Review

Structure-function analysis of apical membrane-linked molecules for treatment and control of schistosome parasites of humans: insights from studies into annexins.

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Neglected tropical diseases are a group of some 17 diseases that afflict poor and predominantly rural people in developing nations. One significant disease that contributes to substantial morbidity in endemic areas is schistosomiasis, caused by infection with one of 5 species of blood fluke belonging to the trematode genus *Schistosoma*. Although there is one drug available for treatment of affected individuals in clinics, or for mass administration in endemic regions, there is a need for new therapies. A prominent target organ of schistosomes, either for drug or vaccine development, is the peculiar syncytium that forms the body wall (tegument) of this parasite. This dynamic layer is maintained and organized by concerted activity of a range of proteins, among which are the abundant tegumentary annexins. In this review, we will outline advances in structure-function analyses of these annexins, as a means to understanding tegument cell biology in host-parasite interaction and their potential exploitation as targets for anti-schistosomiasis therapy.
Neglected tropical diseases

Neglected tropical diseases (NTDs) include a range of lesser known chronic infections that affect poor and disenfranchised people, primarily, but not exclusively, in developing nations (Hotez et al., 2009; Hotez et al., 2007). Chronic infections caused by NTDs lead to many adverse outcomes in affected populations and contribute substantially to human morbidity. In addition to the microbial and protozoan diseases, NTDs include a number of helminth infections, such as diseases caused by flatworm parasites, notably schistosomiasis, echinococcosis and liver fluke diseases, as well as roundworm parasites, such as the major soil transmitted helminth infections (ascariasis, trichuriasis and hookworm diseases). Although no individual NTD rivals the major infection threats of HIV, malaria or tuberculosis in terms of global impact of disease, collectively, the NTDs contribute substantially to morbidity throughout the world (Engels et al., 2006).

A number of factors present major challenges for the development of new treatments for NTDs. Firstly, NTDs are chronic diseases, which may reside in affected people as life-long infections. Secondly, NTDs are not always associated with human mortality and the impact of these diseases can be hidden among other measures such as poor developmental outcomes and adverse cognitive abilities. Thirdly, as stated, NTDs often affect the poorest of the poor. Hence, these diseases receive less attention than other, immediately life-threatening infectious diseases.

Among the NTDs of major interest is human schistosomiasis. This suite of diseases is caused by infection with one of a number of species of the genus Schistosoma, a taxon of the digenean trematodes, commonly known as blood flukes (and historically, the agents of Bilharzia)(Ross et al., 2002). Five species are the main contributors to human schistosomiasis, Schistosoma mansoni, S. japonicum, S.
mekongi, *S. intercalatum* and *S. haematobium*. Transmission of the parasites to humans takes place in freshwater, typically in regions of poor sanitation where human exceta contaminate water bodies. Schistosomiasis infects over 200 million people in approximately 74 nations, the majority of which occur in Sub-Saharan Africa (Steinmann et al., 2006). Distinct foci of schistosomiasis also occur in Asia (China, the Philippines, along the Mekong River, and Indonesia), as well as South America (notably Brazil and some Caribbean Islands). Disability-adjusted life years lost to human schistosomiasis in 2010 were measured at 48/100,000, an increase of 20% on measures in 1990 (Murray et al., 2013).

There is a distinct dichotomy in schistosomiasis in relation to the host responsiveness to various life stages. On one hand, the invasive larvae and adult parasites are largely able to avoid immuno-surveillance of the hosts. To that effect, these parasites employ a series of strategies including rapid development, stealth-like host interfaces and immune-suppression (Wilson, 2009). On the other hand, active secretion of immunogenic molecules by eggs provokes an intense immune response (Burke et al., 2009). This phenomenon is characterized by a strong granulocytic response around the egg in affected tissues that may lead to fibrosis particularly in the liver. The intense response is used to support the escape of the eggs from the host. The cellular infiltrate forces the schistosome egg across the vascular endothelium and into tissues of luminal organs, such as the intestinal lining, the bladder wall or genitalia, forcing them ultimately into the lumens from which they are voided into the environment. The bulk of chronic disease in schistosomiasis is related to host responses against parasite eggs deposited in the blood vessels surrounding the gut (*Schistosoma mansoni* and *S. japonicum*) or bladder and genital organs (*S. haematobium*) (Ross et al., 2002). However, it has proven more effective to direct
control towards killing adult worms or the invasive larvae that establish infection so that the deposition of immunogenic and pathogenic eggs is stopped.

