Journal of Helminthology (2012) **86,** 141–147
doi:10.1017/S0022149X12000120

Metabolite movement across the schistosome surface

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(Received 20 October 2011; Accepted 23 January 2012; First Published Online 27 February 2012)

Abstract

Intravascular schistosome parasites are covered by an unusual double lipid bilayer. Nutrients, such as glucose and amino acids, as well as other metabolites, are known to be transported across this surface via specific transporter proteins. For instance, the glucose transporter protein SGTP4 is found in the host-interactive tegumental membranes. A second glucose transporter, SGTP1, localizes to the tegumental basal membrane (and internal tissues). Following expression in *Xenopus* oocytes, SGTP1 and SGTP4 both function as facilitated-diffusion sugar transporters. Suppressing the expression of SGTP1 and SGTP4 in juvenile schistosomes using RNA interference (RNAi) impairs the parasite's ability to import glucose and severely decreases worm viability. Amino acids can also be imported into schistosomes across their surface and an amino acid transporter (SPRM1lc) has been localized in the parasite surface membranes (as well as internally). In *Xenopus* oocytes, SPRM1lc can import the basic amino acids arginine, lysine and histidine as well as leucine, phenylalanine, methionine and glutamine. To function, this protein requires the assistance of a heavy-chain partner (SPRM1hc) which acts as a chaperone. Water is transported across the tegument of schistosomes via the aquaporin protein SmAQP. Suppressing SmAQP gene expression makes the parasites less able to osmoregulate and decreases their viability. In addition, SmAQP-suppressed adult parasites have been shown to be impaired in their ability to excrete lactate. Analysis of tegumental transporter proteins, as described in this report, is designed to generate a comprehensive understanding of the role of such proteins in promoting parasite survival by controlling the movement of metabolites into and out of the worms.

Introduction

Schistosomes are intravascular platyhelminths that infect approximately 200 million people globally as well as numerous domestic and wild animals (Skelly, 2008). Juvenile worms migrate to the portal blood vessels where they mate and then travel as couples to egg-laying sites. The adult worms are relatively large and highly mobile within the vasculature. The parasites have an unusual outer covering called the tegument. The tegument lacks lateral membranes and its cytoplasm extends as a continuous unit (a syncytium) around the entire body (Morris & Threadgold, 1968; Smith *et al.*, 1969). The syncytium contains mitochondria and two kinds of inclusion bodies: discoid bodies (DBs) and multilaminate vesicles (MLVs) (Wilson & Barnes, 1974b). DBs are $\sim 40 \times 200$ nm in size, are surrounded by a single lipid bilayer and have a granular, mucopolysaccharide content (Wilson & Barnes, 1974b). MLVs are 150–200 nm in diameter and appear as a mass of tightly packed concentrically arranged membranes. The tegumental cytoplasm is connected by numerous slender processes

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to cell bodies (cytons) that lie beneath the peripheral muscle layers. These contain one or more nuclei as well as Golgi complexes, endoplasmic reticulum and mitochondria (Morris & Threadgold, 1968; Silk *et al.*, 1969). Proteins and carbohydrates for export are synthesized in the cytons and packaged into the secretory inclusions by Golgi bodies for transport along the cytoplasmic connections to the tegumental cytoplasm (Smith *et al.*, 1969).

The external tegument surface of schistosomes has a multilaminate, double lipid bilayer, appearance that can be seen using electron microscopy when uranyl acetate is used as a fixative in addition to the conventional glutaraldehyde and osmium tetroxide (Hockley & McLaren, 1973). This unusual multilaminate feature is interpreted to comprise an inner, conventional plasma membrane that is covered by a membraneous secretion called the membranocalyx. It has been proposed that the tegumental multilaminate appearance arises when MLVs fuse with the inner plasma membrane and release their contents to form the overlying membranocalyx (Wilson & Barnes, 1974a). The unusual tegumental outer covering is considered to be an adaptation for survival by schistosomes in the bloodstream. Internally the tegument is bounded by a basal membrane which is separated from the underlying musculature by a finely granular basal lamina or basement membrane (Silk *et al.*, 1969).

