The *Schistosoma mansoni* host-interactive tegument forms from vesicle eruptions of a cyton network

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SUMMARY

During trans-dermal invasion of the vertebrate host, larval schistosomes (cercariae) transform into schistosomula and become enveloped by a double lipid bilayered, tegumental membrane. The glucose transporter protein SGTP4 is found exclusively in these host-interactive tegumental membranes and in membranous vesicles proposed to be their precursor. In this study, we monitored the appearance and migration of this tegumental marker protein during larval transformation to test the current model of tegumental membrane biosynthesis in parasitic blood flukes. Only minutes after transformation was initiated, SGTP4 began accumulating in a previously unrecognized, bilaterally symmetrical, 'cyton network' beneath the peripheral muscle. Approximately 30 min after the initiation of transformation the marker protein was seen in tubules connecting the network to the surface and erupting onto the surface in discrete patches. After 1 h the patches were regularly arrayed over the schistosomula body and began to cover the anterior organ. By 3 h the staining has largely resolved into a contiguous layer of fluorescence covering most of the worm surface. These findings confirm earlier suggestions, based on electron microscopy, that the parasite's surface tegumental membranes are derived from the migration of membranous vesicles produced within cytons and reveal a new subtegumental architecture interconnecting the cytons.

Key words: schistosome, transformation, membrane biosynthesis, anterior organ, glucose transporter.

INTRODUCTION

Adult *Schistosoma mansoni* parasites reside in male–female pairs within the mesenteric venules of their vertebrate hosts. The outer surface of the adult schistosome – its tegument – is the critical site of host–parasite interaction. The apical surface of the tegument consists of 2 tightly apposed, bilayer membranes (McLaren & Hockley, 1977) while the inner surface of the tegument is bounded by a single lipid bilayer basal membrane that has numerous narrow invaginations. The tegument, lacking lateral membranes, is a syncytium surrounding the entire parasite. Numerous cell bodies (or cytons) associated with the tegument lie beneath the muscle layers and are connected to it by cytoplasmic tubules that are lined by microtubules (Morris & Threadgold, 1968; Silk, Spence & Gear, 1969; Smith, Reynolds & Von Lichtenberg, 1969; Wilson & Barnes, 1974). Morphological data suggest that tegumental membranous bodies (called discoid bodies and multilamellar vesicles) are synthesized within the cytons, transported through the cytoplasmic connecting tubules to the syncytium where they fuse with the apical membranes (Morris & Threadgold, 1968; Silk *et al.* 1969; Smith *et al.* 1969). The formation of the double bilayer membrane commences upon skin penetration of vertebrates by infectious forms called cercariae (Hockley & McLaren, 1973; Stirewalt, 1974). First, the original cercarial trilaminate membrane and its dense fibrillar covering, the glycocalyx, begin to be cast off (Samuelson & Caulfield, 1985). By 60 min, a significant portion of the tegumental outer membrane is already multilamellate. Over the next several hours the double lipid bilayered outer tegumental membrane is formed (Hockley & McLaren, 1973).

Cercariae are known to have at least 3 distinct subtegumental cell types that connect to the tegument via cytoplasmic bridges (Morris, 1971; Hockley & McLaren, 1973; Dorsey, 1976; Dorsey & Cousin, 1995). The 3 types are distinguished on the basis of their location within the parasites and the nature of the multilamellar vesicles they contain. The most common cyton type is designated type 1 and contains membranous bodies measuring 100–125 nm. Type 2 cytons are confined to a small dorsoanterior area of the cercarial body; they also contain multilamellar vesicles of 100–125 nm size but additionally contain large (200–250 nm) membranous bodies that possess an electron-dense core (Dorsey & Cousin, 1995). The anterior region of cercariae is called the anterior organ and it lacks cytons (Cousin, Stirewalt & Dorsey, 1981). Instead a large unicellular entity called the head gland exists in the median region of the anterior organ. The head gland possesses multiple connections to the tegument in the anterior region of the oral sucker (Dorsey, 1976). The gland contains both types of membranous

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bodies described above as well as 'homogeneous dense' bodies. In the case of all 3 subtegumental cell types (type 1 cytons, type 2 cytons and the head gland) the membranous bodies contained within them migrate through cytoplasm connections to the tegument during cercarial transformation. Type 1 cytons as well as the head gland have been observed by electron microscopical observations to deposit membrane at the tegumental surface and type 2 cytons have been proposed to likewise contribute surface membrane material (Dorsey & Cousin, 1995). Thus the tegumental surface of schistosomula may be a chimera of deposits from all 3 cell types.

