A quantitative proteomic analysis of the tegumental proteins from *Schistosoma mansoni* schistosomula reveals novel potential therapeutic targets

Javier Sotillo a,*, Mark Pearson a, Luke Becker a, Jason Mulvenna b, Alex Loukas a

a Centre for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, QLD, Australia
b QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia

**ARTICLE INFO**

**Abstract**

The tegument of *Schistosoma mansoni* plays an integral role in host–parasite interactions, particularly during the transition from the free-living cercariae to the intra-mammalian schistosomula stages. This developmental period is characterised by the transition from a trilaminate surface to a heptalaminate tegument that plays key roles in immune evasion, nutrition and excretion. Proteins exposed at the surface membranes of newly transformed schistosomula are therefore thought to be prime targets for the development of new vaccines and drugs for schistosomiasis. Using a combination of tegumental labelling and high-throughput quantitative proteomics, more than 450 proteins were identified on the apical membrane of *S. mansoni* schistosomula, of which 200 had significantly regulated expression profiles at different stages of schistosomula development in vitro, including glucose transporters, sterols, heat shock proteins, antioxidant enzymes and peptidases. Current vaccine antigens were identified on the apical membrane (Sm-TSP-1, calpain) or sub-tegumental (Sm-TSP-2, Sm29) fractions of the schistosomula, displaying localisation patterns that, in some cases, differ from that in the adult stage fluke. This work provides the first known in-depth proteomic analysis of the surface-exposed proteins in the schistosomula tegument, and some of the proteins identified are clear targets for the generation of new vaccines and drugs against schistosomiasis.

&copy; 2015 The Authors. Published by Elsevier Ltd. on behalf of Australian Society for Parasitology Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

**1. Introduction**

Schistosomiasis is one of the most important neglected tropical diseases affecting more than 200 million people worldwide (Gryseels et al., 2006; Colley et al., 2014), particularly in developing and tropical regions. Three main species of schistosomes are of major medical relevance: *Schistosoma mansoni* and *Schistosoma japonicum*, the eggs of which induce hepatosplenic inflammation and liver fibrosis when trapped in the portal system (Gryseels et al., 2006), and *Schistosoma haematobium* which is linked to bladder cancer in chronically infected people (James et al., 1974; Schwartz, 1981; Mayer and Fried, 2007). Despite the widespread use of the anthelmintic drug Praziquantel for the last 20 years in mass drug administration programs, this parasitic infection still causes a loss of 1.53 million disability-adjusted life years (DALYs) and up to 280,000 deaths annually in sub-Saharan Africa alone (van der Werf et al., 2003; King et al., 2005; Gryseels et al., 2006; Steinmann et al., 2006). Indeed, the DALYs attributed to schistosomiasis may be far greater than initially appreciated due to recent awareness of the morbidity associated with infections that were traditionally classified as “asymptomatic” (King, 2015).

*Schistosoma mansoni* presents a complex life cycle that involves a freshwater snail where the ciliated miracidium undergoes asexual replication through mother and daughter sporocyst stages, eventually shedding thousands of cercariae (Colley et al., 2014). The cercariae exit the snail into freshwater approximately 4–6 weeks after infection and quickly penetrate the skin of the human host. Once in the skin, cercariae shed their tail and transform into schistosomula, which slowly migrate through the skin before entering the blood capillaries en route to the lungs and ultimately into the portal system where they feed on blood and mature into dioecious adult worms (Miller and Wilson, 1980; Gryseels et al., 2006). Schistosome adult flukes live for 3–10 years in their definitive human hosts, mainly due to their ability to avoid immune-mediated clearance (Kusel et al., 2007). Their unique, dynamic, tegumental structure and the complex immuno-evasive...
strategies employed by these parasites help them survive in the harsh intravascular environment (Pearce and MacDonald, 2002).

The tegument of *S. mansoni* participates in different processes such as nutrition, excretion, signal transduction, osmoregulation and immune evasion and modulation, playing a key role in host–parasite interactions (Jones et al., 2004; Mulvenna et al., 2010). The great complexity of this dynamic membrane helps the parasite to transform from a free-living phase into a parasitic stage that migrates through distinct environments in the definitive host (Brink et al., 1977). By replacing its tegumental composition, in just a few hours, the parasite switches from an immune-sensitive to an immune-refractory state (Jones et al., 2004). Different studies have analysed the morphological changes that occur in the outer membrane of *S. mansoni* during development from cercariae to adult worms under different conditions. Hockley and McLaren (1971) described a trilaminate outer membrane in cercariae and a multilaminate membrane in adult worms, and then went on to implicate membrane-bound vacuoles produced by sub-tegumental cells in the formation of the heptalaminate membrane of juvenile parasites (Hockley and McLaren, 1973).

The use of in vitro transformed schistosomula was first studied by Clegg and Smithers (1972), who showed that the growth rate of schistosomula in vitro was identical to the growth rate in vivo for at least 12 days. In addition, the morphological changes that occur in the schistosomula in the first 4 days post-transformation are similar in parasites isolated from the lungs of mice and in parasites cultured in media (Samuelson et al., 1980). The similar morphological features displayed by in vitro and in vivo transformed schistosomula (Brink et al., 1977; Samuelson et al., 1980), opened the possibility of using manually transformed schistosomula for laboratory research instead of lung schistosomula which are notoriously difficult to obtain in large numbers. More recently, gene expression studies revealed the surprising similarities between the two most important human schistosome species, *S. mansoni* and *S. haematobium*.

