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Polymorphism associated with the *Schistosoma mansoni* tetraspanin-2 gene

Pauline M. Cupit[a], **Michelle L. Steinauer**[b], **Bradley W. Tonnessen**[a], **L. Eric Agola**[c], **Joseph M. Kinuthia**[c], **Ibrahim N. Mwangi**[c], **Martin W. Mutuku**[c], **Gerald M. Mkoji**[c], **Eric S. Loker**[a], and **Charles Cunningham**[a,*]

[a]Center for Evolutionary and Theoretical Immunology, Department of Biology, University of New Mexico, Albuquerque, New Mexico, USA

[b]College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, USA

[c]Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi, Kenya

Abstract

A vaccine against schistosomiasis would contribute significantly to reducing the 3-70 million disability-adjusted life years lost annually to the disease. Towards this end, inoculation with the large extracellular loop (EC-2) of *Schistosoma mansoni* tetraspanin-2 protein (Sm-TSP-2) has proved effective in reducing worm and egg burdens in *S. mansoni*-infected mice. The EC-2 loop of *S. japonicum* TSP-2, however, has been found to be highly polymorphic, perhaps diminishing the likelihood that this antigen can be used for vaccination against this species. Here, we examine polymorphism of the EC-2 of Sm-TSP-2 in genetically unique worms derived from six individuals from Kisumu, Kenya.

Keywords

Schistosoma; Polymorphism; Tetraspanin-2; Genotype; Kozak sequence; Vaccine

Schistosomiasis is among the most common human parasitic diseases with an estimated 207 million people infected worldwide of which more than 90% live in sub-Saharan Africa (Hotez and Kamath, 2009). The disease is caused by trematodes of the genus *Schistosoma* and three species in particular - *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum* - are responsible for the vast majority of human infections. The chronic and debilitating nature of the disease results in high costs in public health and economic productivity in developing countries and has prompted the initiation of large scale control programs (Fenwick et al., 2009). The current treatment of choice is praziquantel and while this drug is the least expensive, easiest to use and most readily available of all current antischistosomal drugs (Hagan et al., 2004), it does not readily kill juvenile schistosomes 2-4 weeks after infection of the definitive host (Gönnert and Andrews, 1977). Without rigorous follow-up treatment, this can leave a reservoir of surviving worms to continue the

*Corresponding author. Address: Center for Evolutionary and Theoretical Immunology, Department of Biology, University of New Mexico, Albuquerque, New Mexico, 87131, USA. Tel.: +1 (505) 277 5578; fax: +1 (505) 277 0304. ccunnin@unm.edu .

cycle of infection. This, combined with the high likelihood of reinfection, suggests that praziquantel treatment alone will not significantly impact the numbers infected without also addressing problems associated with sanitation, clean drinking water and local animal reservoirs of disease. Under such circumstances, perhaps the most effective method of controlling schistosomiasis lies with the development of a vaccine.

Tetraspanins are a large superfamily of membrane proteins that are thought to be present in all metazoans. They contain four conserved transmembrane domains (TM1-TM4), intracytoplasmic and C-termini, and a small and large extracellular loop (EC-1 and EC-2, respectively) separated by a short intracellular loop (ICL) (Kovalenko et al., 2005). Tetraspanins can interact with a wide variety of proteins and function as organizers of membrane-signaling complexes (Charrin et al., 2009). While their exact function in schistosomes remains unknown, Tran et al. (2010) were able to demonstrate the importance of *S. mansoni* tetraspanin-2 (Sm-TSP-2) in correct tegument formation and subsequent survival of the parasite in its murine host using RNA interference (RNAi). When mice were immunized with the recombinant EC-2 loop (Glu107-His184) of Sm-TSP-2 followed by challenge with *S. mansoni* cercariae, a significant reduction in adult worm and liver and fecal egg burdens compared with controls was observed (Tran et al., 2006). Furthermore, individuals who were putatively resistant to *S. mansoni* were found to have significantly higher serum levels of IgG1 and IgG3 against Sm-TSP-2 than those of chronically infected individuals, suggesting the molecule's potential as a vaccine antigen.