We have recently identified a glucose transporter protein, designated SGTP4, that is found in the apical membranes of the adult tegument (Skelly *et al*. 1994; Skelly & Shoemaker, 1996). This protein presumably transports glucose from the host bloodstream into the body of the parasite. Using immunogold localization and electron microscopy, SGTP4 is detected only in the outer tegumental membranes of adult parasites and in tegumental discoid bodies and multilamellar vesicles (Jiang *et al*. 1996). SGTP4 is not detected in eggs, sporocysts or cercariae but is detected by Western analysis soon after the transformation of cercariae into schistosomula and remains expressed throughout intra-mammalian lifestages (Skelly & Shoemaker, 1996). Additionally, surface labelling studies using an aqueous biotinylating agent on living adult worms confirms that SGTP4 is present within the outer apical membrane and exposed on the worm surface (Skelly & Shoemaker, 1996). Therefore, SGTP4 is the first example in any platyhelminth of a protein marker that is specific for the double-bilayer, apical membrane at the host–parasite interface. In this study, we monitored the appearance and migration of this specific tegumental marker protein to gain insight into the process of synthesis and deposition of this membrane during the transformation of cercariae into schistosomula.

MATERIALS AND METHODS

Parasites

A Puerto Rican strain of *Schistosoma mansoni* was maintained by passage through *Biomphalaria glabrata* snails and CBA/J mice. Cercariae were obtained from infected snails as previously described (Hackett, 1993). Formation of the double-bilayered membrane commences upon skin penetration of cercariae but was induced *in vitro* by incubating cercariae in RPMI culture medium as described by Brink, McLaren & Smithers (1977).

Immunofluorescence microscopy

Cercariae or schistosomula were fixed in ice-cold acetone for 5 min and were then air dried and stored at −80 °C. Specimens were rehydrated in a small volume of PBS for 20 min. Next they were incubated with PBS containing 1% normal goat serum for 30 min. The rabbit anti-SGTP4 antibodies are directed against a synthetic peptide epitope and, before use, were affinity purified as described (Zhong *et al*. 1995). Cercariae were incubated with anti-SGTP4 antibodies at a 1:40 dilution for 1 h at room temperature. Samples were washed 3 times for 10 min each in PBS containing 2% fetal calf serum. A 1:300 dilution of fluorescein-conjugated $F(ab')_2$ goat anti-rabbit IgG (BioRad) was used to detect bound primary antibody. After washing as above, the slides were mounted in 90% glycerol, PBS, 2% 1,4-diazabicyclo(2,2,2) octane. The sections were examined by conventional epifluorescence microscopy and by laser scanning confocal microscopy using a BioRad MRC600 microscope. In this study confocal microscopy was helpful in clarifying the depth of staining and delineating interior from exterior regions of staining.

RESULTS

Apical tegument biosynthesis and deposition in schistosomula

Most cercariae exhibit either no visible staining or a weak and diffuse internal staining for SGTP4 (Fig. 1, 0 h). Following 60 min incubation in RPMI, the parasite surface stains in a regular but patchy manner (Fig. 1, 1 h). By 3 h incubation the developing parasites exhibit a more uniform surface staining (Fig. 1, 3 h). To examine this process in greater detail parasites were fixed and stained at 10, 30 and 60 min after the initiation of transformation. The results of this experiment are shown in Fig. 2. At the 10 min time-point the marker protein, SGTP4 is clearly detected internally along the length of the parasite (Fig. 2A, D). SGTP4 remains in cytons beneath the surface during this time (Fig. 2G) and our data reveal for the first time that these cytons are interconnected beneath the surface in a 'cyton network' (Fig. 2A, see also Fig. 5). After about 30 min, the protein is detected on the surface of the developing tegument in discrete patches (Fig. 2B, E). In rare cases it is possible to find examples in which staining is visible both within the cyton network and at the surface (Fig. 2H). In these examples the connections between the cytons and the tegument are clearly stained and infer that the membranous bodies containing SGTP4 are in the process of being deposited onto the surface. The rare occurrence of cytons captured in this act of surface deposition (Fig. 2H) suggests that it is a rapid process that we term 'cyton eruption'. Cyton eruptions result in surface membrane deposition and the formation of the patchy staining appearance seen in Fig. 2C. The eruptions are almost always observed to occur simultaneously on adjacent cytons