The tegument is a prime site of host–parasite interaction and there is great interest in understanding the composition and capabilities of the tegumental surface membranes. This is a long-term goal of our laboratory. The molecular composition of the outer surface membranes of intra-mammalian schistosomes has been the subject of much recent study. A series of investigations has used proteomics to characterize by direct analysis the composition of the host–parasite interface (Braschi & Wilson, 2005; van Balkom *et al.*, 2005; Braschi *et al.*, 2006). These studies have involved several different approaches. In some cases tegumental material was preferentially enriched and subjected to proteomic analyses (Braschi & Wilson, 2005; van Balkom *et al.*, 2005; Braschi *et al.*, 2006). In another case, the outer tegument was labelled with biotin and labelled protein was subsequently recovered and analysed by mass spectrometry (Braschi & Wilson, 2005; van Balkom *et al.*, 2005; Braschi *et al.*, 2006). More recently, exposed material has been removed enzymatically from whole worms using trypsin or phospholipase C and these 'tegumental shavings' analysed (Castro-Borges *et al.*, 2011a). Over 50 distinct, major proteins have been identified by these various approaches and these belong to a diversity of protein families, including enzymes, structural proteins and several others that are unique to schistosomes and of unknown function (Skelly & Wilson, 2006). There is reasonable consistency between the different experimental approaches which have greatly expanded the number of known schistosome tegumental proteins. In some instances the newer proteomic analyses corroborate earlier reports defining proteins as being tegumental based on their immunolocalization patterns or activity profiles. In this report, we review work characterizing a number of tegumental components that are involved in transporting select metabolites across the tegument and describing how these proteins contribute to schistosome well-being.

Glucose transport

Despite the fact that schistosomes possess a mouth and a gut and are avid blood eaters, it has long been known that many nutrients are taken up by the parasites directly across the tegument (Fripp, 1967b; Asch & Read, 1975a, b). Thus, one role of the intravascular parasite tegument is to import nutrients from the host bloodstream. Glucose is one metabolite that is copiously consumed; adult *Schistosoma mansoni* are reported to import across their teguments their dry weight in glucose every 5 h (Bueding, 1950) and at a rate three times higher than the glucose uptake by the mucosal border of the rabbit ileum (Gomme & Albrechtsen, 1988). Uptake is not an active process but is via facilitated diffusion (Isseroff *et al.*, 1972; Uglem & Read, 1975; Cornford *et al.*, 1988). These observations suggested that facilitated-diffusion glucose transporter proteins (GTPs) are localized within the schistosome tegument. We employed a degenerate polymerase chain reaction (PCR) approach to clone cDNAs encoding GTP homologues from *S. mansoni* (which we designated SGTP1 and SGTP4) (Skelly *et al.*, 1994). These proteins, both ~55 kDa, exhibited considerable sequence similarity with one another and with facilitated-diffusion glucose transporters of other organisms (Skelly & Shoemaker, 1996; Zhao & Keating, 2007). In addition, they both contained structural features conserved in other characterized members of this sugar transporter family (Skelly *et al.*, 1994). Formal proof that SGTP1 and SGTP4 are sugar transporters was obtained by demonstrating expression of this property within *Xenopus* oocytes following their injection with RNA encoding SGTP1 or SGTP4. Both proteins functioned as typical facilitated-diffusion sugar transporters; they exhibited sodium independence, stereospecificity (transporting D-glucose but not L-glucose), relaxed specificity for other hexoses (also transporting mannose, maltose, galactose and fructose) and both were markedly blocked by the transport inhibitor cytochalasin B (Skelly *et al.*, 1994).

Both SGTP1 and SGTP4 were localized by immunofluorescence and immuno-electron microscopy to the tegument (Zhong *et al.*, 1995; Jiang *et al.*, 1996; Skelly & Shoemaker, 1996). SGTP4 was shown to be present in the host-interactive, apical tegumental membranes, while SGTP1 was found in the tegumental basal membrane. SGTP1 was also widely expressed in the internal tissues of the parasite. Because both SGTPs were functionally active following heterologous expression in *Xenopus* oocytes and both were found in the tegument, we hypothesized that SGTP4 (in the apical membranes) was required for glucose import into the tegument from the external environment and that SGTP1 (in the basal tegumental membrane and elsewhere) was required for the further movement of some of the imported glucose out of the tegument and into the internal tissues (Skelly *et al.*, 1998).

The advent of RNA interference (RNAi) in schistosomes, permitting target gene suppression, provided us with the methodology to examine the importance of these proteins for the parasites and to test directly the hypothesis that these SGTPs were functional sugar importers in the worms. RNAi was employed to knock down expression of both SGTP genes in the schistosomula

