Proteins Exposed at the Adult Schistosome Surface Revealed by Biotinylation

Simon Braschi and R. Alan Wilson

The human blood-dwelling parasite *Schistosoma mansoni* can survive in the hostile host environment for decades and must therefore display effective strategies to evade the host immune responses. The surface of the adult worm is covered by a living syncytial layer, the tegument, bounded by a complex multilaminate surface. This comprises a normal plasma membrane overlain by a secreted bilayer, the membranocalyx. Recent proteomic studies have identified constituents of the tegument, but their relative locations remain to be established. We labeled the most exposed surface proteins using two impermeant biotinylation reagents that differed only in length. We anticipated that the two reagents would display distinct powers of penetration, thereby producing a differential labeling pattern. The labeled proteins were recovered by streptavidin affinity and identified by tandem mass spectrometry. A total of 28 proteins was identified, 13 labeled by a long form reagent and the same 13 plus a further 15 labeled by a short form reagent. The parasite proteins included membrane enzymes, transporters, and structural proteins. The short form reagent additionally labeled some cytosolic and cytoskeletal proteins, the latter being constituents of the intracellular spines. Only a single secreted protein was labeled, implying a location between the plasma membrane and the membranocalyx or as part of the latter. Four host proteins, three immunoglobulin heavy chains and C3c/C3dg, a fragment of complement C3, were labeled by both reagents indicating their exposed situation. The presence of the degraded complement C3 implicates inhibition of the classical pathway as a major element of the immune evasion strategy, whereas the recovery of only one truly secreted protein points to a major element of the immune evasion strategy. The mature worm is covered by a syncytial cytoplasmic layer, the tegument, attached to underlying cell bodies by narrow cytoplasmic connections. The nuclei, ribosomes, endoplasmic reticulum, and Golgi apparatus are located in these cell bodies, and their vesicular products, the discoid bodies and multilaminate vesicles, traffic to the tegument syncytium via the connections. Early ultrastructural studies showed that the apical surface of the tegument has a complex multilaminate appearance that has been interpreted as a plasma membrane overlain by a trilaminate secretion, which was termed a membranocalyx by analogy with the glyocalyx of eukaryotic cells. The membranocalyx is formed when the bounding membrane of the multilaminate vesicle fuses with the apical plasma membrane of the tegument to release its sheetlike contents. These unfold and flow laterally across the plasma membrane. The membranocalyx has some unusual properties including the ability to sequester glycolipids from host erythrocytes. Although initial *in vitro* observations suggested a rapid turnover, *in vivo* studies using erythrocyte antigens as a marker indicated a half-life of approximately 5 days. Given a slow turnover, it is plausible that the membranocalyx provides a physical barrier protecting the underlying plasma membrane, which possesses normal cellular functions, from immune attack.

Methods were devised more than 30 years ago to detach the tegument surface complex for compositional analysis. However, despite separation of the protein constituents by electrophoresis, little progress was made in obtaining their identities. The advent of proteomics has provided the tools to link proteins with their encoding cDNA. Simultaneously the generation of a large *S. mansoni* expressed sequence tag database and release of the draft genome sequence (www.SchistoDB.org) allow us to assign putative functions to many of the transcripts/genes. Two recent studies have reported on tegument protein composition. In the first, an inventory was compiled of proteins present in the
whole tegument syncytium relative to the worm body (13), but the approach provided no information about the position of the constituents within the complex structure. In the second, we reported on the composition of a surface membrane preparation highly enriched by density gradient centrifugation and then subjected to a differential extraction procedure. We were able to identify many components and classify them into cytosolic, cytoskeletal, membrane, and secreted categories (14). However, it was not possible to determine the location of individual constituents within the surface complex and hence to say which are potentially accessible to the external environment of the parasite (including the host immune system).

We report here the use of impermanent biotinylation reagents to label live adult worms \textit{in vitro}. We then recovered the labeled proteins by streptavidin affinity for identification by tandem MS from which we inferred their relative accessibility. The results were interpreted in the context of immune evasion.

**EXPERIMENTAL PROCEDURES**

*Biological Material—* Details of schistosome isolate, life cycle maintenance, and recovery of 7-week-old adult worms were as described previously (14).