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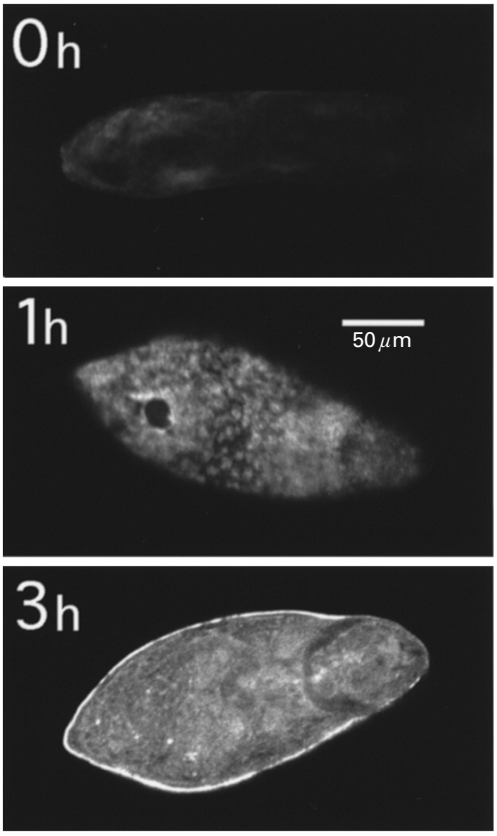

Fig. 1. Epifluorescence immunolocalization of the apical tegument membrane marker protein, SGTP4, at 0, 1 and 3 h post-transformation. Staining was performed as described in the Materials and Methods section on cercariae collected 0 h, 1 h or 3 h after inducing transformation by incubation in rich medium (RPMI).

suggesting that the process is somehow coordinated. Note that the anterior of the parasite (a region called the 'anterior organ') does not stain for SGTP4 during the first 30 min (Fig. 2A, B). After 1 h the patchy surface staining becomes more widespread (Fig. 2C, F, I) and by 3 h has largely resolved into a contiguous layer that includes much of the anterior organ.

Figure 3 shows the progression of staining of the anterior organ of the developing parasites. We never observed subtegumental staining within this organ (Fig. 3A) and the appearance of surface staining was somewhat delayed. Approximately 30 min after transformation commenced, surface patches were first seen on the body but not the anterior organ (Fig. 3B, arrow). Between 30 and 60 min we frequently observed parasites having surface patches on the schistosomulum body but with little or no staining on the anterior. By 60 min the body was extensively stained in a patchy manner and at this time patches began to appear in a regular array on the anterior organ (Fig. 3C, arrow). By 3 h the entire parasite was coated with membrane containing SGTP4 although the extreme anterior of the organisms continued to stain poorly or not at all (arrow, Fig. 3D).

Coalescence of deposited surface membrane

Following cyton eruption SGTP4-staining membrane appears at the surface of the developing parasites after about 30 min as discrete patches that are less than 1 μm in diameter (arrowheads, Fig. 4). By 60 min the surface was extensively stained although the sizes of the surface patches are not uniform. Some patches were 1 μm or less in diameter while neighbouring patches appeared to be coalescing and could extend 5 or more μm across the surface (arrows, Fig. 4). By 90 min large areas of continuously stained surface could be observed and by 180 min most of the surface was stained, although some areas appeared unstained on a single focal plane because of the uneven and pitted nature of the surface.

The cyton network

We have previously noted the apparently ordered array of SGTP4-staining patches that are observed 1 h following transformation to schistosomula (Fig. 1, 1 h) (Skelly & Shoemaker, 1996). As we have shown that these patches derive from cyton eruptions, it is therefore the cyton connections between the cyton network and the tegument that must be regularly arrayed to produce this result. Observation of many schistosomula stained for SGTP4 has indicated the existence of an additional pattern in these parasites; a crude bilateral symmetry of the cyton network. The thin white line in Fig. 5A indicates the line of symmetry in this example.

The cyton network consists of a variety of different types of branches and interconnections and it is often not possible to identify individual cytons within the network. The example shown in Fig. 5B highlights this observation; arrows point to 3 discrete branches emanating from a central circular staining area. Through examination of many stained organisms at different focal planes we infer that the network is entirely interconnected to comprise a single unit. The individual branches appear to vary significantly in their breadth. If we assume that the stained material defines the network then the width of the projections that extend along the length of the parasite vary from less than 1 μm to approximately 10 μm.