That Sm-TSP-2 might also be effective against *S. japonicum* infections was suggested by the finding that Sm-TSP-2 and *S. japonicum* TSP-2 (Sj-TSP-2) shared more than 90% sequence identity over the first 116 amino acids (Tran et al., 2006), however, this analysis did not include the Sj-TSP-2 EC-2 domain. Cai et al. (2008) found significant sequence divergence when comparing the EC-2 domain of both species and reported that there are seven Sj-TSP-2 subclasses (Sj-TSP-2a-g) based on sequence divergence within the variable region of the EC-2 loop. When mice were immunized with a recombinant version of Sj-TSP-2c no protection against infection with *S. japonicum* cercariae was achieved, whereas immunization of mice with a mixture of recombinant proteins of all seven subclasses resulted in only a limited reduction in worm and egg burden. Cai and colleagues (2008) concluded that '…the high polymorphism of this molecule must affect its potential as a vaccine candidate'. Similarly, in an Anhui Province isolate of *S. japonicum*, significant variation at the transcriptional level in the genes encoding Sj-TSP-2 was found and vaccination of mice with a single subclass failed to provide consistent protection (Zhang et al., 2011). In striking contrast, Yuan and colleagues (2010) report that inoculation with an Sj-TSP-2 protein that is identical to Sj-TSP-2d offered a level of protection in mice infected with *S. japonicum* that is comparable to that described for Sm-TSP-2 and *S. mansoni*-infected mice.

While it is not yet clear whether Sj-TSP-2 will prove useful as a vaccine antigen, this does not rule out the possibility that Sm-TSP-2 will be successful in combating *S. mansoni* infections. Success, however, may depend on the level of polymorphism associated with Sm-TSP-2, particularly within the EC-2 domain, which is being developed as a recombinant vaccine antigen for use in phase I trials in the near future (Hotez et al., 2010). Here, we report the level of polymorphism associated with the transcript and gene sequences of 20 unique *S. mansoni* male worms derived from Kenyan field isolates. Populations of *S. mansoni* in Kenya are among the most genetically diverse in the world (Morgan et al., 2005), and thus should provide a good initial representation of Sm-TSP-2 diversity.

Since our goal was to determine the presence of nucleotide polymorphism, we first decided to establish the fidelity of the reverse transcriptase and DNA polymerase used for first strand

cDNA synthesis and subsequent PCR, respectively. These initial experiments were conducted using *S. mansoni* PR-1 harvested by perfusion of Swiss Webster (SW) mice 6 weeks p.i.. After perfusion, the parasites were washed, individual worms sliced with a clean razor blade, approximately 75% stored in RLT buffer (Qiagen) and the remaining portion in 100% ethanol for downstream RNA and genomic DNA isolation, respectively. All animal experimentation was performed in accordance with protocols approved by the University of New Mexico, USA, Institutional Animal Care and Use Committee.

Initial cloning of 30 *tsp2* transcripts from total RNA derived from 20 *S. mansoni* PR-1 using MultiScribe reverse transcriptase (Applied Biosystems) and Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) resulted in the observation of a homopolymeric tract within the sequence encoding EC-2 that was highly prone to the insertion of an extra adenine nucleotide. This was present in exactly half of the transcripts sequenced, producing a frame shift mutation and the generation of an early downstream stop codon. In order to determine whether this insertion was an artifact generated by slippage of the reverse transcriptase or DNA polymerase, the *S. mansoni* PR-1 *tsp2* transcript from 10 individual worms was recloned using AccuScript High Fidelity Reverse Transcriptase and *PfuUltra* II fusion HS DNA polymerase (Agilent Genomics, USA). Sixty *tsp2* transcripts were sequenced and no insertion was observed. The sequence obtained was confirmed through *tsp2* gene sequencing. All subsequent first strand syntheses and DNA amplifications were therefore carried out using these enzymes. In addition, to verify that a mutation was authentic rather than an artifact, at least six independent cDNA and genomic DNA clones from individual schistosomes were sequenced and mutations that were present as single events were discounted. Such mutations were, however, rare.

Although the nucleotide insertion was no longer present in the cloned PR-1 *tsp2* sequences, three other nucleotide changes were observed in all 60 sequences compared with the published coding sequence (GenBank Accession No. **AF521091**). These were C261T, C660G and C697A where nucleotide 1 is the first base of the coding sequence. While C261T and C697A were synonymous changes, C660G resulted in a stop codon that shortened the published inferred amino acid sequence of Sm-TSP-2 by 20 residues. This would have the effect of truncating the carboxy-tail of the protein but would likely not directly impact the EC-2 loop. Each of these observed mutations was also present in the published gene sequence (Accession No. **NW_003038521**). Since the TSP-2 vaccine candidate immunogen is derived from *S. mansoni* PR-1 EC-2, the *tsp2* transcript and gene sequences from individual PR-1 worms provide a framework with which to compare the field isolates.