The schistosomula stage is critical for sexual maturation and parasite establishment, and its surface plays an important role in host–parasite interactions, being the most susceptible target for vaccines and drugs against *Schistosoma* spp. (Jones et al., 2004; Loukas et al., 2007). Using a combination of tegumental labelling and high-throughput quantitative proteomics techniques we have identified a number of proteins that are highly expressed on the tegument of *S. mansoni* schistosomula at different stages of development, and highlight the utility of some of these proteins for the design of novel therapeutics against this important neglected tropical disease.

2. Materials and methods

2.1. Parasite material

*Schistosoma mansoni* (Puerto Rican strain) -infected *Biomphalaria glabrata* snails were provided by the National Institute of Allergy and Infectious Diseases (NIAID) Schistosomiasis Resource Center for distribution through BEI Resources, NIAID, National Institutes of Health (NIH), USA: *S. mansoni*, Strain NMRI Exposed *B. glabrata*, NR-21962. Cercariae were collected from the snails as described previously (Tucker et al., 2013), and immediately transformed into schistosomula by vortexing followed by a Percoll gradient to separate tails from bodies (Colley and Wikel, 1974; Tucker et al., 2013). Transformed schistosomula were incubated in Basch medium at 37 °C, 5% CO₂ (Basch, 1981) at a density of ~30,000 schistosomula in 4 mL of medium for 3 h, 2 days and 5 days. As a control, non-cultured schistosomula were processed immediately after transformation from cercariae.

2.2. Biotinylation of schistosomula tegument

The method used for tegumental biotinylation is a modified version of the method followed by Mulvenna et al. (2010). Approximately 60,000 schistosomula were used at each time point studied. The parasites were cultured for defined periods as described in Section 2.1, washed in Hank’s Buffered Salt Solution (HBSS; Invitrogen, USA) twice at room temperature (RT) and incubated with 1 mM EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fischer Scientific, USA) for 30 min at 4 °C. Biotin was then removed and parasites washed three times at RT in RPMI 1640 with free amino acids (Invitrogen) to quench any remaining biotin. The parasites were then snap-frozen in liquid nitrogen and kept at −80 °C until further use. Teguments were removed using the freeze/thaw/vortex technique (Roberts et al., 1983), where schistosomula were slowly thawed on ice, washed in TBS (10 mM Tris/HC1, 0.84% NaCl, pH 7.4) and incubated for 5 min on ice in 10 mM Tris/HC1, pH 7.4, before vortexing for five 1 s bursts. The tegmental extract was pelleted at 1000g for 30 min and solubilised three times in 200 µL of solubilising solution containing 0.1% (w/v) SDS, 1.0% (v/v) Triton X-100 in 40 mM Tris, pH 7.4 with pelleting at 15,000g between each wash. The washes were combined and incubated with 240 µL of streptavidin-agarose beads (GE Healthcare, UK) for 2 h at RT with gentle head-over-head mixing. After pelleting the beads, the supernatant was collected and proteins that were not bound to streptavidin beads were retained as the “unbound” fraction. Proteins bound to streptavidin were eluted by incubating the beads three times with 300 µL of 2% SDS for 10 min at 95 °C followed by vortexing for 2 min. Proteins eluted from streptavidin in SDS were retained as “biotinylated” proteins.

2.3. Immunofluorescence and microscopy analysis

For microscopy purposes, parasites were biotinylated with 1 mM EZ-Link Sulfo-NHS-SS-Biotin as described in Section 2.2. Paraoxas were then washed three times in HBSS, incubated with streptavidin-FITC for 30 min at RT and washed twice more with HBSS. Negative control parasites were not labelled with biotin and were instead incubated with just streptavidin-FITC. Biotinylated parasites that were not subjected to streptavidin-FITC were also analysed to detect autofluorescence from the biotin label. Samples were visualised using a Zeiss AxioImager M2 ApoTome fluorescence microscope (Zeiss, Germany) equipped with an AxioCam MRm at 60× magnification.

To visualise the extent of biotin internalisation in schistosomula, biotinylated and unbound (control) parasites were fixed in 4% paraformaldehyde in 0.1 phosphate buffer, pH 7.4, for 30 min at RT followed by thorough washing in PBS. Samples were embedded in paraffin, cut and stained with streptavidin–Alexa-555 and counterstained with DAPI before visualisation with a LSM 780 confocal microscope (Zeiss) at 100× magnification.

2.4. Protein digestion and iTRAQ labelling

Two biological samples for each time point were processed and analysed as follows: ITRAQ labelling, reduction, alkylation and digestion was performed on biotinylated and unbound samples
according to the manufacturer’s protocol (AB Sciex, USA). Briefly, 35 µg of protein from each sample was denatured with 2% SDS, reduced with 50 mM Tris-(2-carboxyethyl)-phosphine (TCEP) at 60 °C for 1 h, and cysteine residues were alkylated with 10 mM methyl methanethiosulfate (MMTS) solution at RT for 10 min. Proteins were digested using 1 µg of trypsin (Sigma–Aldrich, USA) at 37 °C for 16 h, and each sample was labelled with different iTRAQ reagents having distinct isotopic compositions. A HiTrap SP HP column (GE Healthcare) was used to remove excess iTRAQ label according to the manufacturer’s instructions and desalting and cleanup of samples were performed prior to electrofocusing using a Sep-Pak C18 cartridge (Waters, USA).

2.5. OFFGEL electrophoresis

Labelled peptides were separated based on pI using a 3100 OFFGEL Fractionator (Agilent Technologies, USA) with a 24 well setup as per the manufacturer’s protocol. Briefly, 24 cm long, 3–10 linear pH range IPG gel strips (GE Healthcare) were rehydrated with IPG Strip Rehydration Solution for 15 min. The tryptic peptides were diluted in 3.6 ml of peptide-focusing buffer and 150 µl was loaded in each well. Samples were focused with a maximum current of 50 µA until 50 kVh was reached, and peptide fractions were harvested and each well rinsed with 150 µl of a solution of water/methanol/formic acid (45%/50%/1%) for 15 min. Solutions were pooled with their corresponding peptide fraction and all fractions were evaporated using a vacuum concentrator. Prior to mass spectral analysis, samples were desalted using ZipTip (Millipore, USA) according to the manufacturer’s protocol, followed by centrifugation under vacuum.

