software. For assays with adult *S. mansoni*, freshly isolated worms were treated with PKI 14-22 amide and H-89 at the following concentrations: PKI 14-22 amide: 50, 25, 10 and 1 μM;

H-89: 1 μM. H-89 was dissolved in DMSO and PKI 14-22 amide was dissolved in water. Individual adult worm pairs (6 pairs per concentration) were placed in the wells of 24 well tissue culture plates containing 1 mL total of DMEM (with 10% fetal bovine serum and 5% penicillin/streptomycin) and appropriate concentration of inhibitor. Equal concentrations of appropriate vehicle alone were added to the wells containing control worms. The maximum concentration of DMSO was less than 1 % and had no visible effect on control worms. Medium containing inhibitor or vehicle was replaced every 24 h. Worms were incubated at 37 °C in 5 % CO_2 and observed every 24 h for egg production for a period of 6 d. Data are representative of two independent experiments.

In vivo restoration of schistosome fecundity in RAG-1$^{-/-}$ mice

Lipopolysaccharide (LPS) (*E. coli* K12 strain) was purchased from InvivoGen and dissolved in PBS at a concentration of 20 μg/100 μL. Two groups of RAG-1$^{-/-}$ mice and one group of wild type C57BL/6 mice were infected with *S. mansoni.* One group of RAG-1$^{-/-}$ mice was injected i.p. twice a week for 6 weeks with 20 μg of LPS, while the other group of RAG$^{-/-}$ mice received injections of PBS. Adult worms were isolated from infected mice at 6 weeks post infection. To quantify egg production, livers of infected mice were homogenized in 0.05% trypsin in PBS (50 mL), eggs were counted under a dissecting microscope and total liver eggs were normalized relative to the number of worm pairs obtained from each mouse. Data are representative of two independent experiments.

Statistical analyses

The statistical significance of differences between treatment groups in PKA activity assays was calculated using one-way ANOVA of repeated measures. The statistical significance of differences between Kaplan-Meier survival curves was calculated using the logrank test. Student's T test was used to test the significance of differences in SmPKA-C mRNA detected by real-time PCR and to compare numbers of eggs/pair produced in control and worms treated with PKA inhibitors *in vitro*. Kruskal-Wallis tests followed by Dunns' multiple comparison tests were used to determine the significance of SmPKA-C mRNA levels and egg production in the LPS treatment experiments. *P* values ≤ 0.05 were considered statistically significant. GraphPad Prism software version 4.0 was used for all statistical analyses.

Results

Differential regulation of SmPKA-C expression in larval stages of S. mansoni

We previously showed that SmPKA-C mRNA is detectable in all *S. mansoni* life cycle stages [21], suggesting that some level of SmPKA-C expression may be required throughout the parasite life cycle. To determine whether SmPKA-C expression is differentially regulated during the schistosome life cycle, quantitative real time PCR was used to investigate the relative abundance of SmPKA-C mRNA in different life cycle stages. In the extramammalian stages of the life cycle, SmPKA-C mRNA was expressed at the

highest levels in sporocysts and cercariae, with levels at least five-fold higher in cercariae when compared to sporocysts (Fig. 9A).

To determine whether SmPKA-C expression correlated with levels of PKA activity, PKA activity assay were conducted on extracts of cercariae using a fluorescent peptide substrate-based assay. Consistent with the relative abundance of SmPKA-C mRNA in cercariae, PKA activity was readily detectable in cercarial protein lysate, with fluorescent reaction product accumulating to similar levels seen with control recombinant human PKA- Cα (Fig. 9B).

PKA-C activity is required for cercarial viability.

A previous study showed that when miracidia were treated with the PKA-C inhibitors H-89 and PKI 14-22 amide, these inhibitors impaired miracidial locomotion in a dose-dependent manner [20]. To determine the effect of PKA inhibition on cercariae, cercariae were treated with H-89 and PKI 14-22 amide at similar concentrations to those used by Matsuyama et al. [20] and observed for 3 h for any phenotypic changes. Treating cercariae with H-89 at 100 μM resulted in 100% mortality within 1 h (Fig 9C). Incubation of cercariae with H-89 at 50 μM resulted in 75% mortality within 1 h and 100% mortality by 2 h (Fig 9C). Cercariae treated with H-89 at 10 μM resulted in 75% mortality by 2 h and 100% mortality at 3 h (Fig 9C). 1 μM concentration of H-89 did not produce mortality in treated cercariae, but cercarial motility was severely decreased when compared to the controls (data not shown). Treating cercariae with PKI 14-22 amide at 100 μM resulted in 100% mortality within 1 h (Fig 9D). Incubation of cercariae with

PKI 14-22 amide at 50 μM resulted in 50% mortality within 1 h and 100% mortality by 2 h (Fig 9D). Cercariae treated with PKI 14-22 amide at 10 μM resulted in 20% mortality by 2 h and 100% mortality at 3 h (Fig 9D). As with H-89, 1 μM concentration of PKI 14-22 amide did not produce mortality in treated cercariae, but the same motility-impaired phenotype was observed in the treated cercariae (data not shown).

Differential regulation of SmPKA-C expression and PKA activity in the intramammalian stages of S. mansoni

In the intramammalian stages, SmPKA-C mRNA was expressed at the highest levels in adult females, where levels were almost twenty-fold higher when compared to adult males and newly transformed schistosomula (Fig. 10A). However, measurement of PKA activity in male and female protein extracts revealed there was relatively more PKA activity per microgram of protein in males than in females (Fig. 10B; $P < 0.0001$), suggesting there is tighter translational and/or post-translational control of baseline PKA activity in females compared to males.

PKA-C activity is required for S. mansoni egg production in vitro

We previously showed that suppression of PKA-C activity, using chemical inhibitors or RNAi of SmPKA-C, was lethal for adult *S. mansoni* worms *in vitro* [21]. To gain insights into the biological roles PKA-C plays in adult worms, we treated freshly isolated adults with a range of sub-lethal concentrations of H-89

and PKI 14-22 amide and observed them *in vitro* for 5 days. All worms remained alive at all the inhibitor concentrations tested, for the duration of the 5 day observation period. However, adult pairs treated with as little as 1 μM H-89 produced significantly fewer eggs on days 1 and 2 when compared to the controls ($P < 0.0001$) and stopped producing eggs entirely by day 3 (Fig. 11A). Higher concentrations of H-89 caused an immediate cessation of all egg production (Fig. 11A). Similarly, all concentrations of PKI 14-22 amide tested caused an immediate and significant reduction in egg production by day 1 (Fig. 11B). Adult worm pairs treated with 25 and 50 μM of PKI 14-22 amide stopped producing eggs altogether on days 4 and 3 respectively (Fig. 11B). Worm pairs treated with concentrations of 1 and 10 μM PKI 14-22 amide stopped producing eggs by day 5 (Fig. 11B). These data suggest that PKA may play a specific role in parasite egg production.

SmPKA-C mRNA expression and PKA activity in adult S. mansoni correlate with reproductive activity in vivo

We have previously shown that the normal development and reproductive activity of *Schistosoma* worms is positively influenced by immune signals and that parasite development and reproduction is compromised in immunodeficient mice, such as RAG-$1^{-/-}$ mice, that lack an adaptive immune system [24,25]. To further investigate the potential role of SmPKA-C in *S. mansoni* reproduction *in vivo*, we measured PKA activity and SmPKA-C mRNA expression in adult *S. mansoni* from infected RAG-$1^{-/-}$ mice, which are reproductively mature but

produce fewer eggs than worms of the same age from infected wild type mice [24]. As shown in Fig. 12A, SmPKA-C mRNA expression in worms from RAG-1$^{-/-}$ mice (P = 0.0007) was decreased to approximately 30 % of the levels detected in the worms from wild type mice. Furthermore, baseline PKA activity was significantly decreased (P < 0.0001) in adult *S. mansoni* from RAG-1$^{-/-}$ mice when compared to adult worms from wild type mice (Fig. 12B). To test whether adult *S. mansoni* from RAG-1$^{-/-}$ mice possess additional inducible PKA activity, worms from RAG-1$^{-/-}$ mice were exposed to 100 μM of the adenylyl cyclase agonist forskolin for 2 h prior to measurement of PKA activity. PKA activity increased significantly after forskolin treatment (P < 0.0001) when compared to untreated worms from RAG-1$^{-/-}$ mice (Fig. 12C), and reached levels comparable to those seen in untreated worms from wild type mice (Fig. 12B). Thus, worms from RAG-1$^{-/-}$ mice express lower levels of SmPKA-C mRNA and have lower baseline PKA activity immediately *ex vivo*, but possess additional PKA activity that can be induced by raising intracellular cAMP concentrations.

In vivo restoration of egg production also restores SmPKA-C mRNA expression and PKA activity

In separate studies (Lamb *et al*., submitted), we have shown that induction of innate immune responses in RAG-1$^{-/-}$ mice restores reproductive activity in *S. mansoni*. Therefore, we hypothesized that, if SmPKA-C is involved in parasite egg production, SmPKA-C mRNA expression and PKA activity in worms from RAG-1$^{-/-}$ mice would also be restored by immune response induction. To test this

hypothesis, we treated *S. mansoni*-infected RAG-1$^{-/-}$ mice with 20 μg *E. coli* LPS twice weekly for 6 weeks and quantified SmPKA-C mRNA and PKA activity in the isolated worms. SmPKA-C mRNA in worms from the LPS treatment group was significantly increased ($P < 0.05$) compared to worms from the control treatment group (Fig. 13A) and was no longer significantly different from the levels found in worms from wild type mice. Furthermore, the PKA activity in worms from the LPS-treated RAG-1$^{-/-}$ mice was significantly higher ($P < 0.0001$) when compared to PBS-treated control RAG-1$^{-/-}$ mice, and reached levels comparable to these found in worms from wild type mice (Fig. 13B). Consistent with previous results, (Lamb *et al*., submitted), egg production was also significantly restored by LPS treatment ($P < 0.05$) compared to the control RAG-1$^{-/-}$ mice (Fig. 13C), although restoration was not complete as egg production was still reduced compared to that observed in wild type mice (Fig. 13C).

Discussion

In this study, our aim was to further define the biological role of SmPKA-C in the *S. mansoni* life cycle, focusing particularly on the adult male and female worms. We showed in a previous report that SmPKA-C is an essential gene in adult worms, at least *in vitro*, and could therefore represent an attractive therapeutic target for the treatment and control of schistosomiasis [21]. In this study, we wished to explore the role of SmPKA-C in greater detail, to understand which aspects of parasite biology would be most affected by targeting SmPKA-C. Here we show that SmPKA-C expression is developmentally regulated, with

cercariae and adult females expressing the highest levels of SmPKA-C transcript, suggesting a significant role for SmPKA-C in these life cycle stages.

Consistent with an important role for SmPKA-C in cercariae, PKA activity was readily detectable in cercariae and cercariae were susceptible to the PKA-C inhibitors H-89 and PKI 14-22 amide, as was shown for miracidia [20]. However, in contrast to miracidia, where PKA-C inhibition merely disrupted miracidial locomotion, PKA-C inhibition produced lethality in cercariae within an hour of exposure. These results suggest that, in contrast to miracidia, PKA is required for viability of the cercarial transmission stage. Consistent with this differential requirement for PKA-C activity in cercariae, SmPKA-C mRNA was also expressed at relatively high levels in daughter sporocysts, but not in eggs (Fig. 1A). We were unable to compare PKA activity in miracidia and cercariae as we could not obtain sufficient quantities of miracidial protein lysate, but our hypothesis is that PKA activity is higher in cercariae than in miracidia.

Interestingly, SmPKA-C mRNA levels decreased dramatically in schistosomula compared to cercariae suggesting a lack of requirement for PKA immediately after infection of the mammalian host. A parallel situation may occur during the miracidium-mother sporocyst transformation, as stimulation of PKA activity in miracidia inhibited the transformation to mother sporocysts [19]. However, as infection of the mammalian host proceeds, SmPKA-C again appears to assume an important role, as SmPKA-C mRNA was highly expressed in adult female worms. Indeed, SmPKA-C mRNA was relatively more abundant in female worms than males, despite higher levels of PKA activity in males,

suggesting a greater degree of translational and/or post-translational regulation of PKA activity in females. In addition to host signals [24], female schistosomes require the presence of male schistosomes for normal physical growth and development, sexual maturation and reproductive capacity [26]. The necessity to transduce these various signals may account for the greater regulation of PKA activity in females compared to males. Indeed, males and females were allowed to separate for up to 2 h *in vitro*, in order to measure sex specific activity, and PKA activity in females may therefore have decreased without the constant stimulation from the male worms.