*Surface Labeling of Live Worms—* The scheme from parasite labeling through recovery of biotinylated proteins for MS is set out as a flow chart (Fig. 1). We first established the reaction conditions using BSA as a model protein before applying them to the parasite. Worms were washed five times in Hanks’ balanced salt solution (Invitrogen), and any that showed damage were removed under 10× magnification. Two sulfo-NHS-biotin reagents, differing in the length of their spacer arms (Table I), were used to label worms, namely sulfo-N-succinimidyl-6-(biotinamido) hexanoate (long form reagent; EZ-Link™ sulfo-NHS-LC-biotin) and sulfo-N-succinimidobiotin (short form reagent; EZ-Link sulfo-NHS-biotin; Pierce). For each experiment the parasite load from 40 mice, ~2000 worm pairs (no attempt was made to separate male and female worms), was incubated in 15 ml of Hanks’ balanced salt solution containing 890 μM labeling reagent for 30 min at 4 °C with gentle agitation (15). The labeling solution was removed, and any unbound reagent was quenched using RPMI 1640 medium (Invitrogen) containing free amino acids. Worms were then washed three times in RPMI 1640 medium, plunged into liquid nitrogen, and stored at −80 °C with protease inhibitors (protease inhibitor mixture, Sigma). Uniformity of labeling and integrity of the surface membrane was examined by exposing a sample of live, labeled worms in RPMI 1640 medium to streptavidin-conjugated FITC (Sigma) for 30 min at room temperature. After incubation the worms were washed five times in Hanks’ balanced salt solution containing 890 μM labeling reagent for 30 min at 4 °C with gentle agitation (15). The labeling solution was removed, and any unbound reagent was quenched using RPMI 1640 medium (Invitrogen), and the resulting supernatants were pooled to give a sample volume of 600 μl. The solubilizing regimes were: Extract 1, 40 mm Tris, pH 7.4, vortexed for 1 min and allowed to stand for 20 min on ice; Extract 2, 5 M urea (BDH, VWR International, Dorset, UK), 2 μl thiourea (BDH, VWR International) in 40 μl Tris, pH 7.4, 25 °C; Extract 3, 5 M urea, 2 μl thiourea, 4% CHAPS (Sigma), 2% N-decyl-N,N-dimethyl-3-ammonio-1-propane sulfate (SB 3-10; Sigma) in 40 μl Tris, pH 7.4, 25 °C; Extract 4, 0.1% SDS, 1% Triton X-100 in 40 μl Tris, pH 7.4, 25 °C; Extract 5, 0.2% SDS, 1% Triton X-100 in 40 μl Tris, pH 7.4, 25 °C. Protease inhibitor mixture (Sigma) was added to each pooled supernatant prior to isolation of the extracted labeled molecules.

*Processing the Biotinylated Proteins—* The biotinylated material in each of the five extracts was isolated by incubation with 60 μl of a prewashed slurry of streptavidin immobilized onto agarose beads (ImmunoPure® immobilized streptavidin, Pierce) for 2 h at room temperature with head-over-head mixing. The streptavidin-bound complex was pelleted by centrifugation for 1 min at 3000 × g, and the supernatant (unbound material) was recovered. The beads were then washed for 5 min in 500 μl of 0.1% SDS, 1% Triton X-100 in PBS by head-over-head mixing at room temperature to remove any further unbound material and pelleted as before. This wash procedure was repeated four times. The bound material was recovered from the washed streptavidin beads by addition of 100 μl of 2% SDS, heating to 90 °C for 10 min, vortexing at maximum speed for 2 min, and pelleting of denuded beads by centrifugation as above. The supernatant containing the biotinylated macromolecules was recovered.

---

1 The abbreviations used are: NHS, N-hydroxysuccinimide; 1-D, one-dimensional; CRP, complement-regulatory protein; SB 3-10, N-decyl-N,N-dimethyl-3-ammonio-1-propane sulfate; strep-HRP, streptavidin conjugated to horseradish peroxidase; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
the procedure was repeated, and supernatants were combined to yield 200 μl for each of the five extracts.

**SDS-PAGE and Western Blotting**—Proteins from each of the five extracts were precipitated with 10% TCA, 80% acetone and solubilized in SDS sample buffer plus reducing agent (Invitrogen). They were separated by 1-D SDS-PAGE using 7-cm NuPAGE 4–12% bis-Tris gels (Invitrogen) followed by SYPRO Ruby (Bio-Rad) staining and image capture using Molecular Imager FX (Bio-Rad). The biotin content of the bound and unbound material after the incubation with streptavidin-agarose beads was examined by Western blotting. An aliquot of the samples was separated by 1-D SDS-PAGE and transferred onto a PVDF membrane using an XCell II blot module (Invitrogen). The membrane was blocked in 4% casein, probed with streptavidin conjugated to horseradish peroxidase (strep-HRP; Amersham Biosciences), and TMB peroxidase substrate (Kirkegaard & Perry Laboratories) was added to visualize labeled bands.