and adult worm life stages. In our laboratory we employ target gene specific, synthetic 27-mer short inhibitory RNAs (siRNAs) for RNAi, which we deliver to the parasites by electroporation (Ndegwa *et al.*, 2007; Bhardwaj *et al.*, 2011). Gene suppression at the RNA level is routinely examined 2 days after treatment by quantitative real-time PCR (qRT-PCR). Suppression at the protein level is routinely monitored ~7 days after treatment by Western blotting. Both SGTP genes were effectively suppressed (either alone or in combination) as assessed at the RNA and protein levels (Krautz-Peterson *et al.*, 2010). Suppression is gene specific; when just SGTP1 is targeted, SGTP4 expression remains unaltered and vice versa. In glucose-uptake assays, SGTP1-suppressed or SGTP4-suppressed parasites exhibited an impaired ability to import radiolabelled sugar compared to control worms. This effect was compounded by suppression of both transporter genes simultaneously (Krautz-Peterson *et al.*, 2010). The reduced ability of SGTP-suppressed schistosomes to import glucose demonstrates unequivocally that the parasites do use both SGTP1 and SGTP4 to take in sugar efficiently. Not surprisingly, worms treated with the sugar transport inhibitor, cytochalasin B, are also greatly impaired in their ability to take up radiolabelled glucose (Krautz-Peterson *et al.*, 2010).

To determine if an inability to import glucose by the SGTP-suppressed parasites had a detrimental impact on the worms, their viability was compared with that of control parasites *in vitro* and *in vivo*. When suppressed parasites are maintained in standard culture medium with a high glucose concentration (10 mM) they exhibit no noticeable phenotypic differences compared with controls. However, when SGTP-suppressed parasites are cultured in medium containing a low glucose concentration (0.05 mM), significantly fewer of them survive relative to controls (Krautz-Peterson *et al.*, 2010). In the low-sugar environment, it appears that a diminished ability to import glucose impacts parasite metabolism and this leads to decreased worm viability. In a similar manner, when SGTP-suppressed parasites are used to infect mice, significantly fewer of them survive to maturation compared with controls (Krautz-Peterson *et al.*, 2010). This outcome shows that the SGTPs are essential for normal parasite development in the mammalian host.

Amino acid transport

In addition to importing glucose across the tegument, schistosomes have long been known to be capable of transporting amino acids from the external environment directly across their body surface (Fripp, 1967a; Asch & Read, 1975b). In early studies of amino acid transport into adult male schistosomes, at least five amino acid transport systems were implicated (Asch & Read, 1975a), indicative of the presence of several specific tegumental membrane amino acid transporter proteins. This is consistent with work in other cellular systems which employ multiple different amino acid transporter systems, some having a specialized role and others a more general role (Castagna *et al.*, 1997). So far, a single amino acid transporter has been characterized in schistosomes. This protein – designated schistosome permease 1 light chain, SPRM1lc – belongs to the glycoprotein-associated family of transporters (Mastroberardino *et al.*, 1998; Skelly *et al.*, 1999). SPRM1lc is a ~55 kDa protein that is found in both larval and adult schistosomes and in a variety of tissues (Skelly *et al.*, 1999). When expressed in *Xenopus* oocytes, SPRM1lc promoted amino acid uptake but, in initial work, only when co-expressed with the human glycoprotein, h4F2hc (Mastroberardino *et al.*, 1998). In this context, SPRM1lc facilitated the transport of the basic amino acids arginine, lysine and histidine as well as leucine, phenylalanine, methionine and glutamine (Mastroberardino *et al.*, 1998; Skelly *et al.*, 1999). The h4F2hc protein acted as a chaperone and was necessary for SPRM1lc to reach the oocyte plasma membrane and function as an amino acid permease. A disulphide bond links h4F2hc and SPRM1lc (Pfeiffer *et al.*, 1998). In schistosome extracts of all life-cycle stages examined, SPRM1lc was found to be associated into a high molecular weight complex that could be disrupted by reducing agents (Skelly *et al.*, 1999). We interpreted this to mean that a large fraction of endogenous SPRM1lc is linked via a disulphide bond to the schistosome h4F2hc homologue. Analysis of the schistosome genome led to the identification of a single h4F2hc homologue. We call this ~72 kDa protein the schistosome amino acid permease heavy chain or SPRM1hc (Krautz-Peterson *et al.*, 2007). When SPRM1lc is co-expressed with SPRM1hc in *Xenopus* oocytes, the pattern of amino acid uptake detected is very similar to that reported for oocytes expressing SPRM1lc with h4F2hc (Krautz-Peterson *et al.*, 2007), showing that heavy-chain influence is minor and that it is the light-chain partner that is most responsible for the uptake characteristics of the heterodimer.