The high expression levels of SmPKA-C mRNA in female worms are suggestive of a role for SmPKA-C in female-specific processes, such as egg production. Consistent with a role for signal transduction pathways in egg production, a previous study showed that tyrosine kinase signaling may be important for schistosome fecundity, by demonstrating that exposure to the broad spectrum tyrosine kinase inhibitor herbimycin A significantly inhibited parasite egg production *in vitro*, in a dose-dependent manner [27]. Furthermore, PKA in the free-living nematode *Caenorhabditis elegans* has been shown to play a critical role in egg production, as RNAi knock-down of *C. elegans kin*-1 (a PKA-C subunit homologue) expression resulted in abnormal egg laying [28,29]. Likewise in *S. mansoni*, PKA inhibition in adult worm pairs with sub-lethal concentrations of H-89 and PKI 14-22 amide significantly decreased egg production *in vitro*, in a dose-dependent manner. These data suggest that, at least *in vitro*, PKA signaling is important for maintaining normal reproduction.

To determine whether a role for PKA signaling in egg production could be substantiated *in vivo*, SmPKA-C expression and PKA activity were assessed in adult *S. mansoni* from infected RAG-1$^{-/-}$ mice, as parasite egg production is significantly impaired in these animals [24,25,30]. Consistent with this hypothesis, PKA activity and SmPKA-C mRNA are significantly decreased in adult worms from RAG-1$^{-/-}$ mice, in comparison to adult worms from wild type mice at the same stage of infection. Furthermore, while baseline PKA activity is decreased in worms from RAG-1$^{-/-}$ mice, it can be activated significantly by the adenylyl cyclase agonist forskolin, indicating that the cAMP/PKA pathway is functional in RAG-1$^{-/-}$-derived parasites but is not fully activated, perhaps due to the absence of specific immune signals required for egg production [31,32,33]. To test this latter possibility, SmPKA-C mRNA expression and PKA activity were assessed in worms from RAG-1$^{-/-}$ mice where schistosome development had been restored through induction of innate immune responses with the Toll-like receptor 4 (TLR 4) ligand LPS [34]. Restoration of parasite egg production *in vivo* resulted in restoration of SmPKA-C mRNA expression and PKA activity to levels comparable with those seen in worms from wild-type mice. Thus, expression of SmPKA-C mRNA and PKA activity in adult *S. mansoni* correlates with egg production, both *in vitro* and *in vivo*, suggesting a significant role for PKA signaling in the reproductive activity of adult schistosomes.

In conclusion, the results we present suggest that, in addition to maintaining schistosome viability, PKA signaling in adult worms is specifically involved in the production of eggs by female worms. These results suggest that,

even at sub-curative levels, targeting PKA in schistosomes could possibly be useful in controlling the transmission of schistosomiasis by preventing or limiting the shedding of eggs in the feces of infected hosts. Furthermore, as the pathology associated with schistosome infection is caused by the deposition of parasite eggs in host tissues, inhibition of egg production through targeting PKA activity could be useful in reducing egg-induced pathology and morbidity.

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Figure 9: SmPKA-C expression and PKA activity in the larval stages of *S. mansoni.*

A, Relative SmPKA-C mRNA levels in *S. mansoni* larval stages, as determined by real-time PCR. Data were normalized to the levels detected in a standard preparation of adult male *S. mansoni* RNA. B, Detection of PKA activity in lysate of *S. mansoni* cercariae using a fluorescence-based kinase assay. ♦, Cercarial lysate, ●, recombinant human PKA-Cα. C and D, Survival of *S. mansoni* cercariae in the presence of PKA-C inhibitors. Cercariae in groups of 20 were maintained in water containing varying concentrations of H-89 (C) or PKI 14-22 amide (D). Survival in the presence of each inhibitor was plotted against time. Inhibitor concentrations are as follows: 0 and 1 μM (●); 10 μM (♦); 50 μM (▲); 100 μM (■). Data are representative of two independent experiments.

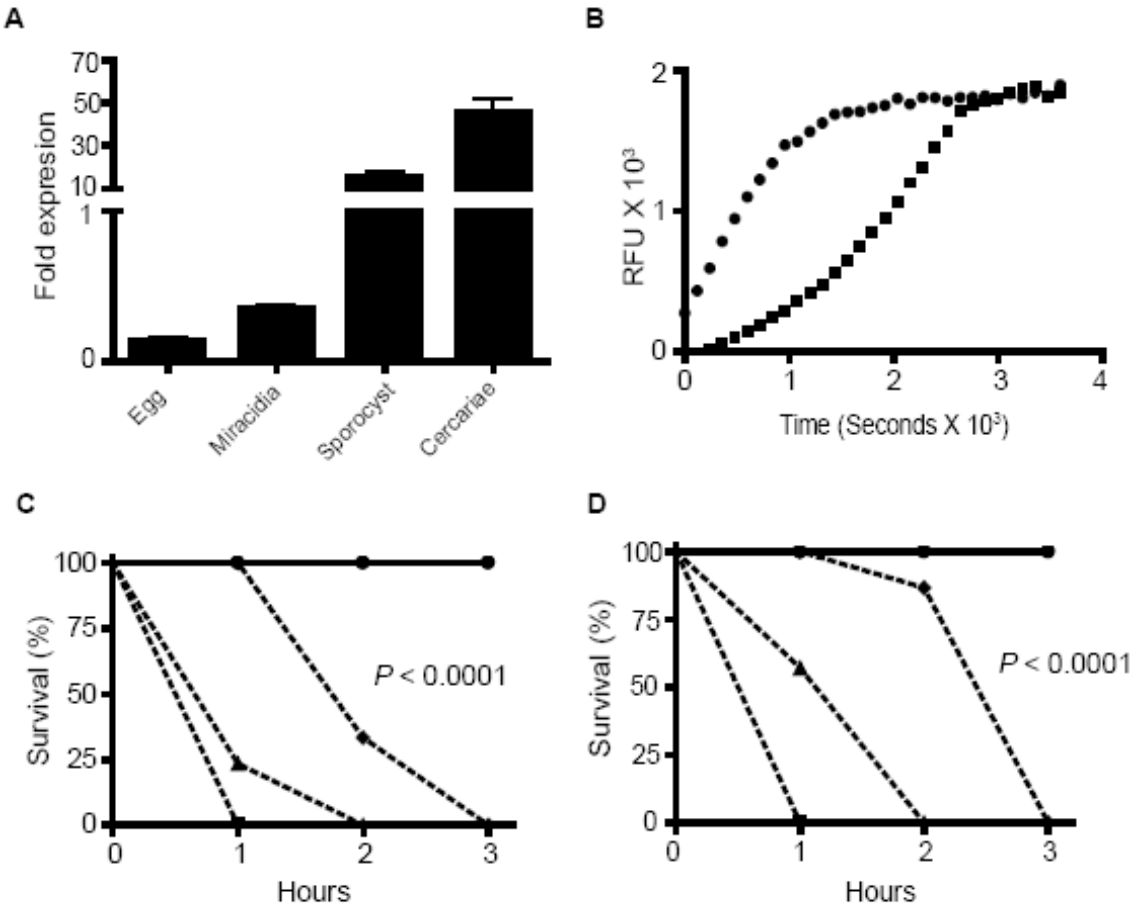

A
Fold expresion
70
50
30
10
1
0
Egg
Miracidia
Sporocyst
Cercariae
B
RFU X 10³
2
1
0
0
1
2
3
4
Time (Seconds X 10³)
C
Survival (%)
100
75
50
25
0
P < 0.0001
0
1
2
3
Hours
D
Survival (%)
100
75
50
25
0
P < 0.0001
0
1
2
3
Hours

Figure 10: SmPKA-C expression and PKA activity in the intramammalian stages of *S. mansoni.*

A, Relative SmPKA-C mRNA levels in adult male and female *S. mansoni* worms and schistosomula, as determined by real-time PCR. Data were normalized to the levels detected in a standard preparation of adult male *S. mansoni* RNA. B, Detection of PKA activity in lysates of adult male and female *S. mansoni* worms, using a fluorescence-based kinase assay. ●, recombinant human PKA-Cα, ▲, male lysate, Δ, female lysate. Data are representative of two independent experiments.

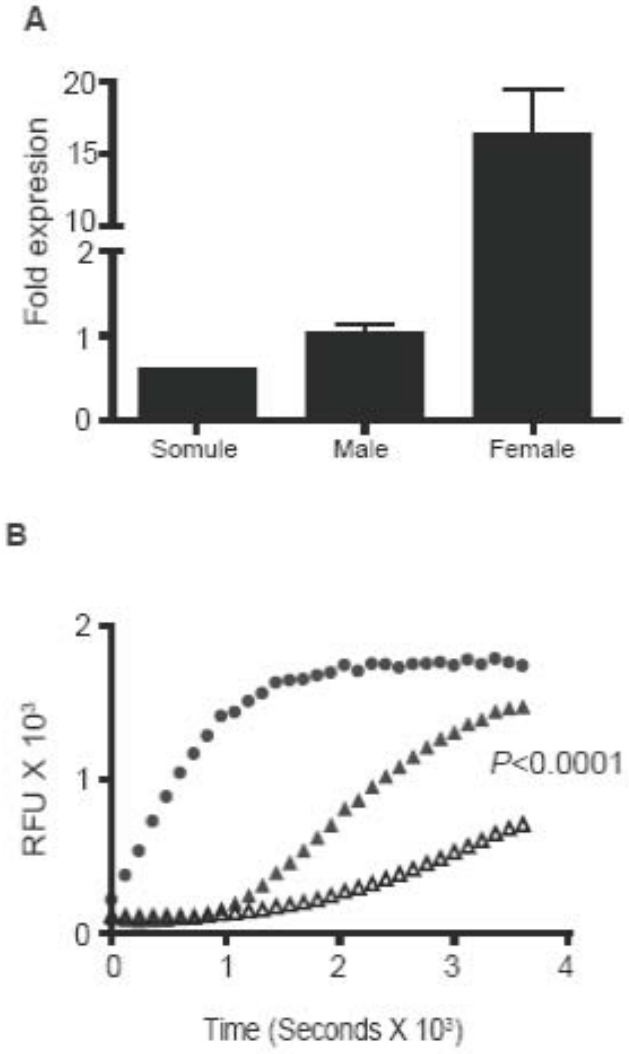

A
Fold expresion
20
15
10
2
1
0
Somule
Male
Female
B
RFU X 10³
2
1
0
P<0.0001
0
1
2
3
4
Time (Seconds X 10³)

Figure 11: Effects of sub-lethal PKA inhibition on egg production by *S. mansoni* worm pairs *in vitro*.

Adult *S. mansoni* worm pairs were maintained in medium containing varying concentrations of H-89 (A) or PKI 14-22 amide (B). Eggs produced per pair in the presence of each inhibitor were plotted against time. Treatment and control groups containing 6 worm pairs each were used for each inhibitor concentration. Data are representative of two independent experiments. *, $P < 0.05$ relative to controls.