Schistosomes belong to the Clade Lophotrochozoa of the Kingdom Animalia. The multicellular animals form a monophyletic grouping (Walker et al., 2011) and there are substantial similarities among the many cellular, biochemical and molecular adaptations in different animal clades. The search for effective treatments against schistosomiasis thus needs to exploit key molecular and conformational differences between target molecules of these parasites and their hosts. This review explores work focused on the search for novel molecular targets of therapeutics and prophylactics, and examines new insights from studies of a primary site of host interaction, the schistosome tegument.

Treatments for Schistosomiasis- Drugs

There currently exist few drugs for treatment of schistosomiasis: praziquantel, metrifonate, oxamniquine and artemether (Bartley et al., 2008; Cioli et al., 1995; Ross et al., 2002). All of these drugs have proven useful for therapeutic treatment of individuals in the clinic or of communities in mass drug administration. Of the four drugs, oxamniquine is only effective against schistosomiasis mansoni, and drug resistance is known and the mechanism of resistance elucidated (Valentim et al., 2013). Metrifonate is only effective against urinary schistosomiasis, caused by S. haematobium, and its use is hampered by a complex administration schedule with multiple doses required over a 2-week period. Frequently, this therapy is met with a low rate of compliance among patients. Furthermore, the drug is labile in warm climates and is thus less useful in field settings. Combination therapy using praziquantel and metrifonate has been effective for urinary schistosomiasis. (Danso-Appiah et al., 2009).
Artemether is a beta-methyl ether derivative of artemesinin, a compound derived from the sweet wormwood Artemesia annua. Artemesinin and its derivatives are highly effective against haematophagous parasites, notably malaria, but they have also proven effective against schistosome infection (Liu et al., 2012). One recent report suggests that artemesinin is acted upon by elemental iron in the iron-rich environment of haematophagous parasites and the complex, in turn, inhibits calcium pump (Shandilya et al., 2013). Concerns about artemesinin-derivative resistance to the more insidious human disease of falciparum malaria, has precluded the use of artemether against schistosomiasis where the two diseases are co-endemic (Bergquist et al., 2005; Utzinger et al., 2007).

The current drug of choice for treatment of schistosomiasis is praziquantel. This drug has been used in mass treatment campaigns in many countries and remains a primary tool in the war against the disease (Knopp et al., 2013). The mode of action of praziquantel remains unknown, although recent developments strongly suggest a role for the drug in calcium homeostasis in the parasites and notably in calcium transport complexes (Greenberg, 2005; You et al., 2013). Despite the lack of mode-of-action data, the drug remains highly effective for a wide range of flatworm diseases of humans and domestic animals. Praziquantel has been deployed for mass drug administration in endemic regions and has been successful in pushing the disease from high to low endemicity. This major achievement has been facilitated in part by reductions in costs associated with manufacture of the drug, and the development of public-private partnerships that have led to the distribution of the drug to many impoverished communities where schistosomiasis is endemic.

Despite its high efficacy, praziquantel has limitations. The drug is only effective against adult or pre-adult forms (Greenberg, 2005). Furthermore,
praziquantel confers no protection against subsequent infection and people may become reinfected within days of treatment (Ross et al., 2002). Treatment failures for S. mansoni and S. haematobium infections have been observed and the presence of resistant strains have been demonstrated experimentally (Greenberg, 2013). While widespread resistance to praziquantel has not been observed clinically, the application of the drug in mass treatment campaigns may result in new resistant forms emerging.