2.6. Reverse-Phase (RP) LC–MS/MS analysis

Fractions recovered from OFFGEL electrophoresis were reconstituted in 10 µl of 5% formic acid. Six microlitres of sample was injected onto a 50 mm 300 µm C18 trap column (Agilent Technologies) and the samples were desalted on the trap column for 5 min at 30 µL/min using 0.1% formic acid (aqueous). Peptides were then eluted onto an analytical nano HPLC column (150 mm × 75 µm 300SBC18, 3.5 µm, Agilent Technologies) at a flow rate of 300 nL/min and separated using a 35 min gradient of 1–40% buffer B (90/10 acetonitrile/0.1% formic acid) followed by a steeper gradient from 40–80% buffer B for 5 min. The mass spectrometer operated in information-dependent acquisition mode (IDA), in which a 1 s TOF MS scan from 350–1400 m/z was performed, and for product ion ms/ms 80–1400 m/z ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion. Analyst 1.6.1 (AB SCIEX) software was used for data acquisition and analysis.

2.7. Database searching and bioinformatic analysis

All searches for the bionylated and unbound fractions were performed similarly against the peptide sequences predicted from the S. mansoni genome data (Berriman et al., 2009) using Mascot search engine v4.0 (Matrix-Science). Mascot searches were done with trypptic specificity, allowing a mass tolerance of ±0.5 Da and two missed cleavages. Methionine oxidation, carbamidomethylation and iTRAQ standard modifications were used as variable modifications. The results from the Mascot searches were validated using the X!Tandem search engine with the program Scaffold Q+ (version Scaffold_4.2.1) (Searle, 2010). Peptide and protein identifications were accepted if they could be established at greater than 95% and 98% probability, respectively, as specified by the Peptide Prophet algorithm (Keller et al., 2002), and contained at least two identified peptides (proteins) (Nesvizhskii et al., 2003). Proteins containing similar peptides that could not be differentiated based on MS/MS analysis were grouped to satisfy the principles of parsimony. A false discovery rate (FDR) of <0.2% and <0.3% for the bionylated and unbound fractions, respectively, was calculated using protein identifications validated by the Scaffold Q+ program.

Scaffold Q+ was used to quantify the isobaric tag peptide and protein identifications. Channels were corrected in all samples according to the algorithm described in i-Tracker (Shadforth et al., 2005) and acquired intensities in the experiment were globally normalised across all acquisition runs. Individual quantitative samples were normalised within each acquisition run, and intensities for each peptide identification were normalised within the assigned proteins. The reference channels were normalised to produce a 1:1-fold change. All normalisation calculations were performed using medians to multiplicatively normalise data. Differentially expressed proteins were determined using Kruskal–Wallis Test analysis and results expressed in log, ratios. Only proteins with a P value <0.05 and a significant log fold-change >0.6 or <-0.6 (for upregulated and downregulated proteins, respectively) were taken into consideration for further analysis.

Protein identifications were assigned to subcellular locations using a combination of Uniloc (http://bioapp.iis.sinica.edu.tw/Uniloc/index.html) and literature searches. Blast2Go (Conesa et al., 2005) was used to classify proteins according to Gene Ontology (GO) categories, and protein family (Pfam) analysis was performed using HMMER v3.1b1 (http://hmmer.org/). Putative signal peptides and transmembrane domain(s) were predicted using the programs SignalP (Emanuelsson et al., 2007) and TMHMM (Krogh et al., 2001), respectively. Heatmaps representing the differentially expressed proteins were generated in R using ggplot2 (Ginestet, 2011) and clustering was performed using Euclidean distances.

3. Results

3.1. Biotin binds specifically to the outer membrane of schistosomula

The use of different sulfu-biotin derivatives for the isolation of tegumental proteins in schistosome adults has been validated in the past (Braschi and Wilson, 2006; Mulvenna et al., 2010). To analyse the suitability and the extent of biotin incorporation in schistosomula, manually transformed, live parasites were bionylated on ice using EZ-Link Sulfo-NHS-SS-Biotin and subjected to immunofluorescence analysis. Bionylated parasites were incubated with streptavidin-FITC and a clear and intense labelling was detected over the entire tegument of the schistosomula (Fig. 1A). Control parasites (unbound) did not show any non-specific labelling of streptavidin-FITC (Fig. 1B) and bionylated parasites that were not incubated with streptavidin-FITC did not show any auto-fluorescence (data not shown). To analyse the distribution of biotin on the schistosomula tegument and rule out the potential labelling of the gastrodermis and other internal structures, bionylated parasites were fixed and subjected to immunohistochemical analysis. Due to the morphological differences of the tegument in the schistosomula at different stages, we analysed the tegumental biotinylalation in young (3 h) and 5 day schistosomula. Biotin was not observed in the internal organs and gastrodermis of the parasite, thereby excluding its ingestion during incubation, and confirming that only tegumental proteins were labelled (Fig. 1C and D).