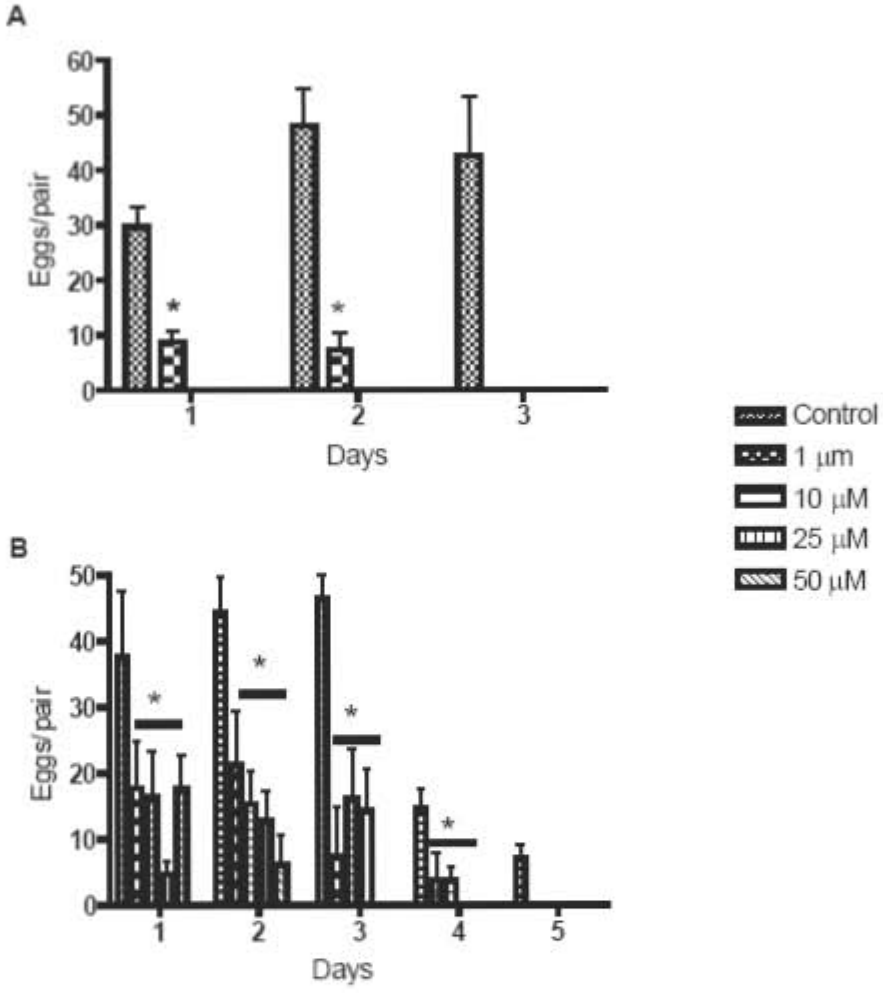

A
Eggs/pair
60
50
40
30
20
10
0
1
2
3
Days
B
Eggs/pair
50
40
30
20
10
0
1
2
3
4
5
Days
Control
1 μm
10 μM
25 μM
50 μM

Figure 12: SmPKA-C mRNA and PKA activity in *S. mansoni* from RAG-1$^{-/-}$ mice.

A, PKA activity in adult *S. mansoni* from RAG-1$^{-/-}$ mice is significantly reduced in comparison to adult *S. mansoni* from wild-type (WT) mice. ▲, Wild-type mice, ●, RAG$^{-/-}$ mice. B, Forskolin increases PKA activity in adult *S. mansoni* from RAG$^{-/-}$ mice. Forskolin-treatment group contained 6 worm pairs (12 worms total) and were performed in triplicate. ▲, forskolin-treated; ●, DMSO control. C, SmPKA-C mRNA is decreased in adult *S. mansoni* from RAG-1$^{-/-}$ mice as determined by real-time PCR. Data are representative of three independent experiments.

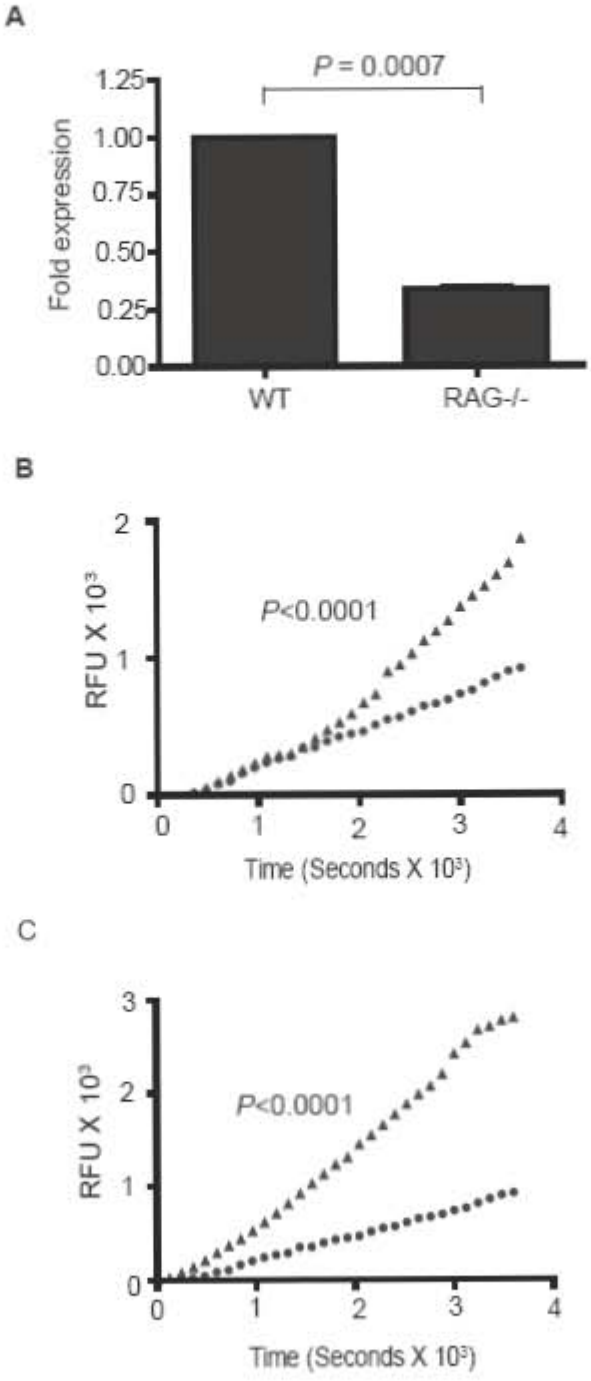
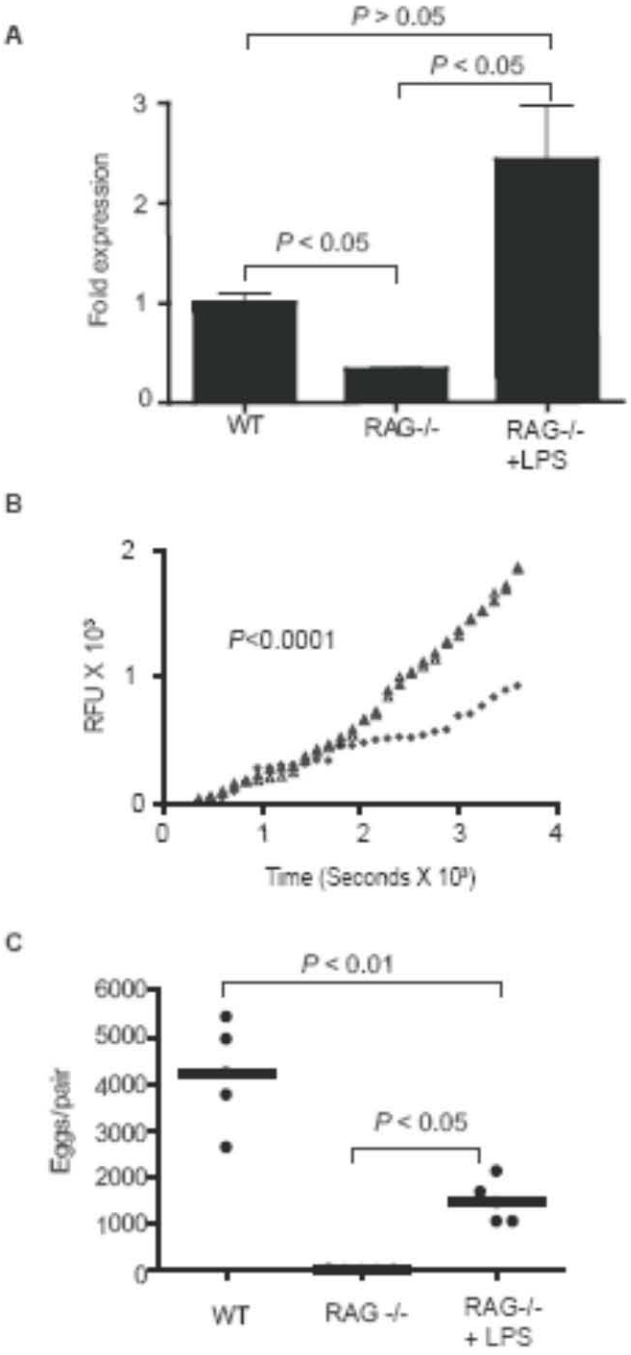

A
P = 0.0007
1.25
1.00
0.75
0.50
0.25
0.00
Fold expression
WT
RAG-/-
B
2
1
0
RFU X 10³
P<0.0001
0
1
2
3
4
Time (Seconds X 10³)
C
3
2
1
0
RFU X 10³
P<0.0001
0
1
2
3
4
Time (Seconds X 10³)

Figure 13: PKA activity and SmPKA-C mRNA in *S. mansoni* from LPS treated RAG-1$^{-/-}$ mice.

A, SmPKA-C mRNA is activated in adult *S. mansoni* from LPS treated RAG-1$^{-/-}$ mice as determined by real-time PCR. B, PKA activity is restored in *S. mansoni* from LPS treated RAG-1$^{-/-}$ mice. Kinase activity was measured in kinase reactions containing 1 µg each of *S. mansoni* protein lysate. ●, PBS treated control RAG-1$^{-/-}$ mice; ▲, wild-type; Δ, LPS treated RAG-1$^{-/-}$ mice. C, Number of eggs per schistosome pair deposited in the livers of wild-type (WT), LPS treated RAG-1$^{-/-}$ mice, and PBS treated control RAG-1$^{-/-}$ mice. Data are representative of two independent experiments.


A
P > 0.05
P < 0.05
P < 0.05
Fold expression
3
2
1
0
WT
RAG-/-
RAG-/-
+LPS
B
RFU X 10³
2
1
0
P<0.0001
0
1
2
3
4
Time (Seconds X 10³)
C
P < 0.01
P < 0.05
Eggs/pair
6000
5000
4000
3000
2000
1000
0
WT
RAG -/-
RAG-/-
+ LPS

Chapter 4

CONSERVATION OF PROTEIN KINASE A CATALYTIC SUBUNIT SEQUENCES IN THE SCHISTOSOME PATHOGENS OF HUMANS

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Manuscript submitted for publication review to Experimental Parasitology as a technical report.

Abstract

cAMP-dependent protein kinases (PKAs) are central mediators of cAMP signaling in eukaryotic cells. Previously we identified a cDNA which encodes for a PKA catalytic subunit (PKA-C) in *Schistosoma mansoni* (SmPKA-C) that is required for adult schistosome viability *in vitro*. As such, SmPKA-C could potentially represent a novel schistosome chemotherapeutic target. Here we sought to identify PKA-C subunit orthologues in the other medically important schistosome species, *S. haematobium* and *S. japonicum*, to determine the degree to which this potential target is conserved and could therefore be exploited for the treatment of all forms of schistosomiasis. We report the identification of PKA-C subunit orthologues in *S. haematobium* and *S. japonicum* (ShPKA-C and SjPKA-C respectively) and show that PKA-C orthologues are highly conserved in the *Schistosoma*, with over 99% amino acid sequence identity shared among the three human pathogens we examined.

Keywords: *Schistosoma haematobium*; *Schistosoma japonicum*; cAMP-dependent protein kinase

Introduction

Schistosomiasis, a disease caused by parasitic blood flukes of the genus *Schistosoma*, afflicts over 200 million people in tropical and sub-tropical regions worldwide and accounts for approximately 280,000 deaths annually in sub-Saharan Africa alone [1]. *S. mansoni* and *S. haematobium* are endemic in sub-Saharan Africa while *S. japonicum* is endemic in Asia, most notably in China. Currently, the anthelminthic praziquantel (PZQ) is the only drug that is used for the treatment of schistosomiasis, due to its ability to kill the adult worms of all the medically important *Schistosoma* species (*S. mansoni, S. haematobium,* and *S. japonicum*) [2]. However, reliance on PZQ alone for both treatment and transmission control efforts may not be sustainable in the long term. PZQ tolerant strains have been selected for in the laboratory [3] and there is indirect evidence of decreased PZQ sensitivity in the field [4,5]. The potential for PZQ resistance is real and current research is focused on the identification of novel anti-schistosome chemotherapeutic targets [6].