**LC-MS/MS and Database Searching**—The SDS-PAGE gels of the bound material were restained with Coomassie BioSafe (Bio-Rad), and each protein band was excised, digested with trypsin, and subjected to LC-MS/MS as described previously (14). Sections of gel lanes where no protein staining was visualized were also analyzed for potential protein content. Briefly peptides were separated on a reversed-phase Monolith column (LC Packings, Dionex, Sunnyvale, CA) using an Ultimate nanoflow HPLC system (LC Packings) and spotted directly onto a MALDI target plate using a Probot (LC Packings). A 4700 Proteomics Analyzer with TOF-TOF optics (Applied Biosystems, Framingham, MA) was used to obtain fragmentation spectra, which were processed by GPS Explorer software version 2.0 (Applied Biosystems) to provide peak lists. These were submitted to MASCOT version 1.9 (16) (Matrix Science, London, UK) and searched against the National Center for Biotechnology Information non-redundant (NCBI) database as well as the S. mansoni genome and transcriptome databases (www.SchistoDB.org and cancer.ibi.ic.unicamp.br/schistodb/, respectively). Mass tolerance was set to ±0.3 Da for both the precursor and product ion spectra, and search parameters allowed for a maximum of three missed tryptic cleavage sites, the carbamidomethylation of cysteine, the possible oxidation of methionine, and the possible modification of lysine and N-terminal residues by the biotinylation reagents. A protein was considered to be identified when two or more peptides, each with a GPS-generated confidence interval of above 99%, were positively matched in the database. Putative functions were assigned using BLAST (17) against annotated proteins in the GenBank database; scores with an expect value <1e−16 were regarded as a significant match. Potential signal sequences and glycosylation sites were predicted using SignalP version 3.0 and NetNGlyc/NetOGlyc version 1.0, respectively (www.cbs.dtu.dk/services), and transmembrane domains were predicted using HMMTOP (www.enzim.hu/hmmtop/).

**RESULTS**

*The Biotin Reagents Uniformly Label the Worm Surface*—When the BSA was subjected to the complete procedure from labeling to tandem MS and compared with unmodified BSA, no dramatic alterations in peptide mass fingerprints were observed. From the labeled BSA, we identified 14 peptides, six of which (43%) contained a biotinylated lysine residue (peptide details can be found in the supplemental table). The extent of labeling of live adult worms was assessed using FITC-conjugated streptavidin. A sharply demarcated line of surface staining was observed on whole mounts (Fig. 2).

![Fig. 2. Optical section of an adult male schistosome labeled with impermeant biotinylation reagent and probed with streptavidin conjugated to FITC.](image)
Optical sectioning through the worm body confirmed that the pattern of staining was confined to the surface with no penetration into internal tissues. No biotin reagent or streptavidin had entered the gut during incubation.

**Streptavidin Beads Recover Biotinylated Proteins from the Differential Extracts**—The labeled tegument surface, detached from worm bodies, was extracted to recover proteins according to their differential solubility. Labeled proteins were then retrieved from each of the five extracts using streptavidin-agarose beads, and the bound material was separated by SDS-PAGE. The SYPRO Ruby-stained images revealed a sharp pattern of bands with strong similarities between the patterns labeled by the two biotin reagents (Fig. 3a). Overall more proteins were retrieved after labeling with the short form rather than the long form reagent. In both instances, the Tris and 0.2% SDS, 1% Triton X-100 extracts (lanes i and v, respectively) appeared devoid of protein with the exception of a single band of ~15 kDa, which was also present in other extracts. Conversely the other three extractions (lanes ii–iv) recovered more complex mixtures of labeled material. Thus, after urea/thiourea treatment (lane ii), nine bands were visible in the long form-labeled extract, and 15 were visible in the short form-labeled extract. After urea/thiourea/CHAPS/SB 3-10 treatment (lane iii) only three bands were present in both long and short form extracts; the ~70-kDa species were absent from lane ii. After 0.1% SDS, 1% Triton X-100 treatment (lane iv), five bands were present in the long form and nine were present in the short form extracts; apart from the ~15- and ~70-kDa species, the remainder were unique.

To verify that the proteins recovered from the beads were biotinylated, an aliquot was separated by SDS-PAGE, blotted onto a PVDF membrane, and then probed with strep-HRP.
Each protein band visualized by SYPRO Ruby could be detected as a matching band on the Western blot (Fig. 3b) with the exception of the aforementioned ~15-kDa protein, which was absent from several lanes. Conversely additional bands of labeled material were detected only on the Western blots. In particular, after short form labeling, a whole array of new bands appeared, especially noticeable in the high molecular weight region of the Tris extract (lane i), whereas the blot of long form-labeled material more closely resembled its corresponding SYPRO Ruby-stained gel (Fig 3b). These data indicate that the sensitivity of biotinylated protein detection by blotting and enzymatic amplification of the signal is much greater than staining with SYPRO Ruby.