Schistosoma mansoni field isolates were recovered from eggs in the discarded fecal samples of six adult males working as car washers in Kisumu, western Kenya. The car washers use the shallow water along the Lake Victoria shoreline to wash cars and trucks and are occupationally in contact with water in an area where snails infected with *S. mansoni* have been found repeatedly (Steinauer et al., 2008a). Eggs were isolated and hatched from these samples and the parasites passaged through laboratory-reared *Biomphalaria sudanica* snails. Cercariae shed from infected snails were then used to infect laboratory-bred mice and adult worms harvested by perfusion. Again, individual worms were sliced with a clean razor blade and preserved for downstream RNA and genomic DNA isolation.

Microsatellite genotyping of *S. mansoni* field isolates was performed in order to distinguish genetically unique individuals and avoid cloning the *tsp2* gene from clone mates. Eleven previously published microsatellite loci were amplified in two multiplexed PCRs (Steinauer et al., 2008b; Table 1). Our criterion for determining a unique individual was that genotypes had to differ by at least three of the loci assayed, a conservative measure that allows for

mutations, PCR artifacts or scoring errors. Of 80 adult male field isolates analyzed, 20 were identified as unique. The probability of identity (the average probability that two unrelated individuals drawn randomly from a population will have the same multilocus genotype) at eight of the loci by chance was calculated as 7.71×10^{-10} using Genetic Analysis in Excel (http://www.anu.edu.au/BoZo/GenAlEx/index.php), therefore it is likely that we indeed sampled 20 unique individuals. The presence of *Schistosoma rodhaini* which shares the same intermediate host as *S. mansoni* in Kenya (Morgan et al., 2003) was excluded based on the fact that our previous work has shown that alleles at two loci (**AF202966** and **AF202968**) are not shared between *S. mansoni* and *S. rodhaini* (Steinauer et al., 2008a). No alleles that characterize *S. rodhaini* were found in our specimens.

The microsatellite data also serve as a measure of the genetic diversity of individuals sampled, which should in turn serve as a comparative measure to gauge the likelihood of sampling different *tsp2* alleles (Table 1). Diversity, measured as the number of alleles sampled per locus, was lower in the current study than a previous one which sampled schistosomes from snails from the same location (Steinauer et al., 2009), but was not significantly so (paired t-test, $P = 0.0632$).

Six independent *tsp2* cDNA clones were derived from RNA extracted from each of the 20 *S. mansoni* field isolates with a unique genotype and each clone was sequenced in both directions. Compared with the published *S. mansoni* PR-1 coding sequence 10 mutations encoding six different alleles were identified. Three of these were the C261T, C660G and C697A mutations accounted for in PR-1 and were present in all cDNA sequences. This together with our own PR-1 *tsp2* data suggests that these are errors in the original sequence rather than true polymorphisms. The remaining seven mutations and their associated alleles are shown in Table 2. Mutations T75C, T312C and A504G are synonymous mutations. C178T, G282A, C488T and C553A produce L→F, M→I, S→L and L→I amino acid changes, respectively. Fig. 1 shows the location of all non-synonymous mutations and of these, only C488T is predicted to be surface exposed within the EC-2 coding region and therefore potentially accessible to antibodies when EC-2 is used as an immunogen. This relatively low level of polymorphism is in contrast to that observed by Cai et al. (2008) who examined sequence variation in isolates of *S. japonicum*. In this report, the analyses revealed seven Sj-TSP-2 homologs based on sequence diversity within the EC-2 loop. Chimeric hybrid *S. japonicum tsp2* cDNAs with the 5′ portion of one cDNA linked to the 3′ portion of another were also recognized. *Schistosoma japonicum tsp2* genes were identified that appeared to be generated by recombination of *S. japonicum tsp2* variants, suggesting that the chimeric transcripts were not artifacts produced by RNA template switching. In addition, one, two or three *tsp2* mRNA subclasses could be expressed in a single *S. japonicum* worm. In the case of *S. mansoni*, each worm was either homozygous or heterozygous for *tsp2* transcripts with no more than two different alleles being present in each worm (Table 3). Each mutation present in the *tsp2* transcripts was confirmed at the gene level as was the number of alleles present in each worm (Table 3). Thus, in contrast to *S. japonicum tsp2*, we have no evidence that *S. mansoni tsp2* undergoes gene recombination or that the transcripts are differentially spliced or form chimeras, although such mechanisms have been demonstrated to account for the polymorphism associated with *S. mansoni* mucins (Roger et al., 2008).