DISCUSSION

The formation of the double lipid bilayer adult tegument

The current model of adult tegument formation is based on an analysis of the process by electron microscopy. The model suggests that tegument biogenesis commences upon skin penetration of cercariae and involves the migration of membranous inclusions from the tegumental cytons beneath the

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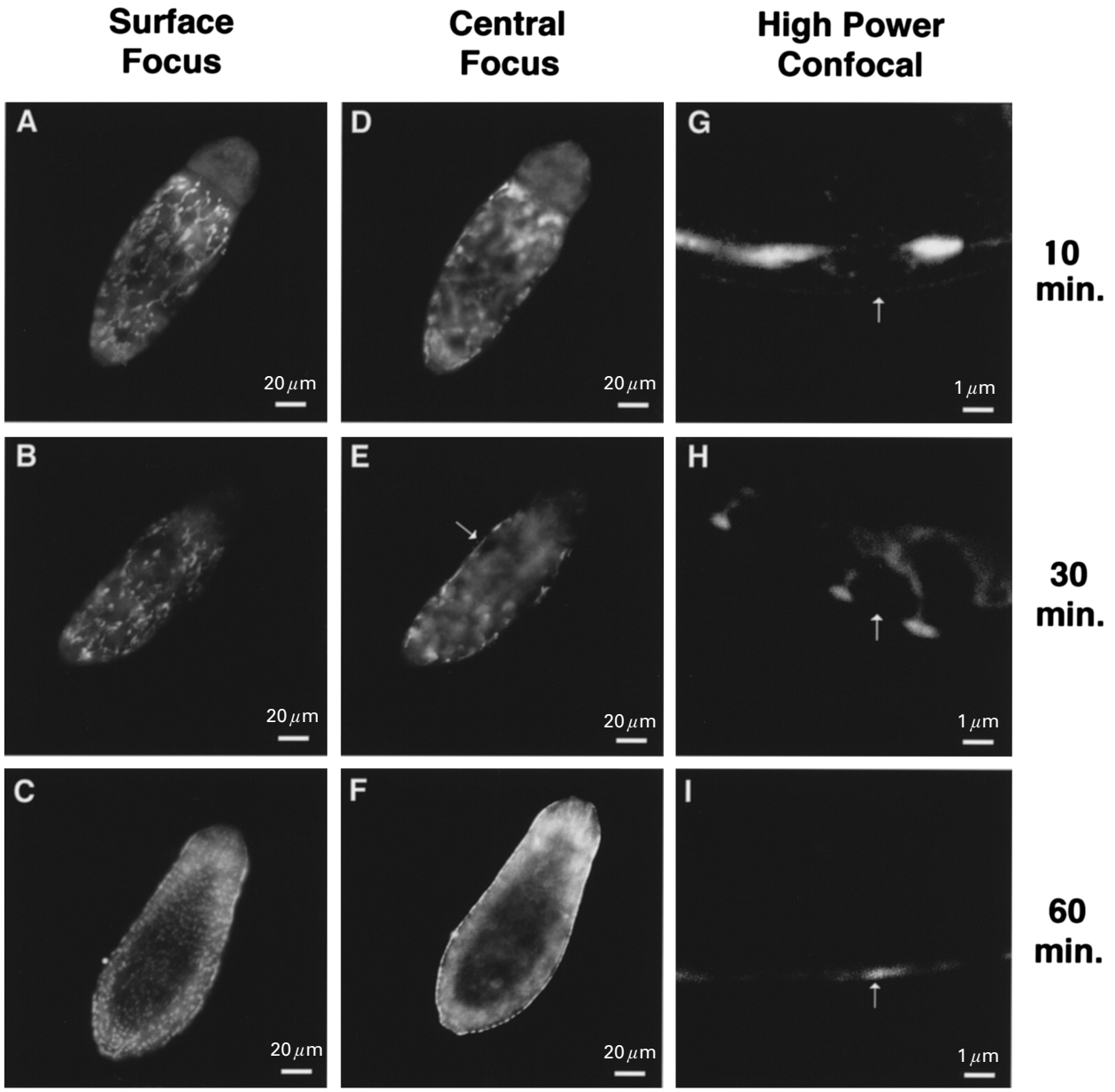

Fig. 2. Schistosomulum tegument morphogenesis as revealed by immunofluorescent staining for SGTP4 during the first hour post-transformation. Immunofluorescent images are of SGTP4 localization following cercarial culture in rich medium for the time-periods indicated at the right. (A, B, C) Images focused at the surface; (D, E, F) images focused centrally to view the outer tegument. Images A–F obtained by epifluorescence microscopy. (G, H, I) High power confocal images. The arrows indicate the postion of the apical tegumental membrane.