**Treatments for Schistosomiasis- Vaccines**

Many experts within the schistosomiasis community argue that continued application of a single drug, praziquantel, for single treatments and as a mass control strategy, is problematic and not likely to lead to effective control of the disease. The alternative, a subunit vaccine, has thus been promoted as an important alternative strategy for the control and elimination of schistosomiasis (Bergquist et al., 2008) (Loukas et al., 2011; McManus et al., 2008). These thoughts have recently led to the formation of think-tanks to evaluate vaccination strategies (Kupferschmidt, 2013).

Optimism for a vaccine rests on observations from the 1970’s on host responses to radiation attenuated cercariae in experimental infections (Bickle et al., 1979a; Bickle et al., 1979b). A cercaria is the larval stage that penetrates human skin to initiate infection. This stage transforms rapidly in the skin to become a host-adapted larva, the schistosomulum, which follows a course of migration over ensuing days through the lung and liver before becoming an adult parasite. It was shown that infection of humans with live, radiation-attenuated (RA) parasites led to strong protection against subsequent challenge infections with normal cercariae. Vaccination with RA cercariae has thus led to an adult worm burden reduction in experimental schistosomiasis of 60 – 70% (Bickle et al., 1979a; Bickle et al., 1979b) (Caulada-
Benedetti et al., 1991) (Coulson et al., 1998). The protection level of attenuated vaccine in animal models is species- and strain-dependent. However, both S. mansoni- and S. japonicum-irradiated cercariae confer high levels of protection in mice, buffaloes and pigs (McManus, 1999). The mechanism of protection appears to result from transcriptional and expression “lethargy” in the attenuated parasites during the early stage of development. The major lessons learned from these studies are that parasite killing is largely dependent upon host-parasite interaction during the host-establishment phase of the parasites, that is, within the first week after infection. During this time, the cercaria undergoes an extensive remodeling of its surface body wall, the tegument, and becomes transcriptionally active, compared with the synthetically active cercaria. Indeed, some of the promising vaccine candidates come from this tissue, and it seems that vaccine targeting of this layer is crucial for parasite killing.

Despite the high level of protection available with radiation-attenuated vaccines, the unstable lifespan, delivery problems and safety problems of these modified cercariae makes them unsuitable for further development as a vaccine (Bergquist et al., 2008). Therefore, efforts have been directed to discover and identify suitable protective antigens from schistosomes, leading to the development of recombinant vaccines, DNA vaccines, peptide-epitope based vaccines, multivalent vaccines and chimeric vaccines (McManus et al., 2008).

Of the vaccines trialed, a number have been promoted for human trials, including the Bilvax vaccine based on a 28 kDa S. haematobium glutathione-S-transferase, which has entered Phase 3 clinical trials, and a S. mansoni tetraspanin (Sm-TSP-2)(Tran et al., 2006), which has entered Phase 1 trials (Kupferschmidt, 2013). Other vaccines presented at a recent vaccine discovery workshop sponsored by
the Bill and Melinda Gates Foundation in the USA (Kupferschmidt, 2013) identified additional vaccines still in experimental phase, including Sm14 a fatty acid binding protein, a calpain (Smp80) from *S. mansoni*, and Sj23, a tetraspanin, a tri phosphate isomerase, an insulin receptor, and paramyosin from *S. japonicum* (Siddiqui et al., 2005; Tendler et al., 2008; You et al., 2012; Zhu et al., 2006; Zhu et al., 2004). An advantage of vaccination strategies against the zoonotic disease schistosomiasis japonica is that the parasite is found in a range of domesticated animals including water buffalo in China. Researchers involved in controlling this species in China and the Philippines have developed vaccines for use in animals as transmission-blocking vaccines, based on modelling of transmission dynamics in endemic regions. Antigen discovery studies are still progressing using a variety of immunomics and proteomics approaches. It is now widely appreciated that targeted approaches are required in antigen discovery, and there is continuing interest in considering fundamental cell biological and developmental understanding with molecular advances.