3.2. Time-course quantitative analysis of tegumental proteins

Tegument of S. mansoni schistosomula was labelled with biotin, extracted following established methods, labelled with iTRAQ,
isotopes and subjected to LC–MS/MS analysis. Two different biological replicates from each time point were analysed and a total of 28,217 spectra were acquired and used to assign unique peptides and unique proteins, leading to the identification of 463 proteins in the biotinylated samples (Supplementary Table S1). The quantification analysis was performed using Scaffold Q+, and only proteins with a $P$ value <0.05 and a log$_2$ fold-change >0.6 or <-0.6 (for upregulated and downregulated proteins, respectively) were taken into consideration for further analysis. A total of 200 proteins had significantly dysregulated expression profiles in at least one of the time points studied (Supplementary Table S2); of these, 22 proteins contained a signal peptide and 28 had a transmembrane domain (Supplementary Table S3). A total of 894 proteins were identified in the unbound fraction (proteins not labelled with biotin and thus present in the schistosomula tegument cytoplasm and sub-tegumental tissues), of which 377 underwent significant expression changes during the experiment (Supplementary Table S4).

The significantly differentially expressed tegumental proteins were subjected to a GO-enrichment analysis using Blast2GO (Conesa et al., 2005), grouped into 10 GO annotation categories and plotted in a clustered heatmap (Fig. 2). Clustering was performed based on protein expression patterns using Euclidean distances and dendrograms were reordered based on mean values. A clear set of proteins was significantly upregulated at all time points studied, while the majority of proteins were downregulated compared with newly transformed schistosomula (Supplementary Table S3). Proteins assigned to peptidase and kinase activities were, in general, significantly upregulated on the tegument of schistosomula over time, whereas heat shock proteins were generally upregulated as the parasites developed (Fig. 2). The significantly dysregulated proteins were also classified into six groups according to their predicted subcellular location using UniLoc and literature searches (Fig. 2). The majority of extracellular proteins were downregulated over time, while the cytoskeletal,
cytosolic and other groups did not follow a specific expression pattern during schistosomula development (Fig. 2).

A more in-depth analysis based on the subcellular location showed some differences in expression profiles of proteins upregulated on the schistosomula surface over time. While more cytoplasmic and nuclear proteins were upregulated on the tegument at 5 days post-transformation compared with earlier time points, the number of upregulated membrane-associated proteins decreased from 3 h to 2 days post-transformation (Fig. 3A). The numbers of upregulated proteins (compared with newly transformed parasites) associated with cytoskeleton, mitochondria and other organelles remained similar throughout the experiment (Fig. 3A). The number of significantly upregulated proteins at each time point was studied and compared with the other time points to show the degree of tegumental protein turnover. Interestingly, more significantly upregulated proteins were found at day 5 (76 proteins) compared with day 2 (67) or 3 h (57) schistosomula, and from these, a total of 41 proteins were common at all time points (Fig. 3B). Seven proteins were uniquely found at 3 h post-transformation and 14 proteins at 5 day schistosomula, while only two unique proteins were present on the schistosomula tegument at 2 days post-transformation (Fig. 3B).

The significantly dysregulated proteins from every studied time point were also subjected to a Pfam analysis. The 10 most represented protein domains from each time point are shown in Fig. 4. Interestingly, proteins with Redoxin, Thioredoxin-like domains and AhpC/TSA domains were upregulated during schistosomula growth but, in contrast, significantly upregulated proteins with an Annexin domain were only found at 3 h and 2 days post-transformation.

4. Discussion

The tegument of schistosomes plays an important role in different processes such as parasite development, nutrition and immune evasion and modulation (Jones et al., 2004; Skelly et al., 2014). As the parasite infects its definitive host, it undergoes different morphological changes to adapt to an intra-mammalian environment, including the switch from a trilaminate outer membrane in cercariae and newly transformed schistosomula to a heptalaminate membrane in adult worms (Hockley and McLaren, 1971). The heptalaminate tegument consists of an invaginated plasma membrane overlain by the membranocalyx, a lipid bilayer formed when the membrane of multilaminate vesicles trafficking from the tegumentary cell bodies fuses with the apical plasma membrane that...
confers protection to the parasite (Morris and Threadgold, 1968; Hockley and McLaren, 1973; Wilson and Barnes, 1977; Braschi and Wilson, 2006). The first few days of life for a schistosomulum in the mammalian host present a critical target for the development of vaccine-mediated protection (McManus and Loukas, 2008; Wilson and Coulson, 2009), since the parasite adopts an immune-refractory state as it rapidly matures on its sojourn through the lungs (Jones et al., 2004). Different studies have elucidated some of the genes and proteins involved in cercaria-schistosomulum transformation and in schistosomulum development; however, these mechanisms are not yet fully understood. The use of mechanically transformed schistosomula can help in the

Fig. 3. Subcellular location of biotinylated proteins from the Schistosoma mansoni tegument. Bar graph showing the abundance of proteins assigned to five different subcellular locations at 3 h, 2 and 5 days (d) (A). Venn diagrams of common proteins upregulated at the different time points by subcellular location (B).

Fig. 4. Protein family (Pfam) analysis of biotinylated proteins from the Schistosoma mansoni tegument. Bar graph summarising 10 most abundant Pfam in the significantly upregulated proteins from the tegument of S. mansoni schistosomula. Proteins with Redoxin, Thioredoxin-like domains and AhpC/TSA domains (in bold) were upregulated during schistosomula growth but, in contrast, significantly upregulated proteins with an Annexin domain (in bold) were only found at 3 h and 2 days post-transformation.
Table 1

Suggested proteins for target purposes. Proteins were analysed according to tegumental expression and regulation, predicted function and/or previous studies. Log2 fold change expression values for each protein at 3 h, 2 days (d) and 5 days post-transformation schistosomula compared to newly transformed schistosomula. Proteins identified in other biotinylation studies are denoted as 1 (Braschi and Wilson, 2006) and 2 (Mulvenna et al., 2010). Protein expression levels were compared with gene expression levels obtained by Gobert et al. (2010). Only expression levels from 3 h to 5 day schistosomula were compared, as controls used by Gobert et al. (2010) were cercariae instead of freshly transformed schistosomula.