Protein kinases have recently been explored as potential anti-schistosome targets [7]. Through the reversible phosphorylation of specific tyrosine (Tyr) and serine/threonine (Ser/Thr) amino acid residues, protein kinases are important mediators in signal transduction pathways [8]. cAMP-dependent protein kinase (PKA) is the major transducer of cAMP signaling and is involved in a wide variety of cellular processes in eukaryotic cells [9]. In its inactive state, PKA is a tetrameric holoenzyme consisting of two identical regulatory subunits (PKA-R) which are bound to two identical catalytic subunits (PKA-C) [10]. Binding of

cAMP to the PKA-R subunits releases and activates the PKA-C subunits. Recently, we provided biochemical and molecular evidence that adult *S. mansoni* have a functional PKA [11]. Furthermore, we identified a cDNA that encodes for a PKA-C subunit in *S. mansoni* (SmPKA-C; GenBank Accession No. GQ168377) and showed that SmPKA-C is required for parasite viability *in vitro* through chemical inhibition and RNA interference studies [11]. As the SmPKA-C protein could potentially represent a new drug target in *S*. *mansoni*, we sought to identify PKA-C subunit orthologues in *S. japonicum* and *S*. *haematobium* to determine the extent to which this potential new target is conserved in the other two medically important schistosome species. Here we report the identification of PKA-C subunit orthologues in *S. japonicum* and *S*. *haematobium* (SjPKA-C and ShPKA-C respectively) which exhibit high levels of nucleotide and protein sequence identity to SmPKA-C.

Materials and Methods

Parasite materials

S. japonicum and *S*. *haematobium* adult worms, isolated from infected mice and hamsters respectively, were kindly provided by Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD).

cDNA cloning and sequence analysis

Total RNA was extracted from adult *S. japonicum* and *S*. *haematobium* using the RNAzol B Method (IsoTex Diagnostics, Inc.). 1 µg ribonucleic acid

(RNA) was used to synthesize complimentary deoxyribonucleic acid (cDNA) using the iScript Select cDNA Synthesis Kit and an oligo $(dT)_{20}$ primer (Bio-Rad). cDNA was then used as template for PCR using PKA-C-specific primers designed from the SmPKA-C sequence. RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RACE) was performed using a 5' 3' RACE Kit (Invitrogen) and internal gene specific primers. RACE and PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen), cloned into pCR4-TOPO vector (Invitrogen), and sequenced using the Big-DyeTerminator cycle sequencing kit (Applied Biosystems). Vector NTI software (Invitrogen) was used to align nucleotide and amino acid sequences. Phylip (Phylogeny Inference Package) software using the dna parsimonony method was used to construct phylogenetic trees based on nucleotide sequence data (http://mobyle.pasteur.fr/cgi-bin/portal.py) [12].

Results and Discussion

Identification of SjPKA-C and ShPKA-C subunit cDNAs

For *S. japonicum*, an incomplete cDNA (GenBank Accession No. AY813860) encoding for a putative PKA-C subunit was identified using the tblastn BLAST program, based on similarity with the SmPKA-C sequence. Approximately 150 nucleotides were missing from the 5' end of this cDNA, resulting in truncation of the kinase domain and loss of the entire amino terminus from the predicted amino acid translation. The complete cDNA sequence of the putative *S. japonicum* PKA-C (SjPKA-C) was obtained using 5' RACE and the

entire open reading frame (ORF) was subsequently amplified from adult worm cDNA. For *S. haematobium*, a 1 kb fragment containing the entire ORF of a PKA-C subunit was amplified from adult *S. haematobium* cDNA using the following primers designed from the SmPKA-C sequence: forward 5'-ATGGGTAATGCACAAGCTGC- 3' and reverse 5'-AAATTCACTAAATTCTTTTGCACATTTCTCTGT- 3'.

The complete ORFs for SjPKA-C and ShPKA-C are both 1053 bp and encode for proteins 350 amino acids in length, each with a predicted molecular mass of 40.4 kDa (GenBank Accession Nos. GU116484 and GU130533 respectively). These parameters are identical to those of SmPKA-C [11]. As with SmPKA-C, both proteins contained all conserved amino acid motifs of a PKA-C subunit, including an intact protein kinase domain (residues 43-297), the ATP-binding site GTGSFRGV (residues 50-57), the Ser/Thr active site RDLKPEN (residues 165 -171), a conserved autophosphorylation site, Thr 197, and a sequence motif which is required for regulatory subunit binding, TWTLCGTPEY (195 - 204) [13,14] (Fig. 14). At the amino acid level, the schistosome PKA-C subunit proteins share 99% identity and only differ at three amino acid residues, all lying outside the regions that are critical for PKA-C function. There is a threonine residue at position 6 in SjPKA-C protein in lieu of an alanine in SmPKA-C and ShPKA-C proteins. ShPKA-C protein differs from SmPKA-C and SjPKA-C proteins at positions 73 (threonine in place of an isoleucine) and 172 (valine in place of an isoleucine) (Fig. 14).

Phylogenetic analysis of PKA-C subunit cDNA sequences from schistosomes and other eukaryotic organisms

There is over 90% nucleotide identity among the three schistosome PKA-C cDNAs. BLAST comparison of SjPKA-C and ShPKA-C cDNA sequences using the non-redundant nucleotide database at NCBI showed that the putative genes shared 70% similarity with PKA-C subunit cDNAs from other eukaryotic organisms (e.g. *Pediculus humanus corporis*, *Hydra magnipapillata*, *Culex quinquefasciatus*, *Xenopus laevis*, and *Homo sapiens*) (Fig. 15). Phylogenetic analysis of these sequences shows that while all three schistosome PKA-C sequences are closely related, the sequences from the two African species (ShPKA-C and SmPKA-C) are most closely related, while the sequence from the Asian species (SjPKA-C) appears more divergent (Fig. 15). This relationship is consistent with that determined from phylogenetic analysis of other sequences from these three species – that the Asian schistosome species, including *S. japonicum*, form a group that is ancestral to the more derived African species groups typified by *S. mansoni* and *S. haematobium* [15]. Extensive conservation of PKA-C sequences among representatives of these three major groupings within the genus *Schistosoma* suggests that PKA-C is likely highly conserved in all *Schistosoma* species.

As PZQ is effective against all three medically important schistosome species, new anti-schistosome chemotherapeutics intended to replace or augment PZQ therapy should ideally have similar broad-spectrum activity. However, efforts to identify novel chemotherapeutic targets in schistosomes have

focused primarily on *S*. *mansoni*, with the hope that targets identified in this species will be translatable to *S. haematobium* and *S*. *japonicum* [6]. In this study, we provide direct evidence that *S. haematobium* and *S*. *japonicum* express orthologous PKA-C subunit proteins that are 99 % identical to the SmPKA-C protein. In our previous study, we showed that inhibition of SmPKA-C expression in adult *S*. *mansoni* by RNA interference produced lethality in treated adult worms, indicating that SmPKA-C is required for adult *S*. *mansoni* viability, at least *in vitro* [11]. Since the nucleotide and amino acid sequences of SjPKA-C and ShPKA-C are almost identical to that of SmPKA-C, we hypothesize that ShPKA-C and SjPKA-C are also essential gene products. RNA interference studies using specific dsRNA to ShPKA-C and SjPKA-C are underway to test this hypothesis. Together these data suggest that a novel anti-schistosome drug that targets schistosome PKA-C subunits may be useful for the treatment of all forms of schistosomiasis.

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Figure 14. Amino acid alignment of PKA-C subunit sequences from *S. mansoni* (SmPKA-C), *S. haematobium* (ShPKA-C) and *S. japonicum* (SjPKA-C).

Underlines indicate conserved amino acid sequences as follows: 1, kinase domain (Phe43 – Phe297); 2, ATP-binding site (Gly50 - Val57); 3, Ser/Thr active site (Arg165 - Asn171); 4, conserved autophosphorylation site (Thr197); 5, Conserved sequence motif for regulatory subunit binding (Thr195 - Tyr204).

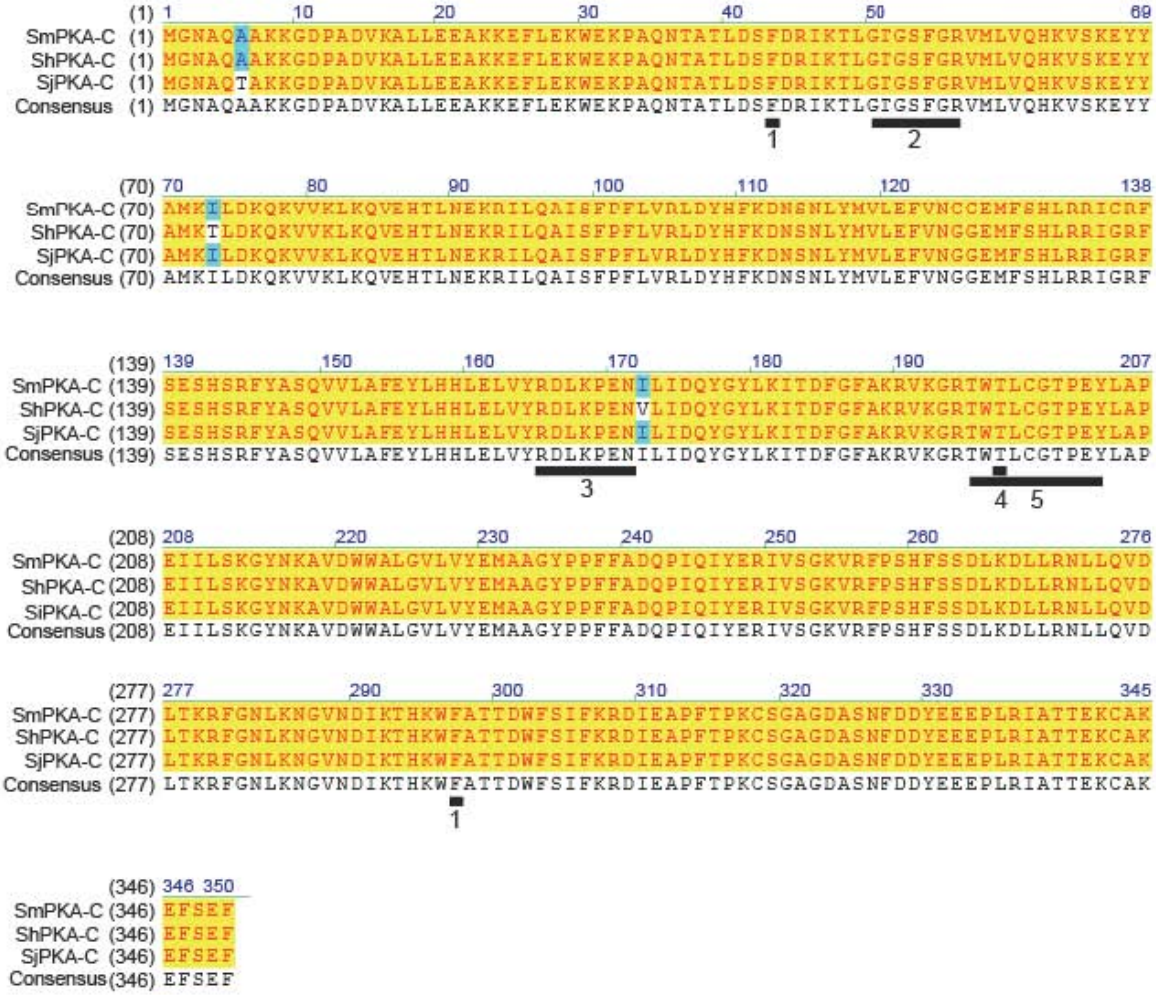
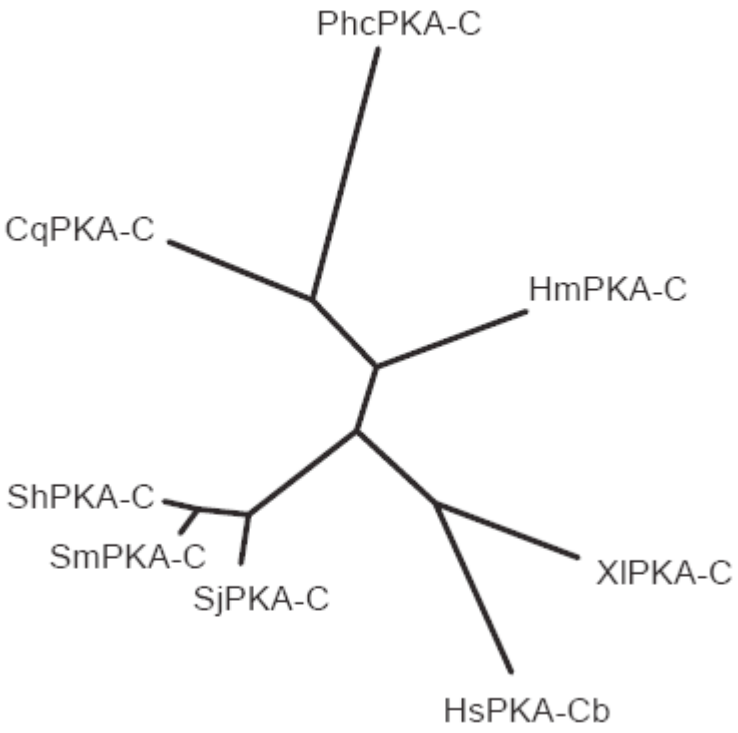