**The tegument of schistosomes**

The tegument, or body wall, of schistosomes is a dynamic host-adapted interface between the parasite and its vascular environment. The tegument is a highly polarized syncytium and possesses functional analogy with transporting epithelia, including the gut lining or the syncytiotrophoblasts of the human placenta. The tegument plays significant roles in nutrient uptake, immune evasion and modulation, excretion, osmoregulation, sensory reception and signal transduction (Jones et al., 2004; Kusel et al., 2007; Wilson, 2011). Given the importance of the schistosome tegument in nutrition and immune evasion, proteins of this surface layer are recognized as prime
candidates to target for vaccine and therapeutic drug development (Loukas et al., 2007).

**Ultrastructure of schistosome tegument**

The tegument is formed as a single syncytium that covers the entire body and is continuous with other epithelia, notably the foregut lining (Silk et al., 1969). This surface layer is a highly ordered region with distinct transporting regions, secretory components and absorptive adaptations. A peculiarity of the layer is the presence of a dual membrane complex that forms the apical extremity of these metazoan parasites (Hockley, 1973; Wilson, 2011). The single unit membrane of the cercaria becomes replaced by a dual membrane system. The outer membraneous structure, termed by some the membranocalyx, is depauperate of proteins, whereas the underlying membrane is decorated with abundant membrane proteins (Braschi et al., 2006).

The tegument is supported by cell bodies that lie embedded in the parasite parenchyma (Gobert et al., 2003; Hockley, 1973). The surface layer and the cell bodies are linked by cytoplasmic bridges that pass through the muscle bundles that lie beneath the parasite body wall (Gobert et al., 2003; Hockley, 1973). Tegumentary cell bodies contain the synthetic machinery of the syncytium, including endoplasmic reticulum and Golgi apparatus, and produce abundant vesicular products that are trafficked to the tegument along cytoplasmic bridges (Figure 3).

The advantage to schistosomes in possessing a syncytial tegument is poorly understood, but appears to be an important strategy that ensures survival of parasites in the vascular environment. Invaginations of the surface membrane complex, as well as in the basal membrane of the cytoplasm (Gobert et al., 2003; Hockley, 1973;
Skelly et al., 2006) are structural evidence of high turnover of these membranes, a process that is related to nutrient uptake and a way of avoiding the host immune response by internalising antibodies and removing possible antigenic molecules from the surface (Skelly et al., 2006). Membrane internalization and translocation events are driven by a complex interplay of multiple membrane proteins including the tetraspanin web (Tran et al., 2010)

**Tegument proteins as vaccine targets**

The schistosome tegument is one of the primary interfaces between host and parasite. Proteins exposed at its surface during intra-mammalian stages, are possibly the most susceptible targets for vaccine development (Loukas et al., 2007). In mice immunised with newly transformed *S. mansoni* schistosomula tegument an induced Th1 type protection has been observed, which damages the adult worm tegument layer, reduces egg number and parasite burden (Smithers et al., 1990). Therefore, it is currently believed that tegument proteins of schistosomes are a priority in antigen discovery. Advances in proteomics vastly increased the speed of identification of tegument proteins (Braschi et al., 2006; Castro-Borges et al., 2011; Mulvenna et al., 2010).

Analysis of the schistosome proteome and transcriptome has resulted in the identification of several molecules associated with the apical parasite membrane of schistosomes (Table 1). These included glucose transport proteins, enzymes and receptors, heat shock proteins, and structural proteins (Castro-Borges et al., 2011; Mulvenna et al., 2010). Among the dominant surface-related proteins is a group of annexins, in particular Anx(Sm)3 (annexin B30) (Castro-Borges et al., 2011) indicating that this annexin and possibly others may have promise as schistosomiasis vaccines development (Hofmann et al., 2010).
Up to five annexins are predicted to occur in the tegument of schistosomes. Following a recent classification of invertebrate annexins based on genome-wide analysis of many species, these annexins are categorized as Annexins B7a, B22, B30, annexin B5a. The abundance, as well as the peculiar features of annexins of some parasite groups, make them potential targets for therapies.