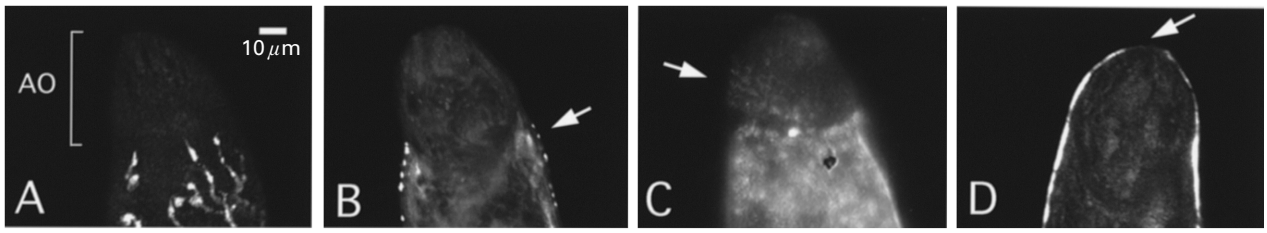

Fig. 3. Epifluorescence immunolocalization of SGTP4 in the anterior organ (AO) at 10 (A), 30 (B), 60 (C) or 180 (D) min post-transformation. Arrows in B and C indicate surface patches and in D, the poorly stained extreme anterior.

muscle layers via microtubule-laden connections to the surface of the parasite (Hockley & McLaren, 1973). On the surface, the deposited membrane exists transiently in 'piles' of multilaminate material that eventually fuse to form the mature intra-mammallian stage apical tegument membrane. In

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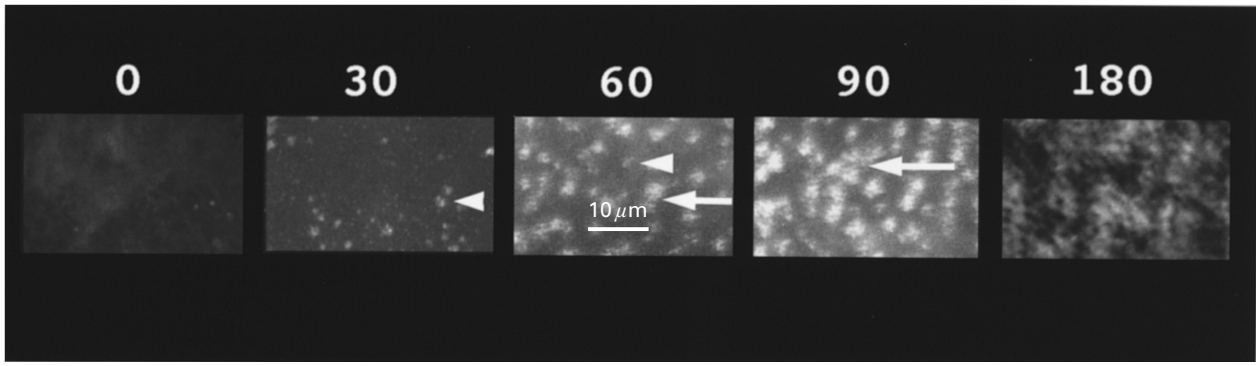

Fig. 4. Appearance and coalescence of the SGTP4 staining surface patches on the tegument surface of newly transformed schistosomula. Epifluorescence surface images are shown of schistosomula stained for SGTP4 at various times (min, as indicated) post-transformation. Arrowheads indicate surface patches arising from cyton eruptions; arrows indicate surface membrane patch coalescence.