(1) 1 10 20 30 40 50 69
SmPKA-C (1) MGNAQAAKKGDPADVKALLEEAKKEFLEKWEKPAQNTATLDSFDRIKTLGTGSFGRVMLVQHKVSKEYY
ShPKA-C (1) MGNAQAAKKGDPADVKALLEEAKKEFLEKWEKPAQNTATLDSFDRIKTLGTGSFGRVMLVQHKVSKEYY
SjPKA-C (1) MGNAQTAKKGDPADVKALLEEAKKEFLEKWEKPAQNTATLDSFDRIKTLGTGSFGRVMLVQHKVSKEYY
Consensus (1) MGNAQAAKKGDPADVKALLEEAKKEFLEKWEKPAQNTATLDSFDRIKTLGTGSFGRVMLVQHKVSKEYY
1
2
(70) 70 80 90 100 110 120 138
SmPKA-C (70) AMKILDKQKVVKLKQVEHTLNEKRILQAISFPFLVRLDYHFKDNSNLYMVLEFVNGGEMFSHLRRICRF
ShPKA-C (70) AMKTLDKQKVVKLKQVEHTLNEKRILQAISFPFLVRLDYHFKDNSNLYMVLEFVNGGEMFSHLRRIGRF
SjPKA-C (70) AMKILDKQKVVKLKQVEHTLNEKRILQAISFPFLVRLDYHFKDNSNLYMVLEFVNGGEMFSHLRRIGRF
Consensus (70) AMKILDKQKVVKLKQVEHTLNEKRILQAISFPFLVRLDYHFKDNSNLYMVLEFVNGGEMFSHLRRIGRF
(139) 139 150 160 170 180 190 207
SmPKA-C (139) SESHSRFYASQVVLAFEYLHHLELVYRDLKPENILIDQYGYLKITDFGFAKRVKGRTWTLCGTPEYLAP
ShPKA-C (139) SESHSRFYASQVVLAFEYLHHLELVYRDLKPENVLIDQYGYLKITDFGFAKRVKGRTWTLCGTPEYLAP
SjPKA-C (139) SESHSRFYASQVVLAFEYLHHLELVYRDLKPENILIDQYGYLKITDFGFAKRVKGRTWTLCGTPEYLAP
Consensus (139) SESHSRFYASQVVLAFEYLHHLELVYRDLKPENILIDQYGYLKITDFGFAKRVKGRTWTLCGTPEYLAP
3
4 5
(208) 208 220 230 240 250 260 276
SmPKA-C (208) EIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYERIVSGKVRFPSHFSSDLKDLLRNLLQVD
ShPKA-C (208) EIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYERIVSGKVRFPSHFSSDLKDLLRNLLQVD
SjPKA-C (208) EIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYERIVSGKVRFPSHFSSDLKDLLRNLLQVD
Consensus (208) EIILSKGYNKAVDWWALGVLVYEMAAGYPPFFADQPIQIYERIVSGKVRFPSHFSSDLKDLLRNLLQVD
(277) 277 290 300 310 320 330 345
SmPKA-C (277) LTKRFGNLKNGVNDIKTHKWFATTDWFSIFKRDIEAPFTPKCSGAGDASNFDDYEEEPLRIATTEKCAK
ShPKA-C (277) LTKRFGNLKNGVNDIKTHKWFATTDWFSIFKRDIEAPFTPKCSGAGDASNFDDYEEEPLRIATTEKCAK
SjPKA-C (277) LTKRFGNLKNGVNDIKTHKWFATTDWFSIFKRDIEAPFTPKCSGAGDASNFDDYEEEPLRIATTEKCAK
Consensus (277) LTKRFGNLKNGVNDIKTHKWFATTDWFSIFKRDIEAPFTPKCSGAGDASNFDDYEEEPLRIATTEKCAK
1
(346) 346 350
SmPKA-C (346) EFSEF
ShPKA-C (346) EFSEF
SjPKA-C (346) EFSEF
Consensus (346) EFSEF

Figure 15. Phylogenetic analysis of PKA-C cDNA nucleotide sequences from schistosomes and other species.

Sequences are labeled as follows: ShPKA-C, *S. haematobium* PKA-C (GU116484) SmPKA-C, *S*. *mansoni* PKA-C (GQ168377); SjPKA-C, *S. japonicum* PKA-C (GQ130553); HsPKA-Cb, *H. sapiens* PKA-Cβ (AB451364); XlPKA-C, *Xenopus laevis* PKA-C (NM_001087227);), HmPKA-C, *Hydra magnipapillata* (XM_002163898); PhcPKA-C, *Pediculus humanus corporis* PKA-C (XM_0024273690); CqPKA-C, *Culex quinquefasciatus* PKA-C (XM_001868347).


PhcPKA-C
CqPKA-C
HmPKA-C
ShPKA-C
SmPKA-C
SjPKA-C
XlPKA-C
HsPKA-Cb

Chapter 5

A PROTEIN KINASE A REGUALTORY TYPE II ALPHA SUBUNIT IS DISPENSABLE FOR SCHISTOSOME VIABILITY

Brett E. Swierczewski and Stephen J. Davies

ABSTRACT

Due to their involvement in critical cellular processes in eukaryotic organisms, protein kinases have been recently explored as novel anti-cancer and anti-parasitic chemotherapeutic targets. The site-selective cAMP analogue, 8-Cl-cAMP, has recently completed Phase I clinical trials for treatment of cancers caused by overexpression of the regulatory type 1 alpha subunit (PKA-RIα) of cAMP-dependent protein kinase (PKA). In this study, we show that adult *S. mansoni* PKA activity is inhibited by treatment with 8-Cl-cAMP and additionally, 8-Cl-cAMP at various concentrations is lethal for adult schistosomes *in vitro*. A cDNA (Sm04765) with an open reading frame of 1137bp coding for a PKA-RIIα subunit was identified in the *S. mansoni* Genome Project database and was cloned and sequenced. The predicted protein was 44% identical to the PKA-RIIα subunit homologue in *Homo sapiens*. RNA interference experiments treating adult *S. mansoni* via electroporation with Sm04765 specific-double stranded RNA showed a decrease in overall PKA activity in treated worms, but had no other observable effects in the treated parasites.

Introduction

Schistosomiasis, a debilitating disease caused by parasitic trematodes of the genus *Schistosoma*, afflicts over 200 million people in tropical and sub-tropical regions of the world and is estimated to cause over 280,000 deaths per year [1]. The complex schistosome life cycle consists of several morphologically distinct stages and alternates between a molluscan intermediate and a vertebrate

definitive host. Current treatment and transmission control efforts for schistosomiasis are based solely on the anthelmintic, praziquantel (PZQ) [2]. PZQ is effective against the adults of all the medically important schistosome species (*S. mansoni*, *S. japonicum*, and *S. haematobium*). However, reliance PZQ as the sole drug for treatment and control of schistosomiasis may not be sustainable in the near future. PZQ-tolerant strains have been created in the laboratory when treated with sub-curative doses of PZQ [3] and there has been evidence of decreased sensitivity and potential PZQ resistance in a number of clinical field settings [4,5]. The emergence of PZQ resistance may be just a matter of time and current efforts are focused on discovering novel anti-schistosome chemotherapeutic targets [6].

Protein kinases have recently been explored as potential new drug targets in schistosomes and other parasites [7,8]. In particular, cAMP-dependent protein kinase (PKA) has garnered particular attention as a potential novel drug target in protozoan parasites, including *Giardia lamblia* and *Plasmodium falciparum* [9,10,11]. PKA, a serine/threonine kinase, is the major transducer of cAMP signaling in eukaryotic cells and is involved in a wide variety of cellular processes, including cellular proliferation, metabolism, gene transcription, and tissue differentiation [12]. In the absence of cAMP, PKA is a tetrameric holoenzyme consisting of two identical regulatory subunits (PKA-R) and two identical catalytic subunits (PKA-C) [13]. Function of PKA is based on the R subunits contained in the holoenzyme. There are four isoforms of PKA-R subunits in mammalian tissues (RIα, RIβ, RIIα, RIIβ respectively) with RI subunits

involved in cellular proliferation and growth processes and RII subunits responsible for anti-proliferative effects [14]. The RI/RII ratio in cells is tightly regulated and an increase in the RIα subunit has been implicated in the development of a number of human cancers and lymphomas [15,16]. For this reason, considerable effort has been invested in the development of RIα subunit inhibitors. Studies have shown that 8-Cl-cAMP, a site-selective cAMP analogue, and a RIα antisense oligodeoxynucleotide (GEM231) are able to downregulate RIα subunit transcription while up-regulating RIIβ subunit production, which reverts cancer cells to a normal anti-proliferative phenotype [15,17]. Both of these drugs are currently in development as anti-cancer agents, with both having completed Phase I clinical trials [18].

In a recent report, our laboratory provided biochemical and molecular evidence of a fully functional PKA (SmPKA) in adult *S. mansoni* [19]. In this report, we identified a cDNA which encoded for a PKA-C subunit homologue (SmPKA-C; GenBank Accession No. GQ168377) in *S. mansoni*. Furthermore, we demonstrated that SmPKA-C is required for parasite viability *in vitro* through chemical inhibition using PKA-C subunit specific inhibitors and RNA interference studies. In the present report, our aim was to identify schistosome and explore the function of PKA-R subunits in *S. mansoni*. Since PKA-R subunits are the major target for PKA-based chemotherapy, we sought to examine the effects of site-selective cAMP analogues on *S. mansoni in vitro* and *in vivo* to determine if PKA-R subunits, like schistosome PKA-C subunits, are required for parasite viability. In this study, we provide evidence that adult *S. mansoni* is susceptible

to 8-Cl-cAMP *in vitro*. However, we also demonstrate by RNAi that a recently identified schistosome PKA-RIIα subunit homologue is not required for parasite viability.

Materials and Methods

Parasite materials

Biomphalaria glabrata snails infected with the NMRI/Puerto Rican strain of *S. mansoni* were obtained from Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD). Cercariae were collected by exposing infected snails to light for 2 h in 50 mL of ultra-filtered water. Adult *S. mansoni* were collected from 6 week-infected C57BL/6 mice that were infected with 150 cercariae using the tail immersion method [20].

