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Tetraspanin-2 localisation in high pressure frozen and freeze-substituted *Schistosoma mansoni* adult males reveals its distribution in membranes of tegumentary vesicles

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ABSTRACT

The tegument, or body wall, of schistosomes is the primary tissue for host interaction and site targeted schistosome vaccination. However, many aspects of the cell biology, particularly differentiation and maintenance, remain uncharacterised. A leading vaccine candidate, *Schistosoma mansoni* tetraspanin 2 has proven efficacy in experimental models, but its function, precise subcellular location in the tegument and role in tegument biology is not well understood. A primary question is whether this molecule is a true surface molecule, that is, whether it appears within the apical membrane of the tegument. Hitherto, the target sequence for antibody localisation studies had not been available for advanced subcellular localisation studies, such as immuno-electron microscopy, due to aldehyde sensitivity. To circumvent this problem, we adapted the methods of high pressure freezing and cryosubstitution with uranyl acetate for immuno-electron microscopy. The tri-dimensional structure of tegument membranes was resolved using electron tomography. Immunolocalisation of *Schistosoma mansoni* tetraspanin 2 demonstrates that the molecule is localised to tegument membrane compartments, but predominantly within internal structures associated with surface invaginations and internal vesicles. Surprisingly, no label was found at the virtual surface of the parasite. The significance of this localisation pattern is discussed.

1. Introduction

Schistosomes are a major cause of morbidity and mortality in humans, particularly in developing and tropical nations. Adult worm pairs are found in the mesenteric veins of their hosts, where female worms produce hundreds to thousands of eggs per day (Gryseels et al., 2006). For *Schistosoma mansoni* and *Schistosoma japonicum* to continue the life cycle, their eggs must pass through the host tissues into the lumen of the intestine and pass with faeces into fresh water. Most of the pathology of hepato-intestinal schistosomiasis develops when eggs are flushed to organs, typically the liver, where they become lodged and induce an intense granulomatous response. Although the adult worms themselves do not induce host immunological reaction, their longevity in the human host and the eggs they produce, both contribute to the chronic pathology of this disease.

The tegument, or body wall, of schistosomes is the primary interface for host–parasite interactions. This layer fulfils a variety of biological roles for the parasite (Gobert et al., 2003; Jones et al., 2004; Loukas et al., 2007). The tegument is a syncytial layer encapsulating the entire parasite. Upon entry into the definitive mammalian host, the tegument transforms from a layer that is adapted to the external aquatic environment of the infectious cercaria, into a host-adapted form of the parasitic adult worm (Hockley, 1973). The cellular aspects of the change involve the replacement of the unilaminate and glycocalyx-adorned apical membrane of the cercaria with the dual membrane complex of the adult (Gobert et al., 2003). Over the life of the adult worm the tegument undergoes constant renewal. Although the exact molecular adaptations orchestrating surface renewal are unknown, it is thought to occur by membrane shedding and sloughing (Skelly et al., 2006).

The tegument is commonly thought to be the primary target for schistosome vaccines with many tegumental antigens showing high efficacy in experimental vaccine trials in animal hosts (Tran et al., 2006; Loukas et al., 2007; Cardoso et al., 2008). Through the application of various proteomics methods, the protein composition of the

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tegument is well understood (van Balkom et al., 2005; Braschi et al., 2006; Pérez-Sánchez et al., 2006; Mulvenna et al., 2010; Wilson, 2012), but the exact functions of these proteins and organelles are mostly unknown. Among these components, a family of tetraspanins have shown promise as candidates against *S. mansoni*, but not *S. japonicum*, infections (Tran et al., 2006; Zhang et al., 2011; Pearson et al., 2012).

Tetraspanins are plasma membrane-bound proteins. The hallmark feature of this family is the presence of four transmembrane regions with two extracellular loops. The first and smaller extracellular loop (ECL1) contains less than 30 amino acids and the second and larger extracellular loop (ECL2) consists of 76–131 amino acids (Charrin et al., 2009). The proposed roles of tetraspanins are many and varied but exact functions are not fully understood. Tetraspanins have been found on the membranes of mammalian exosomes, small membranous vesicles that have roles in cellular communication and transport of selected proteins, mRNAs and microRNAs (Rana and Zoller, 2011). Tetraspanins also form "tetraspanin webs" or tetraspanin-enriched domains and thus interact with a wide variety of proteins (Boucheix and Rubinstein, 2001; Rubinstein, 2011).

One important protein of the schistosome tegument is *S. mansoni* tetraspanin 2 (*Sm*TSP-2). Vaccine trials using *Sm*TSP-2 in mice have shown consistently strong protection using either fusion protein or non-conjugated peptides (Tran et al., 2006; Pearson et al., 2012). However, little is known of the biological role of this molecule in the tegument. In order to better understand this, localisation of this molecule beyond conventional fluorescence is necessary. Subcellular localisation by immuno-electron microscopy (I-EM) would be an ideal tool to define the ultrastructural localisation of the molecule, but to date this approach has been hampered by two problems. The first is that *Sm*TSP-2 loses antigenicity during the processing required for resin sections or frozen sections traditionally used for I-EM. We reason that this has arisen due to the susceptibility of antigenic sites in *Sm*TSP-2 to paraformaldehyde fixation (Tran et al., 2006). The second is that standard resin used for I-EM does not preserve membrane structure (Griffiths et al., 1993). This latter problem is a major consideration in the membrane-rich tegument as the fine detail of molecular location cannot be reliably mapped if not adequately preserved.