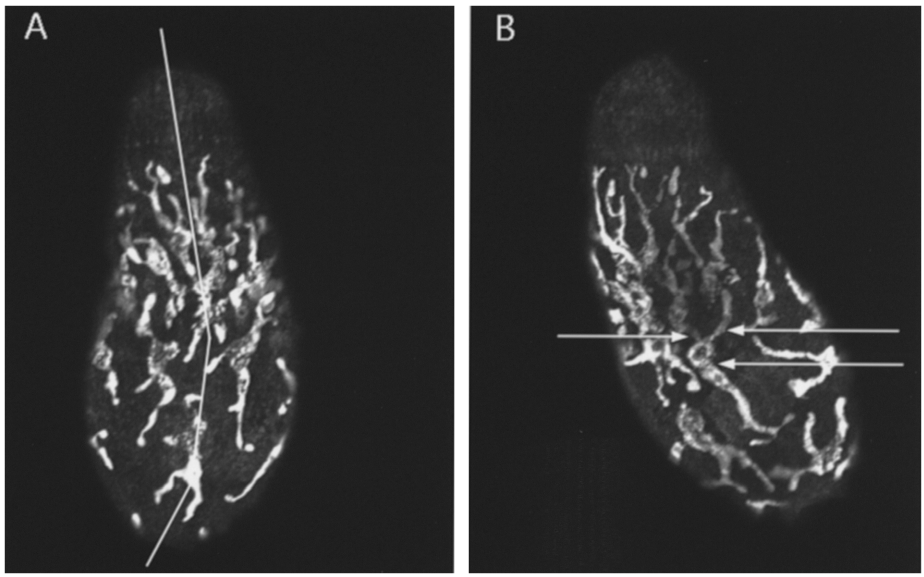

Fig. 5. The cyton network is bilaterally symmetrical and interconnected. Epifluorescence localization of SGTP4 at 10 min post-transformation. (A) Bilateral symmetry is indicated by the thin white line. (B) The interconnected, branching nature of the network is indicated by arrows.

this report, we have attempted to test and extend the current model of tegument biosynthesis by monitoring the appearance and movement of the apical tegumental marker protein SGTP4. In this way, we could examine the membrane transformation process using light microscopy on whole organisms. Immunofluorescence analysis reveals that SGTP4 is first detected within minutes of the initiation of cercarial transformation and is localized to a newly identified cyton network. Next the marker protein is seen in tubules connecting the network to the surface and finally it is deposited at the surface of the maturing schistosomes. In earlier EM localization studies, SGTP4 was found within discoid bodies and multilamellar vesicles (Jiang *et al*. 1996). Our analysis with whole organisms therefore supports the currently accepted model of adult tegument biosynthesis i.e. that surface tegumental membranes are derived from the migration of multilamellar vesicles (now known to contain SGTP4) from within cytons to the surface. Presumably, once the membranous vesicles fuse to the inner apical membrane and deposit their contents at the surface, the outer bilayer forms slowly over the next few hours as the membranous stacks rearrange to form a contiguous covering.

It is noteworthy that discoid bodies are not seen in schistosomula until well after the new tegument has been synthesized (Hockley & McLaren, 1973; Dorsey & Cousin, 1995). This implies that multilamellar vesicles alone can form the new tegument. Since we know that discoid bodies, like multilamellar vesicles, contain SGTP4 (Jiang *et al*. 1996) discoid bodies are likely involved in the maintenance and turnover of the tegument.

The cyton network

The existence of an interconnected network of cytons in transforming parasites has not been previously

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observed. The network is an ordered, bilaterally symmetrical, interconnected entity that extends from the posterior of the cercariae, along the body to the dorsal muscle bundle. Multiple branches can emanate from a single point and they can vary in breadth between 1 and 10 μm. The internal network is never seen to extend into the anterior organ of the parasites. Detailed descriptions of the adult tegument and its subtegumental cytons describe few or no subtegumental connections (Morris & Threadgold, 1968; Smith *et al*. 1969). Once the accumulated SGTP4 within the cyton network has erupted to the surface, there is no subsequent staining of the subcellular regions and so we have no evidence that the network is retained as the schistosome matures. It has previously been reported that, during cercarial tegument formation (when subtegumental cells have delivered their membranous body payload to the tegument), the attachments to the tegument break down and that new membrane-filled cells then make fresh connections to the surface (Hockley, 1972). A similar scenario may apply for the cyton network described here.