PKA cAMP analogue assays

Freshly isolated adult *S. mansoni* were collected and homogenized in cell extraction buffer (Invitrogen) containing protease and phosphatase inhibitor cocktails (Sigma) and *S. mansoni* protein lysate (Sm lysate) was prepared as described previously [19]. Sm lysate protein concentrations were determined using the Quick Start™ Bradford Protein Assay and adjusted to a final concentration of 0.2 µg/µL with additional cell extraction buffer. 8-Cl-cAMP and Rp-cAMPS were purchased from Invitrogen. 8-Cl-cAMP and Rp-cAMPS were dissolved in ammonium hydroxide and water respectively. PKA activity was measured on a Spectramax M2 microplate fluorometer (Molecular Devices) using

the Omnia® Lysate Assay for PKA kit (Biosource). 1 μg total Sm lysate was used as a Source of adult *S. mansoni* PKA and 2 ng of recombinant human PKA-Cα catalytic subunit (Invitrogen) was used as a positive control. PKA activity reactions were performed according to the manufacturer's instructions and as described previously [19], in the presence of 8-Cl-cAMP and Rp-cAMPs or appropriate vehicle control at the following concentrations: 500, 100, and 10 μM. PKA activity measured in relative fluorescence units (RFUs) was plotted using GraphPad Prism software version 4 (Graphpad Software, Inc.). Symbols at each time point represent the means of three biological replicates and all experiments were performed twice.

In vitro treatment of adult S. mansoni with cAMP analogues

Effects of 8-Cl-cAMP and Rp-cAMPs on adult worms were assessed at the following concentrations *in vitro*: 500, 250, 100, 50, 25, 10, and 1 μM. Briefly, individual adult worm pairs (6 pairs per concentration) were isolated and placed immediately in the wells of 24 well tissue culture plates containing 1 mL total of pre-warmed Dulbeccos's Modified Eagle Medium (DMEM) (with 10% fetal bovine serum and 5% penicillin/streptomycin) and appropriate concentration of inhibitor or vehicle. Treated worms and controls were incubated at 37 °C in 5 % CO_2 and observed every 24 h for a period of 7 d. Medium containing inhibitor or vehicle was replaced daily. Worms were considered to be dead when all evidence of motility, including gut peristalsis, had ceased. Experiments were performed twice. Kaplan-Meier survival curves were generated using GraphPad

Prism software. Photomicrographs of treated and control worms were obtained using a Zeiss CL1500ECO dissecting microscope.

Treatment of S. mansoni infected mice with 8-Cl-cAMP

8-Cl-cAMP-Na^+ was purchased from Alexis Biochemicals and dissolved in water at a concentration of 10 mg/mL**.** Two groups consisting of five C57BL/6 mice each were infected with *S. mansoni.* At six weeks post-infection, one group of mice was injected intraperitoneally (i.p.) once a day for 7 d at a dose of 100 mg/kg of 8-Cl-cAMP, while the control group received injections of water. After a one week treatment regimen, *S. mansoni* were collected and number of worm pairs and total number of worms for each mouse were recorded. GraphPad Prism software version 4 was used to plot the data and experiments were conducted twice.

Phylogenetic analysis of PKA-RIIα subunit amino acid sequences

The *S. mansoni* Genome Project website maintained by the Sanger Institute (http://www.genedb.org/genedb/smansoni) was used to identify nucleotide sequences that coded for putative PKA-R subunits. The sequence, Sm04765, coded for a putative PKA-RIIα subunit. To confirm the presence of a corresponding cDNA in adult parasites, PCR using Sm04765-specific primers and 1 μg *S. mansoni* adult cDNA was used to amplify a 950 bp fragment of Sm04765 corresponding to the nucleotide positions 120 - 1086. Amplicons were visualized by gel electrophoresis, cloned into the pCR2.1-TOPO vector

(Invitrogen), and sequenced using the Big-Dye Terminator cycle sequencing kit (Applied Biosystems). Sequences were compared to other genomic sequences using blast servers available at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Vector NTI software (Invitrogen) was used to align the amino acid sequences of Sm04765 and PKA-RIIα subunits from other eukaryotic organisms.

PKA-RIIα subunit silencing in S. mansoni using RNAi

A 950 bp fragment of Sm04765 was generated from adult *S. mansoni* cDNA using PCR with Sm04765 gene-specific primers flanked by T7 polymerase sequences (underlined): forward
5' - TAA TAC GAC TCA CTA TAG GGT CAA TGA CGA TGA AGG ACG AA - 3' and reverse 5'-TAA TAC GAC TCA CTA TAG GGT GAA GCA GCA CGT GGA TGA C- 3'.

Single-stranded RNA was transcribed using the T7 polymerase MEGAscript RNA transcription kit (Ambion). Sm04765 dsRNA was generated and purified using the MEGAscript RNAi kit (Ambion) according to the manufacturer's instructions. For a positive control, a non-schistosome control dsRNA was generated from the pCR-II TOPO vector as described previously [21]. Integrity of the final dsRNA products was analyzed by agarose gel electrophoresis. 40 µg of Sm04765 or control dsRNA, diluted in 100 µl of electroporation buffer (Ambion), were delivered to groups of 12 adult worms (6 males; 6 females) via electroporation as described previously using the GenePulser Xcell Electroporation system (Bio-

Rad) [22]. Adult worms were transferred to 4 mm cuvettes and pulsed at room temperature with a single 20 ms square wave pulse at 125 V. Adult worms were transferred immediately after electroporation to pre-warmed DMEM (containing 10% heat-inactivated fetal bovine serum and 5% penicillin/streptomycin) and incubated at 37 ºC. RNA was extracted from six worms in the treated and control groups respectively after 3 d using the using the RNAzol B Method (IsoTex Diagnostics, Inc.). First-strand oligo cDNA was synthesized using 1 μg RNA and an oligo $(dT)_{20}$ primer using the iScript™ Select cDNA Synthesis Kit (Bio-Rad). Real time PCR was used to assess the efficacy of Sm04765 mRNA knockdown using the following primers that hybridized outside of the targeted region corresponding to nucleotide positions in Sm04765 (54 – 1198): forward 5' - GTT GAT TAT TCA AAC CTC AAA ACC - 3' and reverse 5' - TGC TTC ATA ATC ATC AAT GTT CC - 3'. The accumulation of PCR product was monitored in real time by detection of SYBR Green fluorescence, using a M.J. Research Chromo4 PCR cycler (Bio-Rad). Relative Sm04765 mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method [23] and *S. mansoni* alpha tubulin mRNA was used as the internal control transcript. Primers to amplify a 100 bp fragment of the alpha tubulin gene (GenBank Accession No. S79195) corresponding to nucleotide positions 1711 – 1824 are as follows: forward 5'-GGTTGACAACGAGGCCATTTATG-3' and reverse 5'-TGTGTAGGTTGGACGCTCTATATCT-3'. At 7 d, the remaining worms were incubated with 100 μM of the adenylyl cyclase agonist, forskolin, for 2 h at 37 ºC, protein lysate was prepared and PKA assays to compare PKA

activity in the treated and control worms was conducted as described previously [19].

Statistical analyses

The statistical significance of differences between treatment and control groups in the PKA activity assays was calculated using one-way ANOVA of repeated measures. The statistical significance of differences between Kaplan-Meier survival curves was calculated using the logrank test. *P* values ≤ 0.05 were considered statistically significant. Student's T test with Welch's correction was used to test the significance of differences in Sm04765 expression levels detected by real-time PCR. GraphPad Prism software was used for all statistical analyses.

Results

Parasite PKA activity is sensitive to cAMP analogues

In a previous report, we showed that *S. mansoni* PKA activity is sensitive to alterations of intracellular cAMP concentrations through the inhibition or activation of adenylyl cyclase, suggesting that parasite PKA-R subunits are involved in the activation of the PKA-C subunits [19]. To further define the role of parasite PKA-R subunits in PKA activation, we treated Sm lysate with the cAMP analogues, Rp-cAMPS and 8-Cl-cAMP. 10 μM 8-Cl-cAMP significantly decreased PKA activity in treated Sm lysate when compared to the untreated controls ($P < 0.0001$) (Fig. 16A). Identical results were obtained using 100 and

500 μM 8-Cl-cAMP (data not shown). Conversely, treatment of Sm lysate with Rp-cAMPS had no effect on parasite PKA activity at 10 μM (Fig. 16C), 100 μM or 500 μM (data not shown). As expected, the activity of the recombinant control PKA was not affected by 8-Cl-cAMP or Rp-cAMPS, as this preparation does not contain any PKA-R subunits (Figs. 16B and 16D).

In vitro treatment with 8-Cl-cAMP is schistosomicidal

Treating worms with 8-Cl-cAMP at concentrations of 50 – 500 μM resulted in 100 % mortality within 48 h (Fig. 17A). Worms treated with 8-Cl-cAMP at 25 μM resulted in 75 % mortality by Day 3 and 100 % by Day 4 (Fig. 17A). Incubation of schistosomes with 8-Cl-cAMP at 10 μM resulted in 100% mortality by Day 6 (Fig. 17A). All control worms and worms treated at 1 μM 8-Cl-cAMP survived the 7 d assay. Worms treated at all concentrations of 8-Cl-cAMP resulted in the disassociation of male and female schistosomes, while control pairs remained *in copula* (Fig. 17B). Similar to the PKA activity assays, *in vitro* treatment with Rp-cAMPS had no effect on treated schistosomes at any concentration (data not shown).

8-Cl-cAMP in the treatment of schistosomiasis in vivo

Because 8-Cl-cAMP was lethal to *S. mansoni in vitro*, we treated *S. mansoni*-infected mice with 8-Cl-cAMP to determine if it was efficacious *in vivo*. 54 total worms were recovered from the 8-Cl-cAMP treatment group in comparison to 56 total worms recovered from the control group (Fig. 17C). 20

worm pairs were recovered from the 8-Cl-cAMP treatment group in comparison to 24 total worms pairs recovered from the control group (Fig. 17D). We can conclude from these data that 8-Cl-cAMP had no effect on adult schistosome viability *in vivo*.

Identification of a S. mansoni PKA-RIIα subunit cDNA

To identify cDNA sequences that might encode for PKA-R subunits in *S. mansoni*, BLASTX searches of the *S. mansoni* genome database were performed using PKA-R amino acid sequences from other organisms. The sequence, Sm04765, with partial similarity to other PKA-R subunits was identified. Using Sm04765 gene-specific primers and adult *S. mansoni* cDNA, we confirmed that a cDNA corresponding to Sm04765 was present in *S. mansoni* cDNA. The full-length cDNA is 1754 bp long and contains a complete ORF of 1137 bp, encoding for a protein of 378 amino acids in length and with a predicted molecular mass of 43.0 kDa. The ORF encoded for a putative PKA-R subunit which contained the two cAMP binding sites, Site A (Phe186 – Leu202) and Site B (Phe310 – Tyr326) that are typically characteristic of PKA-RIIα subunits [12,13] (Fig. 18). Additionally, the ORF contained residues for a PKA-C pseudosubstrate site (Arg76 – Ala82), but lacked an intact dimerization region in the amino terminus. BLAST comparison of the amino acid sequence with the non-redundant protein sequence database at NCBI showed that the putative *S. mansoni* PKA-RII protein shared 20% overall identity to PKA-RIIα subunits from other eukaryotic organisms (*Aplysia californica, S. japonicum, Caenorhabditis*

elegans Mus musculus, *Onchocerca volvulus,* and *Homo sapiens*) (Fig. 18). Sm04765 at the amino acid level was 94% identical to a putative PKA-RIIα subunit from *S. japonicum* (GenBank Accession No. AAW24538) and 44% identical to the human PKA-RIIα subunit homologue (GenBank Accession No. NP_004148) suggesting that Sm04765 would likely code for PKA-RIIα subunit.

Silencing of the putative PKA-RIIα subunit (Sm04765) by RNAi

To determine the effect of silencing Sm04765 expression in *S. mansoni*, adult worms were treated via electroporation with 40 µg of Sm04765 dsRNA or control dsRNA. There was no mortality observed in Sm04765 dsRNA treated worms (data not shown). Analysis by real time PCR showed that Sm04765 mRNA levels at 3 d post-electroporation were significantly reduced in the Sm04765 dsRNA treated worms when compared to control dsRNA treated worms ($P = 0.0007$) (Fig. 19A). To determine whether the Sm04765 gene product is required for regulation of *S. mansoni* PKA activity detected in Sm lysate, PKA activity was measured in lysates prepared from worms that were treated with Sm04765 or control dsRNA 7 days previously. PKA activity was significantly reduced in the Sm04765 dsRNA treated worms as compared to the control dsRNA treated worms, suggesting that downregulation of this particular subunit results in decreasd catalytic activity ($P < 0.05$) (Fig. 19B).