Here we report on the use and validation of a method for preparing adult schistosome worms, which ensures improved antigenicity and retention of surface membrane proteins such as *Sm*TSP-2. This involved the combination of the preparative methods of high pressure freezing (HPF) for electron microscopy and cryosubstitution in uranyl acetate solutions. This method follows cryopreparative methods used to characterise eggs of *S. japonicum* (Jones et al., 2008), and the human liver fluke *Opisthorchis viverrini* (Khampoosa et al., 2012), and I-EM studies of the taeniid tapeworm *Taenia ovis* (Jabbar et al., 2010b). This method, applied here to whole adult *S. mansoni* worms, allows for superior membrane preservation and successful localisation of a variety of proteins. *Sm*TSP-2, which, as noted, is aldehyde sensitive (Tran et al., 2006, 2010), labelled strongly on sections prepared by this method, enabling us to define its subcellular location in the tegument of adult male worms. We also describe the improved resolution of morphological features of the male tegument after cryosubstitution, employing methods of electron tomography to visualise membrane interactions.

2. Materials and methods

2.1. Life cycle maintenance

The Puerto Rican strain of *S. mansoni* is maintained in ARC Swiss mice and *Biomphalaria glabrata* snails at the Queensland Institute of Medical Research (QIMR, Australia) from stocks originating from the National Institute of Allergy and Infectious Diseases Schistosomiasis Resource Centre, Biomedical Research institute (Rockville, MD, USA). *Schistosoma mansoni* is maintained at QIMR under permit from the Australian Department of Agriculture, Fisheries and Forestry Biosecurity (DAFF). For cryopreparation, mice were transferred to the Centre for Microscopy and Microanalysis Laboratories, Australia in the Queensland Biosciences Precinct under permit from DAFF. All animal work in this study was approved by the Animal Ethics Committee of QIMR (Project No. P1289) and the Animal Welfare Unit of The University of Queensland. Adult worms at 6 weeks p.i. were perfused from these mice into perfusion buffer containing 145 mM sodium chloride (w/v) and 60 mM sodium citrate (w/v) prior to cryofixation. This buffer was used because it preserves the ionic balance of tissues in culture.

2.2. Cryopreparation

Adult parasites were transferred to copper membranes (Leica Microsystems, Wetzlar, Germany), flooded with 20% BSA (Fraction V, w/v) in PBS and transferred immediately to the specimen chamber of a Leica EM PACT2 High Pressure Freezer (Leica Microsystems, Australia). One parasite only was added to a single membrane. The albumin acts as a cryoprotectant and is present at the surface of the parasites for less than 1 min. After HPF, samples were transferred in cryotubes under liquid nitrogen to a Leica EM AFS freeze-substitution apparatus (Leica Microsystems) for fixation and dehydration in (i) 2% (w/v) osmium tetroxide and 0.5% uranyl acetate (w/v) in 100% anhydrous acetone, or (ii) in 0.2% uranyl acetate (w/v) and 5% water in acetone.

For samples substituted in osmium tetroxide, the tissues were cryosubstituted for 3 days, according to the following protocol. The temperature of the substitution chamber was increased from −160 to −85 °C over a 2 h period and maintained at −85 °C for 48 h, after which the samples were brought to room temperature. The fixative solutions were then replaced with anhydrous acetone corresponding to the fixation conditions at room temperature. After further changes of acetone, osmium-fixed samples were infiltrated with Epon resin (ProSciTech, Australia). Final infiltration of resin was facilitated in a Pelco 34700 Biowave Microwave Oven (Ted Pella Inc., USA) following the method of Khampoosa et al. (2012). Uranyl acetate fixed samples in acetone were transferred to Lowicryl HM20 resin (ProSciTech) at −20 °C and the resin polymerised under UV light at −20 °C in the cryosubstitution apparatus. Ultrathin sections (60 nm) were cut onto formvar-carbon coated copper slot grids. Unstained sections were examined in a JEM1011 transmission electron microscope equipped with an Olympus Morada side-mounted digital camera (Olympus, USA).

2.3. Electron tomography

For electron tomography, 300 nm thick sections of uranyl acetate fixed and Lowicryl-embedded worms were cut using Leica EM UC6 ultramicrotome (Leica Microsystems). Dual-axis tilt-series data was collected on an FEI Tecnai F30 FEG-TEM (FEI Company, USA) operating at 300 kV, over a tilt range of ±66° at 1.5° increments (*a*-axis) and 3° increments (*b*-axis), using SerialEM software (Mastronarde, 2005). Tilt series were reconstructed with the R-weighted back projection algorithm using IMOD/Etomo software (The Boulder Lab for 3D Electron Microscopy, USA) as previously described (Kremer et al., 1996). Segmentation was also done using IMOD software, specifically with "Drawing Tools" and "Interpolator", as described (Noske et al., 2008).

2.4. Ultrastructural localisation

Indirect immunocytochemistry was used to localise a series of molecules within the tegument of adult male *S. mansoni* worms.