The Unbound Material Is Almost Exclusively Non-biotinylated—The supernatant recovered after each of the five extracts that had been incubated with streptavidin beads (i.e. the unbound material) was profiled for both protein and biotin content by SDS-PAGE and Western blotting, respectively. The SYPRO Ruby-stained 1-D gel revealed large numbers of protein bands, reducing in complexity from extract 1 to extract 5 (Fig. 4a, lanes i–v). The blotted material showed traces of biotinylation in experiments with both reagents (Fig. 4b) that was more evident when the short form was used. In both cases the labeled material was almost exclusively confined to the urea/thiourea extract (lane ii) with only the faintest traces visible in 0.1% SDS, 1% Triton X-100 extract (lane iv). Notably the few biotinylated protein bands in the unbound material were also detected in the gels and blots of the bound material (Fig. 3).

Both Parasite Surface and Host Proteins Are Biotinylated—Bands detected on 1-D separations of fractions recovered from the agarose beads were excised and trypsinized, and the

![Fig. 4. Unbound material. a, SYPRO Ruby-stained 1-D SDS-PAGE separation of proteins that did not co-precipitate with the streptavidin beads. b, the corresponding Western blots probed with strep-HRP. Lanes i–v represent extracts 1–5, respectively.](image-url)
resulting peptide mixture was fractionated by reversed-phase chromatography before identification by tandem MS. In total, 117 peptide fragmentation spectra were matched to genome sequences/expressed sequence tags, but only one revealed the presence of a modified lysine residue (peptide details can be found in the supplemental table). A total of 28 identities was obtained plus streptavidin in all five extracts (Table II). These comprised 13 proteins in samples recovered after use of the long form reagent and the same 13 plus a further 15 after use of the short form reagent. It should be noted that almost invariably a single gel band contained two or more proteins, making it impossible to draw inferences about the relative amounts of individual constituents in the preparation. Furthermore some proteins were identified in several gel bands of different molecular weights.

Four host proteins, three distinct immunoglobulin heavy chains and complement component C3, were consistently recovered from SYPRO Ruby-stained gels after surface biotinylation. The heavy chains for IgG1 and IgG3 were present in a single band of an apparent molecular mass of 55 kDa. The four peptide hits for IgG3 originated in constant domains CH1, CH2, and CH3 (two peptides), whereas six peptides for IgG1 originated in the variable domain framework regions VH1 and VH3 and the constant domains CH2 and CH3 (two in each). The heavy chain for IgM was present in a gel band of apparent molecular mass 75 kDa with peptide hits originating in the constant domains CH2 and CH4. Complement component C3 was identified in a gel band of apparent molecular mass 69 kDa, and the two peptide hits both originated in the α chain C3dg region; the apparent molecular mass is consistent with the polypeptide recovered being the partial degradation product of the C3 molecule comprising C3c/C3dg.

### Table II

| Protein name                        | Accession no. | Band number(s) |
|-------------------------------------|---------------|----------------|
| **Host proteins**                   |               |                |
| IgM heavy chain                      | gi|70048         | 4              |
| IgG1 heavy chain                     | gi|440121         | 7              |
| IgG3 heavy chain                     | gi|1304160        | 7              |
| Complement C3 (C3c/C3dg fragment)   | gi|28175786       | 6              |
| Secreted protein                     |               | 11             |
| Sm29                                | Sm09193       | 8              |
| **Membrane structural proteins**     |               |                |
| Tetraspanin B (TE736)               | Sm04463       | —              |
| Tetraspanin D (CD63-like tetraspanin)|Sm12366       | 16, 17         |
| Annexin                             | Sm03987       | 4, 8           |
| Dysferlin                           | Sm10433       | 2, 3, 5        |
| **Membrane enzymes**                |               |                |
| Calpain                             | Sm08542       | —              |
| Alkaline phosphatase                | Sm00962       | 11, 14         |
| ATP-diphosphohydrolase              | Sm12745       | 15             |
| Phosphodiesterase                   | Sm03458       | 12, 15         |
| **Membrane (other)**                |               |                |
| 200-kDa Surface protein             | Sm03865       | —              |
| **Transporters**                    |               |                |
| Voltage-dependent anion channel     | Sm00707       | —              |
| Sodium/potassium transporter (SNaK1)|Sm08331       | —              |
| **Cytoskeletal proteins**            |               |                |
| Fimbrin                             | Sm13240       | —              |
| Actin                               | Sm01276       | —              |
| Severin                             | Sm04123       | —              |
| **Cytosolic proteins**              |               |                |
| Glycerol-3-phosphate dehydrogenase 2|Sm08702       | —              |
| Heat shock protein 70               | Sm09042       | —              |
| Carbonic anhydrase 4                | Sm04975       | —              |
| **No homology**                     |               |                |
| Unknown                             | Sm11517       | —              |
| Unknown                             | Sm01030       | —              |
| Unknown                             | Sm13096       | —              |
| Unknown                             | Sm00749       | —              |
| Unknown                             | Sm11921       | 9              |
| Unknown                             | Sm07392       | 15             |
| **Protein from agarose beads**      |               |                |
| Streptavidin                        | gi|1364156       | 1, 10, 13, 18, 19 |