Discussion

In this study, we used the cAMP analogues 8-Cl-cAMP and Rp-cAMPS to examine the role of parasite PKA-R subunits in the regulation of *S. mansoni* PKA activity. Rp-cAMPS, had no effect on PKA activity in Sm lysate and had was not lethal for adult *S. mansoni in vitro*. This was a bit of a surprise as Rp-cAMPS has been shown to decrease PKA activity in previous studies [24]. Rp-cAMPS inhibits PKA activation by binding to the cAMP binding sites on PKA-R subunits, keeping the holoenzyme intact and preventing disassociation [25]. There are several reasons why Rp-cAMPS seemingly had no effect on schistosome PKA activity. First, the cell permeability of Rp-cAMPS varies considerably in different systems [25] and it is possible that Rp-cAMPS was unable to penetrate and gain access into the parasites *in vitro*. Second, as Rp-cAMPS is a competitive inhibitor of intracellular cAMP, it has been shown that, at high concentrations, cAMP can still bind to PKA-R subunits even in the presence of Rp-cAMPS [26]. This could explain why Rp-cAMPS had no effect on the PKA activity in Sm lysate as cAMP is contained in the kinase reaction buffer used in the assay. Furthermore, Sm lysate might also contain high concentrations of endogenous cAMP that out-competed the Rp-cAMPS. Second generation derivatives of Rp-cAMPS such as Rp-8-Br-cAMPS and Rp-8-Cl-cAMPS have been found to have greater cell permeability and PKA inhibition in comparison to Rp-cAMPS [27]. Studies using these newer Rp-cAMP isomers should be undertaken.

In contrast to Rp-cAMPS, 8-Cl-cAMP inhibited PKA activity in Sm lysate and was lethal for adult *S. mansoni in vitro*. Though 8-Cl-cAMP has been used

for over 30 years, its mechanism of action is still not understood. 8-Cl-cAMP preferentially activates PKA-RIα subunits due to its moderately high binding affinity for both sites A and B on these subunits [28]. In contrast, 8-Cl-cAMP binds with high affinity to site B, but has a low affinity for site A on PKA-RII subunits, thus keeping these holoenzymes intact. This preferential binding to RI subunits results in their downregulation and subsequent upregulation of RII subunits, leading to an increased RII/RI ratio. This phenomenon has been observed in a number of human cancer cell lines and in Phase I clinical trials using 8-Cl-cAMP [15,17,18]. It is thought that after PKA-I holoenzyme disassociation, 8-Cl-cAMP induces truncation of the RIα subunits from a 48kDa to 34 kDa protein through the activation of an unknown protease, facilitating the upregulation of RIIβ subunits [29]. In contrast, other studies have proposed that the metabolic by-product of 8-Cl-cAMP, 8-Cl-adenosine (8-Cl-ADO), is responsible for the anti-proliferative effects observed with 8-Cl-cAMP, independent of PKA activation and RI/RII isotype switching [30,31]. Finally in addition to downregulation of RIα subunits, 8-Cl-cAMP has been shown to cause downregulation of PKA-Cα subunits as well, resulting in decreased PKA activity in cancer cells when compared to non-treated cells [32]. As no nucleotide sequences coding for PKA-RI subunits have been identified in the *S. mansoni* genome database to date, either of these two latter mechanisms may account for the decrease in PKA activity observed in 8-Cl-cAMP-treated Sm lysate and the deleterious effect on *S. mansoni* worms *in vitro*. Alternatively, the *S. mansoni* genome may encode a PKA-RI subunit that has not yet been identified.

PKA-R subunits in eukaryotic organisms do not share the same high degree of conservation that PKA-C subunits share [13,33]. Hence the *S. mansoni* PKA-RIIα subunit amino acid sequence is only 20% identical to eukaryotic PKA-RIIα subunits. Due to this high degree of divergence, cAMP analogues such as 8-Cl-cAMP may have different binding affinities for the *S. mansoni* PKA-R, in comparison to mammalian PKA-R subunits. The identification of an RIα subunit in *S. mansoni* would allow comparison of the amino acid sequences of sites A and B to determine their relative suitability for 8-Cl-cAMP binding or perhaps other site-selective cAMP analogues.

While 8-Cl-cAMP was lethal for adult schistosomes, inhibiting expression of the putative PKA-RIIα subunit by RNAi produced no obvious adverse effects in adult schistosomes. Thus we hypothesize that PKA-RI subunits that have yet to be identified are required for adult schistosome viability, the PKA-RII subunit is not essential. Consistent with this hypothesis, RNAi knock-down of Sm04765 mRNA levels resulted in a decrease in PKA activity when compared to the control worms, but PKA activity was not ablated. We hypothesize that, as in other organisms, inhibition of RI subunit expression in schistosomes might result in greater loss of PKA activity and be toxic to the parasite. To test these hypotheses, we are currently attempting to identify putative PKA-RI subunit sequences in the *S. mansoni* genome.

The ability of 8-Cl-cAMP to kill adult schistosomes in vitro suggested this drug may be useful in treating schistosome infection. However, using a mouse model of schistosomiasis, we were unable to detect any measurable effect of 8-

Cl-cAMP on adult schistosomes *in vivo*. Continuous infusion of 8-Cl-cAMP intravenously is the preferred method of administration for 8-Cl-cAMP in clinical studies [34,35,36]. In our study, we used a dose of 100 mg/kg that was determined to be well below the maximum tolerated dose that produced a reduction in tumor weight in treated mice [36]. Future studies could be undertaken with 8-Cl-cAMP in which continuous IV infusion using mini-pumps are used to administer the compound rather than bolus. Alternatively, the PKA-RI subunits of schistosomes may be sufficiently divergent from those of mammals that 8-Cl-cAMP is no longer efficacious in inducing holoenzyme dissociation. Identification of the nucleotide and amino acid sequences of these subunits in schistosomes will be critical to evaluating whether, as in other eukaryotes, PKA-RI represents a viable target for chemotherapy in schistosomiasis.

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Figure 16: Effects of cAMP analogues on schistosome PKA activity

Kinase activity was measured in kinase reactions containing Sm lysate (A and C) and recombinant human PKA-Cα protein (B and D) and 8-Cl-cAMP (A and B) and Rp-cAMPS (C and D). ▲, inhibitor-treated; ●, no inhibitor. A-D, each time point represents the mean of three biological replicates. Data are representative of two independent experiments

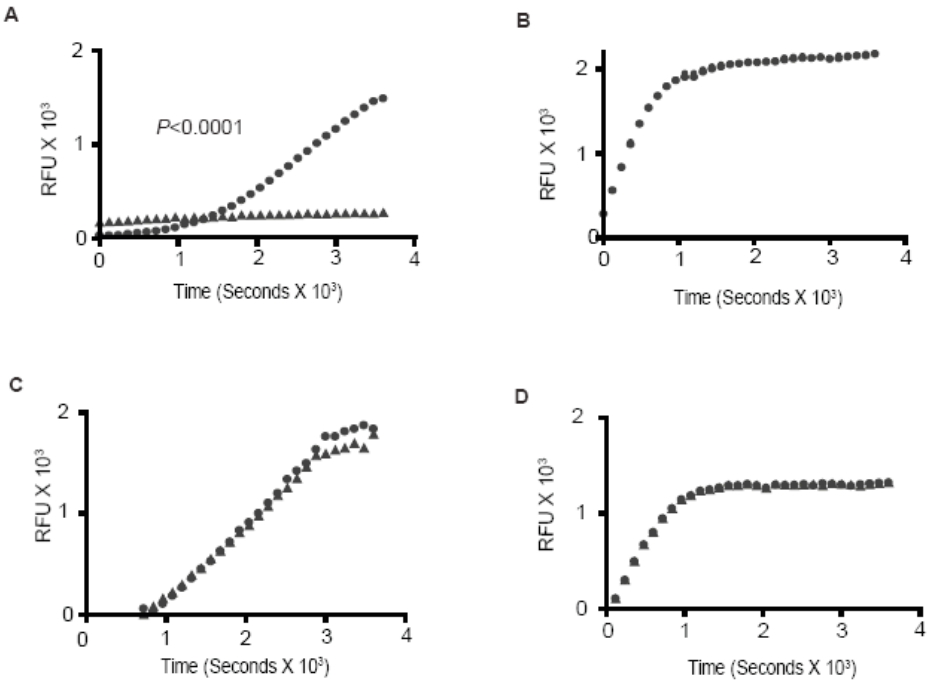

A
B
C
D
RFU X 10³
Time (Seconds X 10³)
P<0.0001
0
1
2
3
4

Figure 17: Effects of 8-Cl-cAMP on adult *S. mansoni* viability *in vitro* and *in vivo*.

A, Adult *S. mansoni* worm pairs were maintained in medium containing varying concentrations of 8-Cl-cAMP. Survival in the presence of 8-Cl-cAMP was plotted against time. Concentrations of 8-Cl-cAMP are as follows: 0 and 1 μM (●); 10 μM (♦); 25 μM (■); 50, 100, 250, and 500 μM (▼). Treatment groups containing 6 worm pairs each (12 worms total) were used for each concentration. B, Micrographs of representative worm pairs incubated in the presence of 500 μM 8-Cl-cAMP or vehicle control. Scale bars = 1 mm. 8-Cl-cAMP was administered i.p. at 100 mg/kg for 1 week to *S. mansoni* 6 week post-infected C57BL/6 mice. C, Total no. of worms isolated from treated and control mice. D, No. of worms pairs isolated from treated and control mice. Data are representative of two independent experiments.

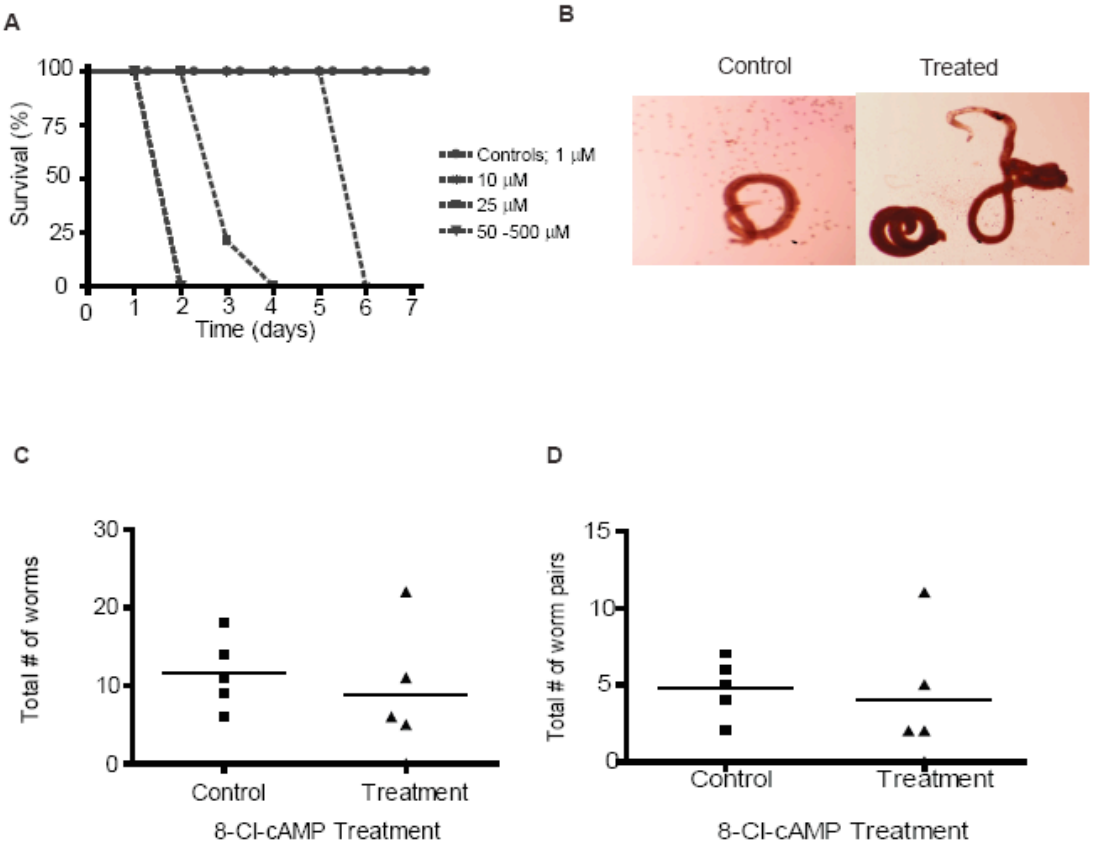

A
100
75
50
25
0
Survival (%)
0 1 2 3 4 5 6 7
Time (days)
Controls; 1 µM
10 µM
25 µM
50 -500 µM
B
Control
Treated
C
30
20
10
0
Total # of worms
Control
Treatment
8-Cl-cAMP Treatment
D
15
10
5
0
Total # of worm pairs
Control
Treatment
8-Cl-cAMP Treatment