Both SPRM1lc and SPRM1hc are detected in all life stages examined. This is consistent with their role in amino acid uptake into cells, an important function that would likely be required by all life stages and all tissues. Both proteins have been shown to be widely expressed in the adult parasites and both localize to the tegumental membranes. Localization of these proteins at the host/parasite interface has been confirmed for SPRM1lc, which has been shown to be available for surface biotinylation on living worms (Skelly *et al.*, 1999; Braschi & Wilson, 2005), and for SPRM1hc, which has been identified by proteomics in isolated tegumental membranes (Skelly *et al.*, 1999; Braschi & Wilson, 2005). These data strongly suggest that the SPRM1hc/SPRM1lc heterodimer functions not only to import amino acids from the external environment into the tegument but also to move amino acids from there into the internal tissues.

Water transport

Aquaporins are integral membrane proteins that act as water channels. Some aquaporins are capable of also transporting other small uncharged solutes, such as glycerol and urea, across a membrane and these are known more precisely as aquaglyceroporins (Gonen & Walz, 2006). Aquaporins/aquaglyceroporins are widespread in nature, where they occur as tetramers in plasma

membranes. Proteomic analysis of the schistosome tegumental membranes revealed the presence of an aquaglyceroporin homologue at the host-interactive surface (Skelly *et al.*, 1999; Braschi & Wilson, 2005). The cDNA encoding the protein, which we designated SmAQP, was cloned and characterized (Faghiri & Skelly, 2009). It encodes a predicted ~33 kDa protein that is found largely in the schistosomula and adult parasite tegument, as determined by immunolocalization. SmAQP has highest relative expression in the intravascular life stages, as assessed by quantitative real-time PCR (Faghiri & Skelly, 2009). It is the most abundant constituent of adult schistosome tegumental membranes (Castro-Borges *et al.*, 2011b). To explore the function of SmAQP, parasites were treated with siRNAs targeting the SmAQP gene in order to silence its expression. This treatment results in potent (>90%) SmAQP gene suppression (Faghiri & Skelly, 2009). The suppressed parasites, lacking tegumental water channels, resist rapid swelling when placed in hypotonic medium, unlike their control counterparts which quickly double in volume (Faghiri & Skelly, 2009). Additionally, SmAQP-suppressed parasites, unlike controls, resist shrinkage when incubated in hyperosmotic solution (Faghiri & Skelly, 2009).

Formal demonstration of the ability of SmAQP to act as a water channel was seen following its expression in *Xenopus* oocytes. Oocytes expressing SmAQP rapidly swell and burst in less than 3 min when transferred from an isotonic to a hypotonic solution whereas non-injected oocytes survive at least until the end of the experiment (at 90 min). The water osmotic permeability (P_f) in oocytes expressing SmAQP was $202 \pm 36 \times 10^{-4}$ cm/s (Faghiri *et al.*, 2010).

These experiments reveal a new function for the schistosome tegument – through SmAQP the tegument can act to control water movement into and out of the parasites and thus it plays a central role in parasite osmoregulation. The vital importance of SmAQP function is clear because the suppressed parasites exhibit lower viability in culture relative to controls and those that survive the RNAi treatment generally exhibit a stunted appearance following prolonged suppression (Faghiri & Skelly, 2009).

As noted earlier, aquaglyceroporins can act as conduits for substrates other than water. For instance, some aquaporins have been shown to play a role in controlling the exchange of metalloids (like arsenic and antimony) between an organism and its environment (Sanders *et al.*, 1997; Liu, 2010). Some such metalloids have been used therapeutically and, until relatively recently, an important treatment option for schistosome infection involved the use of trivalent antimonials such as potassium antimony tartrate (Rodriguez-Molina *et al.*, 1950; Bueding & Mansour, 1957). In the case of the protozoan parasite leishmania, trivalent antimonials that kill the parasites enter through surface aquaporin proteins, and mutations in aquaporin genes confer resistance to the drugs (Gourbal *et al.*, 2004). To test the hypothesis that, likewise in schistosomes, parasite-killing antimonial drugs enter through tegumental SmAQP, we compared the ability of potassium antimony tartrate to debilitate control versus SmAQP-suppressed parasites. The expectation was that parasites with reduced levels of SmAQP following RNAi treatment should have an impaired ability to take up the drug and should therefore exhibit a greater resistance to potassium antimony tartrate-mediated killing. Our data support this hypothesis; significantly fewer SmAQP-suppressed parasites were killed following exposure to potassium antimony tartrate compared to controls (Faghiri & Skelly, 2009). This result provides evidence that SmAQP can act as a conduit for anti-schistosome, antimonial drugs.