**a** The putative identity gained by BLAST analysis against the GenBank™ database.
**b** Accession numbers beginning with Sm correspond to the S. mansoni genome database (www.SchistoDB.org), and those beginning with gi correspond to the NCBInr database.
**c** Band number equating with protein bands in Fig. 3a. Where no number is given (—), the identity was not found in that sample.
The 24 schistosome proteins could be grouped according to their putative functions (Table II). The long form reagent labeled one protein, Sm29, apparently secreted on the basis of SignalP analysis of the amino acid sequence. Three membrane enzymes, alkaline phosphatase, phosphodiesterase, and diphosphohydrolase, capable of hydrolyzing organic phosphates were recovered as well as three structural membrane proteins, annexin, dysferlin, and the tetraspanin D (CD63-like tetraspanin). The short form reagent retrieved a 200-kDa membrane-bound protein, termed “Surface protein” in the original description, plus one further tetraspanin (B; tetraspanin TE736) involved in membrane structure. A membrane protease, calpain, was also labeled, as were two transporters, the sodium/potassium transporter (SNaK1) and a voltage-dependent anion channel. The short form reagent also appeared to have greater penetrating power, labeling three cytoskeletal proteins, actin, fimbrin, and severin, and three cytosolic proteins, carbonic anhydrase 4, heat shock protein 70, and glycerol-3-phosphate dehydrogenase 2. A further four schistosome proteins of unknown function were also labeled by the short form reagent (Table II).

DISCUSSION

To determine the external accessibility of tegument surface proteins, we incubated live adult schistosomes with impermeant biotinylation reagents. The two reagents used label proteins with exposed amine groups either at lysine residues or at the N terminus. Because of their sulfo groups, they are water-soluble and therefore excluded by the lipid bilayer, preferentially labeling secreted proteins and membrane proteins with extracellular loops or domains. It was necessary to keep the duration of the reaction short (30 min) because labeling had to take place in an amino acid-free balanced salt solution rather than culture medium, increasing the possibility of worm degradation. We first optimized the methods for labeling, recovery of proteins, and their identification by tandem MS using purified BSA. The retrieval of biotinylated molecules from agarose-immobilized streptavidin is challenging because of the exceptionally high binding constant of the complex \( K_d \approx 10^{-15} \text{M} \) (18). We found that boiling in 2% SDS was the best method, recovering >90% of the bound protein (some streptavidin was also detached from the agarose). Although tandem MS revealed that 43% of peptides derived from labeled BSA contained biotinylated lysines, there were also numerous unmodified lysines. This suggests either an incomplete reaction or the inaccessibility of some lysines to the reagent. (Similar results were obtained with a second model protein, bovine alkaline phosphatase.\(^2\)) The biotinylation of a lysine residue prevents cleavage by trypsin at that site, altering the composition of the peptide mixture generated for tandem MS, which could result in peptides too long (>4000 Da) for satisfactory fragmentation. This did not create a problem for the identification of BSA (or tegumental proteins) because of the partial biotinylation; furthermore we recovered the intact labeled proteins rather than individual biotinylated peptides.

We utilized two forms of sulfo-NHS-biotin for parasite labeling in the expectation that they would have different physical properties and labeling capabilities, and this proved to be the case. The fact that the only cytosolic and cytoskeletal proteins identified were labeled uniquely by the short form reagent reveals its greater penetration of the tegument surface. The different patterns of protein labeling produced with the long and short form reagents enable us to make inferences about the putative location of individual proteins. Because the long form reagent did not react with any cytoplasmic proteins, we assume that all 13 proteins detected lie within or external to the plasma membrane (Fig. 5). However, as we have no means of separating the plasma membrane from the overlying membranocalyx, we cannot deduce with certainty whether any of the 13 proteins are located in the latter structure. Nine parasite proteins (and four host proteins, discussed below) were identified and placed in secreted, membrane structure, membrane enzyme, and no homology categories. Of the three enzymes, two are membrane-spanning, whereas the third, alkaline phosphatase, is glycosylphosphatidylinositol-anchored. All three hydrolyze organic phosphate substrates, possess large extracellular domains, and, by analogy with other organisms, are likely to be located on the external leaflet of the plasma membrane, features entirely consistent with their labeling. Such a location implies the existence of water-filled “pores” in the membranocalyx to allow the ingress of substrates (and the labeling reagent). However, we surmise that such pores would not be large enough to give antibodies access to the protein domains situated immediately external to the plasma membrane. Similarly it seems unlikely that secreted proteins lying between the two layers would escape via such pores. As plasma membrane enzymes, the three proteins would traffic to the surface anchored in the bounding membrane of the multilaminate vesicle, not its contents.