| Predicted Function | Identified protein | Accession Number | Location | 3 h | 2 d | 5 d | Identified Transcriptomic comparison
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding</td>
<td>Annexin</td>
<td>Smp_074140</td>
<td>Cytoskeleton</td>
<td>0.6</td>
<td>0.2</td>
<td>0.5</td>
<td>1, 2 Downregulated</td>
</tr>
<tr>
<td></td>
<td>Dynamin</td>
<td>Smp_044920</td>
<td>Cytoskeleton</td>
<td>2.4</td>
<td>2.6</td>
<td>1.2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>T-complex protein 1 subunit alpha (tcp-1-alpha)</td>
<td>Smp_017360</td>
<td>Cytoskeleton</td>
<td>0.7</td>
<td>2.1</td>
<td>2.3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Chaperonin containing t-complex protein 1</td>
<td>Smp_004990</td>
<td>Cytosol/Nucleus</td>
<td>2.6</td>
<td>3.5</td>
<td>3.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Guanine nucleotide-binding protein beta 1</td>
<td>Smp_094480</td>
<td>Cytosol/Nucleus</td>
<td>3.2</td>
<td>2.8</td>
<td>3.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Histone H2A</td>
<td>Smp_031720</td>
<td>Cytosol/Nucleus</td>
<td>1.1</td>
<td>1.6</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Histone H3</td>
<td>Smp_082240</td>
<td>Cytosol/Nucleus</td>
<td>2.3</td>
<td>2.7</td>
<td>4.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Histone H4</td>
<td>Smp_053290</td>
<td>Cytosol/Nucleus</td>
<td>1.7</td>
<td>1.7</td>
<td>3.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>P30 brc protein</td>
<td>Smp_056360.5</td>
<td>Cytosol/Nucleus</td>
<td>1.4</td>
<td>3.8</td>
<td>3.7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Proteasome regulatory subunit-related</td>
<td>Smp_058650</td>
<td>Cytosol/Nucleus</td>
<td>1</td>
<td>1.6</td>
<td>2.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Histone H2b</td>
<td>Smp_108390</td>
<td>Cytosol/Nucleus</td>
<td>–0.2</td>
<td>–0.4</td>
<td>3.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Ribosomal protein S9</td>
<td>Smp_119920</td>
<td>Cytosol/Nucleus</td>
<td>–0.4</td>
<td>–0.7</td>
<td>1.2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>605 acidic ribosomal protein P0</td>
<td>Smp_009690.1</td>
<td>Cytosol/Nucleus</td>
<td>–0.1</td>
<td>–0.1</td>
<td>0.6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>14-3-3 protein</td>
<td>Smp_009760</td>
<td>Cytosol/Nucleus</td>
<td>–0.4</td>
<td>1.3</td>
<td>1.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>E1b-55kd-associated protein</td>
<td>Smp_062720.5</td>
<td>Cytosol/Nucleus</td>
<td>–0.6</td>
<td>0.3</td>
<td>1.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Clathrin heavy chain</td>
<td>Smp_154420.1</td>
<td>Membrane</td>
<td>1.3</td>
<td>2.1</td>
<td>1.7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Programmed cell death protein</td>
<td>Smp_136660</td>
<td>Membrane</td>
<td>0.7</td>
<td>1.4</td>
<td>1.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Trimeric g-protein alpha o subunit</td>
<td>Smp_016630.1</td>
<td>Membrane associated</td>
<td>2.1</td>
<td>1.6</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Ankyrin 2,3,unc44</td>
<td>Smp_139110</td>
<td>Membrane associated</td>
<td>–0.1</td>
<td>0.5</td>
<td>0.6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Receptor for activated Protein Kinase C</td>
<td>Smp_102040.1</td>
<td>Membrane associated</td>
<td>–0.1</td>
<td>1.1</td>
<td>1.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Low-density lipoprotein receptor (ldl)</td>
<td>Smp_020550</td>
<td>Membrane associated</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Heat Shock Protein</td>
<td>Succinyl-coa synthetase beta chain</td>
<td>Smp_167330</td>
<td>Mitochondria</td>
<td>3</td>
<td>2.6</td>
<td>0.9</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Calreticulin autoantigen homologue precursor</td>
<td>Smp_030370</td>
<td>Unknown/Other</td>
<td>–0.4</td>
<td>0.9</td>
<td>1.2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cyclinphilin B</td>
<td>Smp_040790</td>
<td>Unknown/Other</td>
<td>0</td>
<td>0.6</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Heat shock protein 70</td>
<td>Smp_106930.1</td>
<td>Cytosol/Nucleus</td>
<td>1</td>
<td>2.1</td>
<td>2.3</td>
<td>1, 2 Downregulated</td>
</tr>
<tr>
<td></td>
<td>Heat shock protein (hsf16)</td>
<td>Smp_148530</td>
<td>Cytosol/Nucleus</td>
<td>1.6</td>
<td>3.8</td>
<td>4.9</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Heat shock protein</td>
<td>Smp_072330.1</td>
<td>Cytosol/Nucleus</td>
<td>0</td>
<td>2.1</td>
<td>2.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Heat shock protein (hsfp60)</td>
<td>Smp_008545</td>
<td>Mitochondria</td>
<td>0.9</td>
<td>1.6</td>
<td>1.2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Heat shock protein 70 (hsf70)</td>
<td>Smp_049550</td>
<td>Unknown/Other</td>
<td>0.8</td>
<td>3.4</td>
<td>0</td>
<td>1.2 Downregulated</td>
</tr>
<tr>
<td>Kinase</td>
<td>Integrin-linked protein kinase 2 (ilk-2)</td>
<td>Smp_079760.1</td>
<td>Membrane associated</td>
<td>–0.9</td>
<td>0.5</td>
<td>1.1</td>
<td>–</td>
</tr>
<tr>
<td>Other enzymatic</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (gapdh)</td>
<td>Smp_056970.1</td>
<td>Cytoskeleton</td>
<td>0.7</td>
<td>1.2</td>
<td>0.6</td>
<td>1, 2 Downregulated</td>
</tr>
<tr>
<td></td>
<td>Thioredoxin peroxidase</td>
<td>Smp_059480</td>
<td>Cytosol/Nucleus</td>
<td>–2.9</td>
<td>0.4</td>
<td>2.2</td>
<td>2 Upregulated</td>
</tr>
<tr>
<td></td>
<td>Adenosylhomocysteinase</td>
<td>Smp_028440.1</td>
<td>Cytosol/Nucleus</td>
<td>–0.9</td>
<td>0.3</td>
<td>0.7</td>
<td>– Upregulated</td>
</tr>
<tr>
<td></td>
<td>Expressed protein</td>
<td>Smp_105680.1</td>
<td>Membrane associated</td>
<td>0.6</td>
<td>1.4</td>
<td>2.7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Protein disulfide-isomerase</td>
<td>Smp_056760</td>
<td>Membrane associated</td>
<td>–0.2</td>
<td>0.8</td>
<td>0.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Dihydrolipoamide succinyltransferase</td>
<td>Smp_104330</td>
<td>Mitochondria</td>
<td>1.3</td>
<td>1.5</td>
<td>1.3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Protein disulfide-isomerase er-60 precursor (erp60)</td>
<td>Smp_079770.1</td>
<td>Unknown/Other</td>
<td>1.3</td>
<td>2.7</td>
<td>2.4</td>
<td>–</td>
</tr>
<tr>
<td>Other/Unknown</td>
<td>Arp2/3 complex 20 kd subunit</td>
<td>Smp_160770.1</td>
<td>Cytoskeleton</td>
<td>–0.7</td>
<td>0</td>
<td>0.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>40s ribosomal protein s10</td>
<td>Smp_066890</td>
<td>Cytosol/Nucleus</td>
<td>0.9</td>
<td>1.2</td>
<td>1.7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Universal stress protein</td>
<td>Smp_043120</td>
<td>Cytosol/Nucleus</td>
<td>1.4</td>
<td>2.3</td>
<td>3.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Glucose transport protein</td>
<td>Smp_012440</td>
<td>Membrane associated</td>
<td>4.2</td>
<td>4.5</td>
<td>4.4</td>
<td>2 Upregulated</td>
</tr>
<tr>
<td></td>
<td>Prohibitin</td>
<td>Smp_075210.2</td>
<td>Membrane associated</td>
<td>1.9</td>
<td>2.7</td>
<td>1.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Prohibitin</td>
<td>Smp_075940</td>
<td>Membrane associated</td>
<td>2</td>
<td>2.5</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Prominin (prom) protein</td>
<td>Smp_130520.1</td>
<td>Membrane associated</td>
<td>1.9</td>
<td>2.5</td>
<td>1.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Sterol reductase-related</td>
<td>Smp_124300</td>
<td>Membrane associated</td>
<td>4.3</td>
<td>4</td>
<td>4.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Stomatin-related</td>
<td>Smp_072640</td>
<td>Membrane associated</td>
<td>2.7</td>
<td>3.1</td>
<td>3.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Venom allergen-like (val) 4 protein</td>
<td>Smp_002070</td>
<td>Membrane associated</td>
<td>–2.8</td>
<td>–3</td>
<td>–4.5</td>
<td>–</td>
</tr>
</tbody>
</table>