Since cercariae have at least 3 distinct subtegumental cell types (type 1 cytons, type 2 cytons and the head gland) that connect to the tegument via cytoplasmic bridges, the question arises as to which of these comprise the cyton network described here (Hockley & McLaren, 1973; Dorsey, 1976; Dorsey & Cousin, 1995). Both type 1 and type 2 cytons are found in the body of the cercariae while the head gland is confined to the anterior organ. The staining pattern for SGTP4 reported in this study clearly demonstrates that the cyton network is composed of (at least) type 1 cells running through the body of the parasites. Type 2 cytons are confined to a small dorsoanterior area of the body and while cytons in this location also stain for SGTP4 we cannot unambiguously say, at this level of observation, that type 2 cytons contain SGTP4.

The anterior organ

The subtegumental region of the anterior organ is never observed to stain for SGTP4 and there is a delayed appearance of surface patches following the initiation of transformation. This raises the issue as to how the anterior region of the parasite acquires its SGTP4-containing double bilayer membrane. Our inability to detect SGTP4 in the head gland of the anterior organ suggests that this gland does not contribute SGTP4 to the surface. Nevertheless, SGTP4 is eventually detected at the surface of much of the anterior organ and the sequence of events resulting in its appearance resembles that observed elsewhere on the parasite i.e. SGTP4 is seen first as a series of small, discrete patches at the surface of the organ followed by the merging of these patches to finally provide a more uniform covering. This seems inconsistent with the report that the head gland deposits membrane only at the oral end of the anterior organ while cytons, probably type 2, extend cytoplasmic connections and deposit membrane into the dorsoposterior tegument of the anterior organ (Dorsey, 1976; Dorsey & Cousin, 1995). We hypothesize that SGTP4-containing membrane is synthesized by type 1 and type 2 cytons in the body of the schistosomula, and that many type 2 cyton cytoplasmic connections extend into, and terminate at the surface of the anterior organ. We suggest that SGTP4 is synthesized in the cyton networks within the body of the schistosomulum and deposited throughout the entire worm. The delayed appearance at the anterior organ may indicate that type 2 cytons erupt later than type 1 cytons or that the tegumental connections to this organ simply form, or open, later than those in the body. The delay in apical tegument formation in the anterior organ may be to avoid early exposure of proteins at the anterior end of the worm where they may be damaged by the release of proteases from the pre- and post-acetabular glands during vertebrate skin invasion. An examination of numerous transforming parasites reveals that the extreme anterior of the anterior organ stains poorly or not at all for SGTP4. Membranous bodies from the head gland are released at this location (Dorsey, 1976) suggesting that there may be limited, or delayed, merging of the differing membranous deposits from the different cell types. The detection of SGTP4 in cytons but not in the head gland highlights the fact that the 100–125 nm multilamellar bodies common to both the head gland and the cytons are biochemically different.

Subtegumental connections

SGTP4 clearly accumulates within a heretofore uncharacterized cyton network before it is suddenly released to the surface. This raises the question as to what transiently impedes the movement of SGTP4-containing multilamellar vesicles into the schistosomulum tegument. The seminal electron microscopical analysis of Hockley & McLaren (1973) provides one possible explanation. They report that there are no connections between cytons and the tegument in free-swimming cercariae and propose that attachments to the surface from subtegumental cells form as transformation proceeds. Indeed, others report that such attachments only appear 30 min after inducing cercarial transformation by incubation in salt solution (Brink *et al*. 1977) which correlates with the timing we find for the release of SGTP4 to the surface. The movement of membrane-containing SGTP4 to the tegument would therefore coincide with the *de novo* laying down of these connections. Other authors, however, document that connections to the tegument already exist in cercariae (Stirewalt, Cousin & Dorsey, 1983; Salafsky *et al*.

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1988). If this is the case, then some other structural or physiological barrier must exist to prevent the premature movement of multilamellar vesicles into the cercarial tegument and this barrier must be removed by about 30 min post-transformation.

When SGTP4 migrates to the surface of the developing schistosomulum, the internal cyton network is no longer clearly detected. This suggests either that SGTP4-containing membranous bodies are rapidly and continuously delivered to the surface once the initial release of accumulated material has taken place or that SGTP4 gene expression is down-regulated shortly following transformation. Northern analysis did not detect a significant drop in SGTP4 mRNA levels between cercariae, schistosomula and adult; in fact, some increases were apparent (Skelly *et al*. 1994). In addition, SGTP4 can be detected in the membranous bodies of schistosomula some 15 h after transformation (Jiang *et al*. 1996). For these reasons we believe that SGTP4 no longer accumulates within the cytons although synthesis continues throughout mammalian-stage parasite maturation and is rapidly deposited at the surface to replace membranes that are lost through turnover or shedding.

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