The long form reagent labeled the three proteins involved in membrane structure. However, these included only one of the three tetraspanins (tetraspanin B) identified in our compositional study (14). It is a member of a large family of proteins encoded by the \textit{S. mansoni} genome and likely to form part of the intramembranous web that provides a scaffold for other components (19). The second structural protein, annexin, normally binds to phospholipids at the cytoplasmic face of membranes, but its labeling by the long form reagent implies an extracellular location. Such an occurrence has been reported in other organisms (20), although the annexin gene does not encode a signal peptide or transmembrane domains. Given annexin’s lipid binding properties, it is tempting to speculate that it provides the molecular “glue” that sticks the membranocalyx to the underlying plasma membrane (Fig. 5). Al-

\(^2\) W. Borges, personal communication.
ternatively its capacity to assemble into molecular sheets (21) could provide a scaffold for the relatively fluid membranocalyx. The third membrane structural protein, dysferlin, facilitates vesicle fusion and repair of the plasma membrane in other organisms (22). Only one true secreted protein, Sm29, was labeled by the long form reagent and would thus be exported in the multilaminate vesicle contents, making it a possible constituent of the membranocalyx or the space between the two lipid bilayers. Given our argument about the size of the pores in the membranocalyx, Sm29 would be unlikely to pass through this layer. Indeed there seems to be little evidence for rapid secretion of proteins from the tegument surface, although the slow turnover of the membranocalyx (6) implies that if it contains significant protein constituents these must enter the bloodstream environment. Both the proteins of unknown function, labeled by the long form reagent, bear membrane-spanning domains and so are most likely to reside in the plasma membrane, not the membranocalyx, because of its origin as the multilaminate vesicle contents (4).

Three of the six intracellular proteins labeled by the short form reagent, actin, fimbrin, and severin, have a cytoskeletal role. Paracrystalline actin is a major constituent of tegumental spines (23), whereas severin and fimbrin are likely determinants of spine structure (Fig. 5), the former cross-linking actin (24) and the latter capping growing filaments (25). The tight apposition of the plasma membrane to the underlying spines (26) would require minimal penetration of reagent to label the three proteins. By inference, the other three cytosolic proteins, carbonic anhydrase, glycerol-3-phosphate dehydrogenase, and HSP70 are likely to be located in the cytoplasm immediately beneath the plasma membrane. The latter is abundant in the cytosol (27), but other proteins such as enolase, aldolase, or glutathione S-transferase, which are equally abundant, were not labeled, and so were presumably not so accessible. In this context, the short form labeling of the calcium-activated protease calpain, which functions in cytoskeleton remodeling, is in accord with its location in other organisms on the inner leaflet of the plasma membrane (28). The labeling of tetraspanin B only by the short form reagent implies a more cryptic location in the intramembranous tetraspanin web than for tetraspanin D. It is notable that only two transporters, SNaK1 and voltage-dependent anion channel, were biotinylated; presumably this reflects the relative accessibility or lysine content of their extracellular loops compared with other transporters identified previously in the tegument (14). The failure of the long form reagent to label the 200-kDa Surface protein is enigmatic, given that it possesses a large extracellular domain. However, its inaccessibility to antibodies prior to praziquantel treatment of worms does indicate a relatively protected location (29). The four proteins with no known homology, labeled by the short form reagent, were identified from partial sequences in the database. Two of them possess predicted transmembrane domains, suggesting a plasma membrane location, but nothing can be inferred about the remaining two. The detection of more bands on Western blots by strep-HRP than in the corresponding gels by SYPRO Ruby staining raises the issue of sensitivity, particularly for the short form reagent. It appears that the amplification step in Western blotting, whether using enzyme-labeled streptavidin or antibody, significantly enhances sensitivity, and a proportion of these proteins are detected at concentrations below the threshold for identification by tandem MS. This may explain why we failed to detect the glucose transporter SGTP4 when others using a similar protocol to biotinylate the worm surface and recover tagged proteins were

Fig. 5. Diagrammatic representation of the tegument and the proposed locations of the proteins identified. Those proteins shown in **blue** are labeled by both the long form and short form biotinylation reagents. The proteins in **red** are labeled only by the short form reagent and are therefore likely to have a more intrinsic location. The schistosome proteins with no homology are not represented as their full-length sequence is unknown.
able to locate it on a blot using specific antibody (30). We also failed to detect SmRK-1, a member of the transforming growth factor-β family of receptor kinases, reported previously as labeled by sulfo-NHS-LC biotin (15, 31), and SCIP-1 (paramyosin), proposed both as an Fc receptor (32) and as an inhibitor of complement C9 polymerization (33).