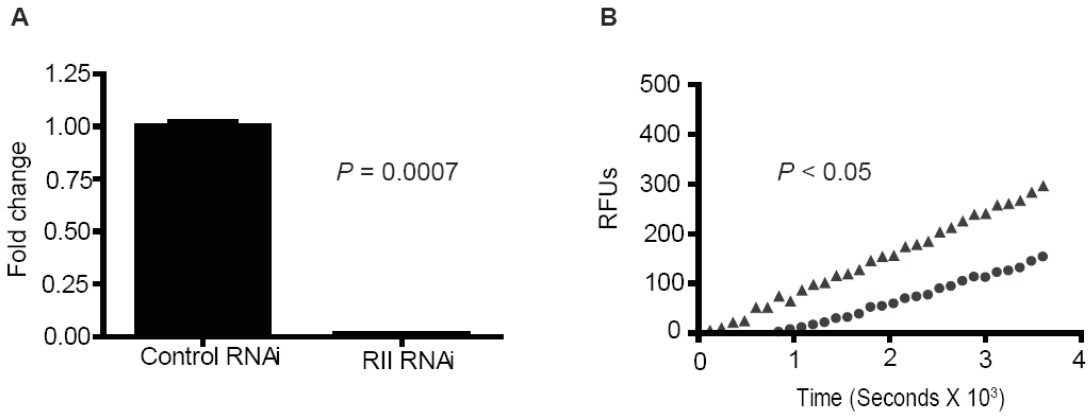
Figure 18: Amino acid alignment of putative *S. mansoni* PKA-RIIα subunit (SmRIIa) and other PKA-RIIα subunits from other organisms.

Sequences are labeled as follows: SjRIIa, *S. japonicum* PKA-RIIα (AAW24538); AcRIIa, *Aplysia californica* PKA-RIIα (AAR26237); HsRIIa, *Homo sapiens* PKA-RIIα (NP_004148); MmRIIa, *Mus musculus* PKA-RIIα (NM_008924); CeR, *Caenorhabditis elegans* PKA-R (J05220); OvR, *Onchocerca volvulus* PKA-R (AY159364).


SmRIIa
SjRIIa
AcRIIa
HsRIIa
MmRIIa
CeR
OvR
Consensus

Figure 19: RNAi of Sm04765 in adult *S. mansoni*.

A, Three days after electroporation, Sm04765 transcript levels were decreased in worms electroporated with 40 µg of Sm04765 dsRNA, while transcript levels were unaffected in control dsRNA-treated worms, as determined by real-time PCR. C, 7 days after electroporation, Sm04765 dsRNA-treated (●) and control dsRNA-treated worms (▲) were incubated for 2 h in 100 µM forskolin. PKA activity was significantly decreased after electroporation with 40 µg Sm04765 dsRNA compared to control dsRNA-treated worms.


A
1.25
1.00
0.75
0.50
0.25
0.00
Fold change
P = 0.0007
Control RNAi
RII RNAi
B
500
400
300
200
100
0
RFUs
P < 0.05
0
1
2
3
4
Time (Seconds X 10³)

Chapter 6: Summary and Future Studies

Overall, the data presented suggest that *S*mPKA, with particular emphasis on the SmPKA-C gene product, could potentially be a novel therapeutic drug target in schistosomes. Through both chemical inhibition and RNA interference, we were able to show that SmPKA is essential for *S. mansoni* viability *in vitro* and may play a critical role in schistosome egg production. Drug development for treatment of cancer in humans has primarily focused on developing inhibitors to the RIα subunits and therefore there is little information on the susceptibility of the catalytic subunit to chemical inhibition [1]. There are few studies in the literature using PKA-C subunit inhibitors in animal models [2]. One study examined morphine tolerance in mice in which PKI 14-22 amide was administered via intracerebroventricular injections into treated mice [3]. Additional studies will therefore be required to assess the feasibility of using H-89 or PKI 14-22 amide in a murine model of schistosomiasis, to establish dosage rates and routes of administration that might be effective. An additional barrier to the use of these compounds as anti-schistosome drugs, is that they were only schistosomicidal at concentrations that are not pharmacologically realistic.

A more productive approach to identifying anti-PKA drug candidates that target the SmPKA-C gene product might be to take advantage of the extensive inhibitor libraries located at the National Institutes of Health, which contain several thousand protein kinase inhibitors, to screen for compounds which have an inhibitory effect on SmPKA-C protein. This type of approach identified oxadiazole-2-oxide analogues such as furoxan as potential novel compounds against the *S*. *mansoni* detoxifying enzyme, thioredoxin-glutathione reductase

(TGR) [4]. These analogues were selected first for their inhibitory effects on recombinant TGR activity and subsequently for potent anti-schistosomal effects. A similar approach could be undertaken for SmPKA-C protein. First, recombinant SmPKA-C protein would be used to screen for compounds that inhibit SmPKA-C activity. Second, effective inhibitors would then be screened using *in vitro* killing assays with *S. mansoni* worms, similar to the H-89 and PKI 14-22 amide experiments conducted in Chapter 2. Lastly, those drugs that are lethal *in vitro* would be tested in a murine model of schistosomiasis.

Numerous attempts have been made in our laboratory to produce recombinant SmPKA-C protein using both prokaryotic and eukaryotic expression systems with mixed results. Using *in vitro* transcription and translation systems for the production of recombinant protein, the most robust expression of recombinant SmPKA-C protein was observed when *Spodoptera frugiperda* insect cell lysate was used. While this is a viable approach to the production of recombinant protein, further experimentation is required to optimize the expression of active, recombinant SmPKA-C protein. A source of enzymatically active recombinant protein would be a valuable reagent for the identification of novel inhibitors that could potentially be used as new anti-schistosome chemotherapeutics, either alone or perhaps in conjunction with praziquantel. Furthermore with the complete *S. mansoni* genome sequence available [5], additional studies to identify and characterize other potential *S. mansoni* PKA-C subunits could be undertaken using similar methodologies to those used in the identification of SmPKA-C in Chapter 2.

In addition to the lethality observed with SmPKA inhibition, we observed a potential correlation between egg production and SmPKA activity both *in vitro* and *in vivo*. As eggs are the source of pathology and transmission, studies focusing on the role of SmPKA-C protein in egg production should be undertaken. A number of recent studies have employed an elaborate method to study the role of schisotosome genes *in vivo* [6,7], whereby schistosomules are treated with specific dsRNA and then used to re-establish infections in mice by intraperitoneal injection [8]. At 6-7 weeks post-infection, adult worms are isolated and parasitological parameters examined, including: 1) total worms; 2) number of schistosome pairs; 3) parasite length; 4) egg production; 5) mRNA levels; 6) enzyme activity. A similar approach could be employed to test the function of SmPKA-C *in vivo*. If defects are observed, these could be attributed to the loss of SmPKA-C protein and subsequently schistosome PKA activity.

To determine whether SmPKA-C has an important function in schistosome egg viability, eggs could be soaked in the presence of PKA-C subunit inhibitors, H-89 and PKI 14-22 amide, and then exposed to bright light to induce hatching. In concert with these studies, we could examine the effects of SmPKA-C knockdown specifically by soaking eggs in the presence of SmPKA-C dsRNA. This was found to be an efficient method of delivering gene-specific dsRNA into schistosome eggs [9]. These studies would allow us to determine if SmPKA-C protein is required for egg viability, as we have previously demonstrated with adults and cercariae [10]. Additionally, immunohistochemistry studies using a specific SmPKA-C antibody would show anatomically where SmPKA-C protein is

most expressed in the parasite life cycle. If the protein is highly expressed in the adult parasites as we suspect, this strengthens our hypothesis that SmPKA-C protein could be a novel therapeutic target.

Furthermore, we identified SmPKA-C orthologues in the other two medically important species, *S. haematobium* and *S. japonicum,* which are 99% identical to SmPKA-C protein at the amino acid level. Due to the high degree of PKA-C subunit conservation shared among the three species, targeting schistosome PKA-C subunits could be a viable strategy for the development of novel chemotherapeutics that would be efficacious against all three medically important species. Similar to the studies we have conducted for *S. mansoni,* RNAi studies using SjPKA-C and ShPKA-C specific dsRNA could be conducted to determine if the requirement for the PKA-C gene product is also essential for parasite viability in these species. The studies described in Chapter 3 aimed at elucidating the biological role of PKA in *S. mansoni* could be undertaken with *S. japonicum* and *S. haematobium*. If these experiments show that PKA is required for egg production in all three schistosome species, these studies would establish schistosome PKA activity as an attractive target for the treatment and amelioration of schistosome infections.

As the SmPKA-C gene product is nearly 80 % identical at the amino acid level to the human PKA-Cα subunit homologue, targeting the schistosome PKA-C subunit specifically may be problematic. With this in mind, we also focused on the identification of PKA-R subunit homologues in *S. mansoni*. As mentioned earlier, PKA-RIα subunits are the primary targets of the cancer drugs, 8-Cl-cAMP

and GEM231 [11], and therefore the characterization of regulatory subunit homologues in schistosomes may be especially relevant to developing PKA as an anti-schistosome target. Indeed, we found that SmPKA activity was inhibited by 8-Cl-cAMP and that this compound was lethal for adult *S. mansoni in vitro* at various micromolar concentrations, suggesting that targeting PKA-R subunits may be a viable approach to disrupting PKA function in schistosomes. We also confirmed that an mRNA encoding a putative PKA-RIIα subunit (Sm04765) that was identified by the *S. mansoni* genome project is expressed by adult worms and that the putative protein is 44 % identical to the human PKA-RIIα homologue at the amino acid level. PKA-R subunits are not as highly conserved as PKA-C subunits, suggesting that identification and development of cAMP analogs and inhibitors that specifically target schistosome PKA-R subunits may be possible. However, despite completion of the S. mansoni and S. japonicum genomes, PKA-R subunits remain poorly characterized in schistosomes. In addition to the SmPKA-RII subunit mentioned above, there are currently only five other nucleotide sequences (Sm09450, Smp_131050, Smp_019280, Sm072273, and Smp_030400) in the *S. mansoni* genome database that are characterized as putative PKA-R subunits. Translation of the nucleotide sequences revealed truncated ORFs in all of the sequences, indicating that these sequences are either incomplete or that the putative coding sequences have been incorrectly predicted from the genomic sequence. Additional 5'- 3'RACE experiments could be used to determine the correct full length ORFs of these sequences. Our hope through these studies would be the identification of a putative schistosome PKA-

RIα subunit, as this is the major target of PKA cancer chemotherapeutics [12]. Interestingly, the PKA-R subunit homologue in *Onchocerca volvulus*, causative agent of river blindness, is being studied as a potential drug target and vaccine candidate [13].

As PKA-R subunits determine the function of holoenzymes, studies to determine the function of PKA-R subunits in schistosome biology should be conducted to complement the studies we have done on schistosome PKA-C subunits. Similar to its PKA-C subunit homologue, the PKA-R subunit of *C. elegans* has been shown to play an important role in egg embryogenesis and hatching during the first larval stage [14]. As we have demonstrated SmPKA-C's essential role in egg production, this suggests that targeting both schistosome PKA-R and PKA-C subunits may be a promising strategy for the development of new drugs for the treatment of schistosomiasis.

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