**Schistosome Annexins**

The survey of group B annexins from different invertebrate taxa revealed that the proteins occur in the vast majority of species studied so far (Cantacessi et al., 2013). The abundant annexin proteins are conspicuously evident in many parasite groups and arthropod vectors, but evidently not in mollusk hosts. Using structure-based amino acid sequence alignments and phylogenetic analyses, the recent analysis provided a robust classification for this protein group, enabling information on structure-functional relationships of these proteins, as well as to assign names to sequences with ambiguous annotations in public databases. It was immediately apparent in phylogenetic analyses that gene duplication in divergent clades was the major evolutionary event in annexin genesis, in particularly in schistosomes. The highest representation of annexins was found in *Schistosoma mansoni* with 13 annexins, many lined up on two chromosomes (Cantacessi et al., 2013). Evidence gained so far suggests that these 13 annexins are expressed differentially in different tissues (Gobert et al., 2009; Jia X. et al., in press). Annexins B7a, B22 and B30 are distinctly associated with the syncytial tegument (Braschi et al., 2006; Mulvenna et al., 2010). Our tissue-specific transcriptomic survey of female *S. japonicum* indicated that different annexins were expressed preferentially by different cell types, with the gut lining expressing annexins B7 and B22, while the vitelline
gland expressed annexin B5 (Gobert et al., 2009). Although S. japonicum is a distinctive parasite, since it diverged early from other species of Schistosoma, similar patterns of annexin expression might reasonably be expected to be conserved. The abundance of annexins B7 and B22 in gut and tegument allows the postulate that these molecules may be epithelial annexins in these parasites, and thus associated with syncytial epithelia. Importantly, both tissues are predicted to have high membrane turnover and reshaping.

Structure-Function Observations of Schistosome Annexins

Observations made by us and others in the recent past point towards a potential use of parasite annexins as therapeutic targets. These observations include: (i) immunoreactivity of some parasite annexins (Gao et al., 2007; Hongli et al., 2002; Leow C.Y. et al., in press; Palm et al., 2003; Weeratunga et al., 2012; Weiland et al., 2003); (ii) localisation of certain parasite annexins to areas of potential exposure and/or structural integrity [REF]; and (iii) the existence of a unique structural feature (an extended helical linker between repeats II and III) in parasite annexins that differentiates them from host annexins (Hofmann et al., 2010; Leow C.Y. et al., in press; Weeratunga et al., 2012).

Schistosome annexins display many canonical features of annexins. Structural analysis of S. mansoni annexin B22 reveals that it maintains the high conservation of the annexin fold (Leow C.Y. et al., in press), and engages in calcium-dependent binding to phospholipid membranes.

Many group B annexins, including those from T. solium, C. elegans annexin B36 (nex-4), and some Group E annexins, including alpha-12- and alpha-19 giardin, possess an unusually long linker segment between repeats II and III on the concave
side of the protein (Hofmann et al., 2010). Whereas the typical length of this linker in annexins is about 10–15 amino acids, the linker peptide found in some anexins of groups B and E comprises between 25 and 38 amino acids (Hofmann et al., 2010). Moreover, secondary structure predictions consistently indicate that this elongated linker region adopts an alpha-helical structure, and the recent crystal structure of S. mansoni annexin B22 provided the anticipated experimental proof. We hypothesise that this additional alpha-helical element on the concave side of the molecule may provide a target for immunological therapeutics (Hofmann et al., 2010). It is tempting to speculate that other parasite annexins with an extended II/III linker peptide may adopt a very similar conformation. A comparison of the extent of the N-terminal domains for annexins with the unique linker shows that such a fold may be possible for most of them.

We have recently determined the crystal structure of annexin B22 from S. mansoni (Leow C.Y. et al., in press), which confirms the presence of the predicted alpha-helical segment in the II/III linker and also reveals a covalently linked head-to-head dimer. Annexin B22 and its homologues from S. japonicum (Cantacessi et al., 2013) and S. bovis (de la Torre-Escudero et al., 2012) are the only B annexins known to date that possess an exposed cysteine residue in the IIDE loop (Cys173), a position where most other annexins possess a serine residue. In annexin B22, the involvement of Cys173 in an inter-molecular disulphide bond as well as several intimate electrostatic side chain interactions add to the stabilisation of the unique head-to-head dimer topology where the dimer interface is exclusively located in module II/III. Structurally, this is significantly different to other annexin head-to-head dimers (Hofmann et al., 2010), where the dimer interface comprises the entire convex surface of both molecules.
In addition, from the calcium-bound crystal structure of annexin B22, canonical as well as novel calcium binding sites can been identified, which seems to be a recurring motif in parasite annexins. Intriguingly, the dimer arrangement observed in the annexin B22 crystal structure revealed the presence of two non-anticipated prominent features: a potential non-canonical membrane binding site and a potential binding groove opposite of the former.