(continued on next page)
collection of samples for transcriptomic and proteomic studies, due to their shared morphology and gene expression patterns (Brink et al., 1977; Samuelson et al., 1980; Protasio et al., 2013); however, very few studies have analysed the protein composition of the schistosomula (De la Torre Escudero et al., 2011; Hong et al., 2011, 2013), and none have specifically focused on the schistosomula tegument stage of the major human-infecting species. Different techniques have been applied to the study of the tegumental proteomic composition from the adult stage of different Schistosoma spp., and the use of biotin for labelling the membrane has proven to be a valid method to locate surface exposed proteins on the apical membrane of adult parasites (Braschi and Wilson, 2006; Mulvenna et al., 2010; Zhang et al., 2013). In the present study we used a short-chain, thiol-cleavable biotin derivative for the analysis of tegumental proteins from the schistosomula stage of S. mansoni, and taking advantage of OFFGEL separation techniques and quantitative mass spectrometry methods, we have identified and quantified a large number of differentially expressed proteins on the apical membranes of the parasites. Some of these proteins are clear targets for the generation of new vaccines and drugs against schistosomiasis (Table 1).

A total of 463 proteins were identified on the tegument of S. mansoni schistosomula, of which 200 were significantly regulated during the experiment. All 463 proteins were also found in the unbound fraction, which might indicate incomplete affinity purification with streptavidin agarose; however, van Balkom et al. (2005) found the majority of tegumental proteins were also present in the body of the adult parasite after the tegument had been stripped, and other authors attributed this to the sub-tegumental location of cell bodies beneath the tegument syncytium, whereby surface proteins are shuttled to the syncytial membranes via complex transport networks (Braschi et al., 2006). From the 36 proteins identified in a previous study to be present in Schistosoma bovis schistosomula (De la Torre Escudero et al., 2011), 24 (66.7%) were also identified in our analysis. In addition, six of the proteins identified in the present study were previously shown to be differentially expressed in S. japonicum schistosomula compared with adult flukes (Hong et al., 2013). Furthermore, the pattern of up- and downregulation of proteins in our study agrees with changes in gene expression profiles from transcriptomic studies (Table 1) (Gobert et al., 2010).