We recovered four host proteins with immunological functions after labeling with both long and short form reagents implying an extrinsic location (Fig. 5). These were the immunoglobulin heavy chains of IgM, IgG1, and IgG3 plus complement component C3. All were present after thorough washing of worms; from this we infer that they were not simple contaminants, particularly as we did not recover albumin, a more abundant plasma protein. We conclude that all three Ig heavy chains were intact from their respective molecular weights on gels and the distribution of peptide hits throughout the molecules. Antibodies have been detected previously at the tegument surface by immunocytochemical methods (34–36). The principle question is whether they bind via their Fc or Fab regions. Fc receptors have been reported at the tegument surface (37) that are capable of binding heterospecific antibody, but we did not detect such host proteins or potential schistosome homologues; the latter do not appear to be encoded in the S. mansoni genome (www.SchistoDB.org). Thus, if schistosomes possess endogenous Fc receptors they will only be identified by functional studies. Indeed the muscle protein paramyosin has been proposed as an Fc receptor in Schistosoma japonicum (32) and reported at the tegument surface by immunofluorescent staining (33) but was not detected in this or our previous study on the S. mansoni surface (14). The presence of IgM at the surface, for which no Fc receptor has been described in schistosomes, may indicate binding to specific antigens via the Fab region. Furthermore the fixation of complement C3, which requires a free Fc region for membrane attack and therefore merit investigation as vaccine candidates. (So far only calpain has received serious attention (42)). Our data suggest the majority of labeled parasite proteins appear to be associated with the plasma membrane rather than the membranocalyx. It must be emphasized that the latter originates as a secretion within the lumen of the multilaminate vesicles where the bilayer structure assembles during vesicle maturation in the tegument cell body (26). Consequently proteins translated from genes encoding a leader sequence, but not transmembrane domains, are the most likely candidates for inclusion. The detection of only one protein in this category, Sm29, suggests that the membranocalyx contains relatively few proteins. It would thus function primarily as an inert barrier between the host immune system and the vulnerable plasma membrane with its assortment of enzymes, transporters, and structural proteins. Our task is to devise new strategies to isolate the membranocalyx and analyze its composition.

Acknowledgment—We are grateful to Ann Bamford for maintenance of the S. mansoni life cycle.

* This work was supported with funds from the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases and The Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biology, University of York, York YO10 5DD, UK. Tel.: 44-1904-328592; Fax.: 44-1904-328599; E-mail: sb212@york.ac.uk.

REFERENCES
1. World Health Organization (2002) TDR Strategic Direction for Research: Schistosomiasis, World Health Organization, Geneva.
2. Harris, A. R., Russell, R. J., and Charters, A. D. (1984) A review of schistosomiasis in immigrants in Western Australia, demonstrating the unusual longevity of Schistosoma mansoni, Trans. R. Soc. Trop. Med. Hyg. 78, 385–388
3. Hockley, D. J., and McLaren, D. J. (1973) Schistosoma mansoni; changes in the outer membrane of the tegument during development from cer-

---

3 S. Braschi and R. A. Wilson, manuscript in preparation.
Biotinylation of the Schistosome Surface

caria to adult worm. Int. J. Parasitol. 3, 13–25

4. Wilson, R. A., and Barnes, P. E. (1977) The formation and turnover of the membranoaxon in the tegument of Schistosoma mansoni. Parasitology
74, 61–71

5. Goldring, D. L., Clegg, J. A., Smithers, S. R., and Terry, R. J. (1976) Acquisition of human blood group antigens by Schistosoma mansoni. Clin. Exp. Immunol. 26, 161–167

6. Saunders, N., Wilson, R. A., and Coullson, P. S. (1987) The outer bilayer of the adult schistosome tegument surface has a low turnover rate in vitro and in vivo. Mol. Biochem. Parasitol. 25, 123–131

7. Roberts, S. M., MacGregor, A. N., Vojvodic, M., Wells, E., Crabtree, J. E., and Wilson, R. A. (1983) Tegument surface membranes of adult Schistosoma mansoni: development of a method for their isolation. Mol. Biochem. Parasitol. 9, 105–127

8. Oaks, J. A., Cain, G. D., Mower, D. A., and Raj, R. K. (1981) Disruption and removal of the tegument from Schistosoma mansoni with triton X-100. J. Parasitol. 67, 761–775

9. Bennett, J. L., and Seed, J. L. (1977) Characterization and isolation of concanavalin A binding sites from the epidermis of S. mansoni. J. Parasitol. 63, 250–258

10. Roberts, S. M., Boot, C., and Wilson, R. A. (1988) Antibody responses of rodents to a tegument membrane preparation from adult Schistosoma mansoni. Parasitology 97, 425–435