Lactate transport

The analysis of SmAQP function explored using RNAi reviewed above focused on the schistosomula life stage. In our analysis of SmAQP function in the adult life stage, we found that robust suppression of SmAQP (unlike the outcome in schistosomula) did not result in any detectable change in adult parasite viability or morphology (Faghiri *et al.*, 2010). However, by serendipity we discovered that cultured adult parasites whose SmAQP expression is suppressed by RNAi treatment, unlike controls, fail to rapidly acidify their culture medium. We noted minimal colour change in the RPMI medium after 96 h in culture. The medium that contained SmAQP-suppressed adult worms looked redder (i.e. retained more phenol red indicator) than medium containing control worms. Direct measurement of the pH of the media confirmed that medium containing control worms was significantly more acidic than medium that contained the SmAQP-suppressed group (Faghiri *et al.*, 2010). A key reason cultured schistosomes rapidly acidify their medium is because they excrete considerable amounts of lactic acid. The lactic acid is generated by the parasites largely following glucose catabolism through glycolysis. Adult schistosomes have long been termed homolactate fermenters because of their central reliance on glycolysis for energy generation (Shapiro & Talalay, 1982; Thompson *et al.*, 1984). The large amount of glucose consumed by adult worms, mentioned earlier, is coupled to the generation of large amounts of lactic acid – the end product of glycolysis. Our observations of the SmAQP-suppressed parasites led to the hypothesis that they were impaired in their capacity to excrete lactic acid. To test this idea, the level of lactate in parasite culture medium was measured and, as predicted, medium from the SmAQP-suppressed group was found to contain significantly less lactate compared to media from control parasites (Faghiri *et al.*, 2010). To directly test the hypothesis that SmAQP can transport lactate, the protein was expressed in *Xenopus* oocytes and radiolabelled lactate uptake assays were performed. It was found that SmAQP-expressing oocytes imported significantly more lactate versus controls (Faghiri *et al.*, 2010). These data unequivocally demonstrated the ability of SmAQP to transport this substrate. The transport of lactate in SmAQP-expressing oocytes follows Michaelis–Menten kinetics with very low apparent affinity ($K_m = 41 \pm 5.\ 8$ mM) (Faghiri *et al.*, 2010). Thus SmAQP functions not only to control water movement but also the movement of the metabolic by-product, lactate. The presence of SmAQP in the tegument of the worms assures an efficient efflux of lactate from

https://doi.org/10.1017/S0022149X12000120 Published online by Cambridge University Press

the animals to the host-medium. Worms in culture can excrete 0.5 mmol/h of lactate in the medium and they can also accumulate a high concentration of lactate (Githui *et al.*, 2006a, b). It is noteworthy that the low affinity of SmAQP for lactate is similar to that of the rat lactate transporter MCT4, expressed in *Xenopus* oocytes (Dimmer *et al.*, 2000). The MCT4 protein is reported to be adapted to the export of lactate from highly glycolytic cells (Dimmer *et al.*, 2000) and, as mentioned, adult schistosomes are also highly glycolytic. The transport of lactate (or water) into SmAQP-expressing *Xenopus* oocytes can be completely inhibited by the drug phloretin. Phloretin was previously shown to inhibit lactate release from cultured adult worms, presumably by acting on tegumental SmAQP (Githui *et al.*, 2006b).

Using the *Xenopus* oocyte uptake assay system, it was found that SmAQP can also transport mannitol and fructose but not glucose (Faghiri *et al.*, 2010). Thus it appears that SmAQP is a versatile tegumental transporter controlling the movement of water, lactate and other metabolites across the parasite tegument.

Proteomic analysis of tegument fractions reveals that the tegument is likely involved in the transport of additional metabolites. For instance, ion transporters with associated ATPases can be found in tegumental membrane extracts (Skelly & Wilson, 2006). Whether lipid is taken up trans-tegumentally is not known. Low-density lipoprotein (LDL) has been reported to bind to schistosomula and adult males of *S. mansoni* (Chiang & Caulfield, 1989; Pereira *et al.*, 2011). However, endocytosis of LDL has not been reported. Further, no clear homologue of known lipid-binding proteins has been described in tegumental proteomic analyses (Skelly & Wilson, 2006). Therefore additional work is needed to clarify how the worms acquire lipid, as well as to fully define other transport capabilities of the unique, intravascular schistosome surface.

Summary

In summary, this work shows that the intravascular schistosome tegument contains several transmembrane proteins that form channels or transporters necessary for parasite survival. Tegumental glucose transporters (SGTP1 and SGTP4) act to import sugar, the permease SPRM1lc (with the assistance of its heavy-chain chaperone SPRM1hc) acts to import some amino acids, and the aquaglyceroporin SmAQP can transport several metabolites (mannitol, fructose) including the metabolic waste product lactate. Impeding these important transport processes by targeting these proteins using chemotherapy or immunotherapy should offer new approaches to controlling schistosome infection.

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https://doi.org/10.1017/S0022149X12000120 Published online by Cambridge University Press