After a GO-enrichment analysis, the majority of proteins were assigned “binding activity”, which includes proteins involved in many different biological processes. Interestingly, most of the proteins with putative peptidase activity (~73%) were rapidly downregulated and only three peptidases were upregulated at least at one time point throughout the experiment (notably at 3 h or 5 days post-transformation) (Supplementary Table S3). A cecral elastase (Smp_006280, a serine protease inhibitor, has been found to regulate by cercariae together with a chymotrypsin-like protease (Salter et al., 2000). In addition, the downregulated protein Smp_062080, a serine protease inhibitor, has been found to regulate the enzymatic activity of cercarial elastase to minimize host tissue damage at the site of larval invasion and prevent severe inflammation (Quezada et al., 2012). Other proteases were detected in the schistosomula apical membrane, including metalloproteases belonging to the Invadolsin (Smp_090100 and Smp_090110) and Leucine aminopeptidase (Smp_030000) families. The Invadolsin Smp_090100 was the first metalloprotease described in the cercarial secretions (Curwen et al., 2006), and, together with the Invadolsin Smp_090110, was significantly downregulated (~2.4 and ~1.6, respectively) in 3 day old schistosomula compared with cercariae (Parker-Manuel et al., 2011).

### Table 1 (continued)

<table>
<thead>
<tr>
<th>Predicted Function</th>
<th>Identified protein</th>
<th>Accession Number</th>
<th>Location</th>
<th>3 h</th>
<th>2 d</th>
<th>5 d</th>
<th>Identified Transcriptomic comparison*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidase</td>
<td>Invadolsin (m08 family)</td>
<td>Smp_090100</td>
<td>Membrane associated</td>
<td>–1</td>
<td>0</td>
<td>–0.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Invadolsin (m08 family)</td>
<td>Smp_090110</td>
<td>Membrane associated</td>
<td>1.3</td>
<td>1.6</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Leucine aminopeptidase (m17 family)</td>
<td>Smp_030000</td>
<td>Membrane associated</td>
<td>0.6</td>
<td>0.3</td>
<td>0.9</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cercarial elastase (so1 family)</td>
<td>Smp_000510</td>
<td>Membrane associated</td>
<td>1.3</td>
<td>0.4</td>
<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Farnesylated-protein converting enzyme 1 (m48 family)</td>
<td>Smp_082620</td>
<td>Membrane associated</td>
<td>0.6</td>
<td>0.4</td>
<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial phosphate carrier protein</td>
<td>Smp_083720</td>
<td>Membrane associated</td>
<td>1.3</td>
<td>0.9</td>
<td>0.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial processing peptidase beta-subunit (m16 family)</td>
<td>Smp_006560.1</td>
<td>Mitochondria</td>
<td>–0.4</td>
<td>0.6</td>
<td>1.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Calpain (C02 family)</td>
<td>Smp_157500</td>
<td>Membrane associated</td>
<td>–2.2</td>
<td>–1</td>
<td>–1</td>
<td>1, 2</td>
</tr>
<tr>
<td>Structural/</td>
<td>Myosin-10 (heavy chain-b) (nnmhc-b) Flotillin-2</td>
<td>Smp_046060.1</td>
<td>Cytoskeleton</td>
<td>2</td>
<td>2.4</td>
<td>1.1</td>
<td>–</td>
</tr>
<tr>
<td>Motor</td>
<td></td>
<td>Smp_033970</td>
<td>Membrane associated</td>
<td>1.4</td>
<td>2.2</td>
<td>2.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Talin 2</td>
<td>Smp_142630</td>
<td>Membrane associated</td>
<td>2.1</td>
<td>3.4</td>
<td>3.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Tubulin beta chain</td>
<td>Smp_079960</td>
<td>Structural/Motor</td>
<td>0</td>
<td>–0.1</td>
<td>1.6</td>
<td>2</td>
</tr>
<tr>
<td>Transporter</td>
<td>Adp, atp carrier protein</td>
<td>Smp_079220</td>
<td>Membrane associated</td>
<td>2.8</td>
<td>2.9</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>Transferase</td>
<td>Conserved hypothetical protein Glutathione S-transferase 28 kda (GST 28) (GST class-mu)</td>
<td>Smp_017450</td>
<td>Cytosol/Nucleus</td>
<td>–0.4</td>
<td>–2</td>
<td>1.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smp_054160</td>
<td>Cytosol/Nucleus</td>
<td>–0.6</td>
<td>0.5</td>
<td>0.8</td>
<td>2</td>
</tr>
</tbody>
</table>

* Due to difficulties in cross matching the protein codes from the present study with the ones from Gobert et al. (2010), the comparison was made by annotations.
The three significantly upregulated peptidases (Smp_082620, Smp_083720, Smp_009650.1) might play roles in the migration of schistosomula through the dermal and sub-dermal tissues en route to the vasculature, although dedicated studies are required to confirm this hypothesis. The GO-enrichment analysis also revealed five heat shock proteins that were upregulated during the experiment (Supplementary Table S3). These highly conserved stress-induced proteins are found in many trematodes and nematodes (Mazier and Mattei, 1991). HSP-70 is immunogenic in *S. mansoni*-infected humans (Hedstrom et al., 1988) and is the target of IgM and IgA responses in other trematode infections in mice (Sotillo et al., 2008). Upregulation of heat shock proteins (HSPs) in the earliest stages of intra-mammalian schistosomula development is likely in response to the dramatic change in niche.