11. Dalton, J. P., Strand, M., Mangold, B. L., and Dean, D. A. (1986) Identification of Schistosoma mansoni glycoproteins recognized by protective antibodies from mice immunized with irradiated cercariae. J. Immunol. 136, 4680–4694

12. Verjovski-Almeida, S., DeMarco, R., Martins, E. A., Guimarães, P. E., Ôjopi, E. P., Paquola, A. C., Piazza, J. P., Nishiya, M. Y., Jr., Kitajima, J. P., Adamson, R. E., Ashton, P. D., Bonaldo, M. F., Coullson, P. S., Dillon, G. P., Farias, L. P., Gregorio, S. P., Ho, P. L., Leite, R. A., Malquias, L. C., Marques, R. C., Miyasato, P. A., Nascimento, A. L., Ohlweiler, F. P., Reis, E. M., Ribeiro, M. A., Sa, R. G., Stukart, G. C., Soares, M. B., Gargioni, C., Kawano, T., Rodrigues, V., Madeira, A. M., Wilson, R. A., Menck, C. F., Setubal, J. C., Leite, L. C., and Dias-Neto, E. (2003) Transcriptome analysis of the acelolamate human parasite Schistosoma mansoni. Nat. Genet. 35, 148–157

13. van Balkom, B. W., van Gestel, R. A., Brouwers, J. F., Krijgsveld, J., Tielens, A. G., Heck, A. J., and van Hellemond, J. J. (2005) Mass spectrometric identification of the Schistosoma mansoni tegumental sub-proteome. J. Proteome Res. 4, 958–966

14. Braschi, S., Curwen, R., Ashton, P., Verjovski-Almeida, S., and Wilson, R. A. (2006) The tegument surface membranes of the human blood parasite Schistosoma mansoni: a proteomic analysis after differential extraction. Proteomics, in press

15. Davies, S. J., and Pearce, E. J. (1999) Atypical post-translational modification and targeting of a Schistosoma mansoni surface receptor, a member of the transforming growth factor β receptor family of cell surface receptors. Mol. Biochem. Parasitol. 104, 299–310

16. Loukas, A., Jones, M. K., King, L. T., Brindley, P. J., and McManus, D. P. (2000) Receptor for Fc on the surfaces of schistosomes. Infect. Immun. 69, 3646–3651

17. Deng, J., Gold, D., LoVerde, P. T., and Fishelson, Z. (2003) Inhibition of the complement membrane attack complex by Schistosoma mansoni paramyosin. Infect. Immun. 71, 6402–6410

18. Kemp, W. M., Damjan, R. T., and Greene, N. D. (1976) Immunocytochemical localization of IgG on adult Schistosoma mansoni tegumental surfaces. J. Parasitol. 62, 830–832

19. Kemp, W. M., Merritt, S. C., and Rosier, J. G. (1978) Schistosoma mansoni: identification of immunoglobulins associated with the tegument of adult parasites from mice. Exp. Parasitol. 45, 81–87

20. Kemp, W. M., Brown, P. R., Merritt, S. C., and Miller, R. E. (1980) Target antigen modulation by adult male Schistosoma mansoni. J. Immunol. 124, 806–811

21. Kemp, W. M., Merritt, S. C., Bogucki, M. S., Rosier, J. G., and Seed, J. R. (1977) Evidence for adsorption of heterospecific host immunoglobulin on the tegument of Schistosoma mansoni. J. Immunol. 119, 1849–1854

22. Pearce, E. J., Hall, B. F., and Sher, A. (1990) Host-specific evasion of the alternative complement pathway by schistosomes correlates with the presence of a phospholipase C-sensitive surface molecule resembling human decay accelerating factor. J. Immunol. 144, 2751–2756

23. Li, B., Salle, C., Dehoff, M., Foley, S., Molina, H., and Holers, V. M. (1993) Mouse Crry/p65. Characterization of monoclonal antibodies and the tissue distribution of a functional homologue of human MCP and DAF. J. Immunol. 151, 4295–4305

24. Kemp, W. M., Damjan, R. T., Greene, N. D., and Lushbaugh, W. B. (1976) Immunocytochemical localization of mouse α2-macroglobulin-like anti-
genetic determinants on Schistosoma mansoni adults. J. Parasitol. 62, 413–419

25. Sher, A., Hall, B. F., and Vadas, M. A. (1978) Acquisition of murine major histocompatibility complex gene products by schistosomula of Schisto-
soma mansoni. J. Exp. Med. 148, 46–57

26. Hota-Mitchell, S., Siddiqui, A. A., Dekaban, G. A., Smith, J., Tognon, C., and Podesta, R. B. (1997) Protection against Schistosoma mansoni infection with a recombinant baculovirus-expressed subunit of calpain. Vaccine 15, 1631–1640