**Annexins in schistosomes**

A variety of roles has been proposed for annexins. In vertebrates, annexins are known to display a broad range of biological activities including response to inflammation, membrane traffic and adhesion, anticoagulation, signal transduction, developmental processes and membrane repair (Bouter et al., 2011; Draeger et al., 2011). In parasites, annexins are suggested to be involved in maintenance of membrane structure (Peattie et al., 1989; Tararam et al., 2010), anti-inflammatory activity (Zhang et al., 2007) and fibrinolytic activity (de la Torre-Escudero et al., 2012). Annexins may thus have distinct roles in enabling survival of parasites when they are within the hosts. Some annexins are speculated to be involved in redox reactions and the regulation of reactive oxygen molecules in plants (Hofmann et al., 2003) (Konopka-Postupolska et al., 2011) and in mammals (Madureira et al., 2011; Madureira et al., 2013; Tanaka et al., 2004).

Localization of *S. mansoni* annexin B22 by fluorescence and electron microscopy in different species of schistosomes (de la Torre-Escudero et al., 2012; Leow C.Y. et al., in press; Tararam et al., 2010) demonstrates that the molecule is strongly associated with the tegument and the apical plasma membrane complex of adult parasites. The
molecule is expressed in human-parasitic phases of the parasite life cycle suggesting a major role in surface membrane dynamics during life in the human host. Although annexin B22 shares many structural similarities with other annexins, its dimeric nature as well as the unique extended linker region suggests that this molecule is co-adapted to function in the peculiar syncytial environment of the tegument of these parasites (Leow C.Y. et al., in press). Among the peculiarities, there is a prominent groove that occurs within the dimeric species. This groove is postulated to enable the annexin B22 dimer to assume an adaptor function, linking the apical membrane complex with proteins.

Annexin B22 possesses another unique feature, namely the external arrangement of the II/III linker that is reflexed over the N-terminal region of the molecule. There is now substantial evidence that annexins, and indeed other molecules of the schistosome tegument, adopt unique conformations that might be exploited for therapeutics or prophylaxis as they distinguish the parasite proteins from homologous proteins in the host.

The question remains as to how these unique regions might be targeted. Undoubtedly, as with all areas of investigation concerning annexins from a wide variety of organisms, more structure-function analyses of the proteins in cells is required. For schistosomes, as with other parasites with divergent epitopes, the peculiarities of the annexins, including the extended II/III linker regions, non-canonical calcium binding sites and other molecular anomalies are of interest, not only for enhancing fundamental understanding of membrane dynamics, but also for designing anti-parasite targets.

Being highly adapted to life within hosts, helminth parasites present considerable difficulties in functional genomics analyses. They are not easily cultivated outside of
the host and require molecular signaling from their host to develop fully. Furthermore, transgenesis studies for schistosomes remain in their infancy, although these parasites are amenable to RNA interfering technologies. Thus, studies of annexins of schistosomes present some challenges. Two important outcome of the recent comparative analysis of invertebrate annexins (Cantacessi et al., 2013) is the occurrence of common structural motifs in some group B (invertebrate) and group E (Giardia) annexins and the growing diversity of annexins among the single celled protists. The encouraging outcomes suggest that there is substantial information to be gained from comparative studies among parasites that are less tractable in laboratory models and readily culturable invertebrate model species and parasites. These comparative structure-function investigations as models for understanding annexin function at the host-parasite interface, as heterologous expression systems of parasite annexins and as targets of inhibitor and drugs assays, will prove invaluable as we move towards developing targeted therapies for parasites of scio-economic importance.

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