**Fig. 5.** Expansion and annotation of the schistosome tegumental protein turnover hypothesis. The tegument is renewed by the fusion of multilaminate vesicles trafficking from the tegumentary cell bodies (Wilson and Barnes, 1977; Skelly and Shoemaker, 2001; Jones et al., 2004). Proteome composition of the *Schistosoma mansoni* schistosomula tegument changes over time. Proteins represented were selected according to level of dysregulation, predicted function and/or previous published studies. DB, discoid bodies; MV, multilaminate vesicles; HSP, heat shock protein; TSP, tetraspanin; VAL, venom allergen protein.
environments, including substantial thermal changes between freshwater and the human body; HSPs from nematodes that transition from free-living to parasitic stages are thought to function in similar ways (Devaney, 2006).

The significantly regulated proteins were also classified according to their predicted subcellular location and 67 of the 200 proteins were predicted to be membrane-associated (Supplementary Table S3). Several authors have placed emphasis on the apical membrane proteins as vaccine candidates (Loukas et al., 2007; McManus and Loukas, 2008; Pinheiro et al., 2011; Wilson, 2012). The expression of Glucose transport protein-1 (SGPT1, Smp_012440) and sterol reductase Lamin B receptor (ERG24, Smp_124300) was upregulated in the tegument of schistosomula over time. RNA interference (RNAi) suppression of S. mansoni sgt-1 expression decreased parasite survival by impairing parasite feeding in S. mansoni schistosomula and adult worms (Krautz-Peterson et al., 2010); moreover, steroids and fatty acids have been proposed to be a good target for anti-schistosome interventions (Parker-Manuel et al., 2011) due to the inability of the parasite to de novo synthesise these molecules (Brouwers et al., 1997; Berriman et al., 2009). Strikingly, the expression of some membrane-associated proteins that have been proposed as vaccine antigens in different Schistosoma spp., e.g. calpain (Ohta et al., 2004), Na+/K+ atpase (Ohta et al., 2004; Yu et al., 2007; Da'dara et al., 2013) and succinate dehydrogenase iron-sulfur protein (Yu et al., 2007) were downregulated during schistosomula development. It is also noteworthy that these proteins were also downregulated over time in the underlying tissues (unbound sample) after tegument removal. The mechanisms of action of these subunit vaccines are not yet fully understood, but their expression profiles and apical tegument membrane location imply key roles in early migration of the parasite and maintenance of tegument integrity and/or turnover.

Two distinct Venom Allergen-Like proteins - VAL-4 (Smp_002070) and VAL-6 (Smp_124050) were downregulated on the maturing schistosomula tegument compared with newly transformed schistosomula. Other members of the VAL family (VAL-10, VAL-16) were also downregulated in the unbound sample. Downregulated expression of val genes (val-4 and val-16) has also been reported in schistosomula compared with expression levels in cercariae and germ balls (Parker-Manuel et al., 2011), and val-6 expression is upregulated in both cercariae and adult worms (Rofatto et al., 2012). These changes in val gene and corresponding protein expression imply distinct functions for these proteins in different aspects of host–parasite biology, including cercarial matrix development and penetration, snail invasion by miracidia, and intra-molluscan sporocyst development (Yoshino et al., 2014). This family of proteins is abundant in different helminth species including gastrointestinal nematodes, where they play various roles in the infective process of parasitic species (Hawdon et al., 1999; Cantacessi et al., 2009; Tribolo et al., 2015). They have been trialed as vaccines against hookworm infection in humans (Dietert et al., 2012), and should be considered as potential vaccine antigens for schistosomiasis.

Tetraspanins (TSPs) are a family of transmembrane proteins that have shown efficacy in small and large animal models of schistosomiasis (Loukas et al., 2007), and were detected in biotinylated and unbound tegument tissues. Sm-TSP-2 is essential for tegument formation (Tran et al., 2010) and is a target of protective immunity in vaccinated mice and naturally resistant human subjects (Tran et al., 2006). Sm-TSP-1 was found on the apical membrane of schistosomula, although its expression levels were not significantly regulated throughout the experiment; Sm-TSP-2 on the other hand was only found in the unbound sample, which agrees with previous findings showing localisation within internal compartments associated with surface invaginations and vesicles in the tegument (Schulte et al., 2013), but is in contrast with other studies that show Sm-TSP-2 on the surface of live 5 day old schistosomula when probed with anti-Sm-TSP-2 antiserum (Tran et al., 2010). Other protective vaccine antigens including GST and several annexins were found upregulated on the apical membrane during schistosomula growth. Annexins have been speculated to “glue” the membranocalyx to the plasma membrane (Braschi and Wilson, 2006), and GST appears to play a central role in the parasite detoxification system (Smith et al., 1986). GST and other antioxidant proteins might play an important role during schistosomula migration, and additional proteins with Redoxin and Thioredoxin-like domains were upregulated at 5 days post-transformation.

The tegument of S. mansoni is a very dynamic structure whose composition is renewed constantly. The number of membrane-associated or cytoplasmic/nuclear proteins uniquely upregulated at the different time points supports the notion of a continuous tegument turnover (Skelly and Shoemaker, 1996, 2001; Skelly and Alan Wilson, 2006), so the identification of key proteins from the apical membrane of schistosomula at the different stages of development is crucial for understanding the biology and potential Achilles heel of the parasite (Fig. 5). Herein we have combined membrane-labelling techniques with state-of-the-art quantitative proteomics to identify tegumental proteins that could be important for the development of new vaccine and drug targets against schistosomiasis.

**Acknowledgments**

The following reagent was kindly provided by the National Institute of Allergy and Infectious Diseases (NIAID) Schistosomiasis Resource Center for distribution through BEI Resources, NIAID, National Institutes of Health, USA: Schistosoma mansoni, Strain NMRI Exposed Biomphalaria glabrata, NR-21962. This work was supported by a program grant from the National Health and Medical Research Council of Australia (NHMRC). AL is supported by a NHMRC Principal Research Fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jipara.2015.03.004.

**References**