The amount of protein synthesized from a particular mRNA is often dependent on the strength of the Kozak sequence of that transcript. We noted two Kozak sequence variants associated with *S. mansoni tsp2;* 5′-GAAAACAUGG-3′ was found associated with alleles 1 and 5 and the PR-1 sequence while 5′-GAAGACAUGG-3′ was found with alleles 1, 2, 3, 4 and 6. Within Kozak sequences some nucleotides are thought to be more important than others in conferring the ‘strength’ of the sequence. Outwith the start codon nucleotides (+1 -

+3) those that lie at positions −3 and +4 (relative to the +1 nucleotide) appear to have the most influence on the sequence strength. For most species an A or G at −3 and a G at +4 are thought to confer the strongest sequences. Should this also prove true for *S. mansoni* then it should be expected that both of the *tsp2* Kozak sequences should be equally adept at initiating translation.

Of the 20 field isolates examined, only one possessed the C488T mutation resulting in the change of a serine to a leucine within the EC-2 loop. This exchange of a non-polar for a polar side chain may affect the structure of the loop and the long-term effectiveness of the vaccine but without further experimentation this remains conjecture. What is clear, however, is that within the cohort of worms tested there is significantly less variation in the EC-2 loop of *S. mansoni* than *S. japonicum*. Tran et al. (2006) noted that many mammalian TSP-2 molecules are able to complex with major histocompatibility complex (MHC) molecules and suggested that tetraspanin molecules in the schistosome tegument may be receptors for host ligands such as the MHC which in turn act to block the host immune response to the parasitic infection. Cai et al. (2008) have expanded on this argument by suggesting that the level of variation seen in the Sj-TSP-2 EC-2 may relate to its life history. As it is the EC-2 loop that interacts with other proteins (Stipp et al., 2003), Cai and colleagues (2008) have hypothesized that the variation in this region of Sj-TSP-2 is an adaption to overcome the polymorphism of MHC molecules from the more than 40 vertebrate reservoirs that host this parasite. The reduced level of polymorphism associated with Sm-TSP-2 may therefore reflect the fact that *S. mansoni* has a much narrower definitive host range than *S. japonicum*, with humans as perhaps the most important vertebrate host (Modena et al., 2008). Should this hypothesis be supported it would suggest that a vaccine based on Sm-TSP-2 might have an excellent chance for success in the prevention of *S. haematobium* infections as this species also predominantly infects humans.

Alternatively, *S. japonicum* may have an inherently high capacity for somatic mutation. Yin et al. (2008) have demonstrated that when snails were infected with a single *S. japonicum* miracidium at least nine multilocus genotypes were found among the worms derived from the infection. These mutations were confirmed by regenotyping and while this high level of somatic mutation has implications for population genetic analyses it may also impact coding sequences. Whether somatic mutation can account for the level of polymorphism seen in the EC-2 loop of Sj-TSP-2 is an open question.

In summary, our analysis of *tsp2* genes and transcripts from 20 genetically unique *S. mansoni* field isolates shows limited polymorphism within sequence encoding the EC-2 loop. Although a more extensive analysis of Sm-TSP-2 polymorphism in worms from a wider geographical area is required, this initial study suggests that this molecule remains a candidate vaccine antigen against *S. mansoni* and perhaps *S. haematobium* infection.

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Highlights

- Polymorphism of the tetraspanin-2 gene of *Schistosoma mansoni* was investigated.
- Twenty genetically unique *S. mansoni* field isolates were identified.
- Tetraspanin-2 genes and transcripts from these isolates were cloned and sequenced.
- There are relatively low levels of polymorphism in the gene investigated.
- Tetraspanin-2 retains its potential as an anti-schistosomal vaccine.

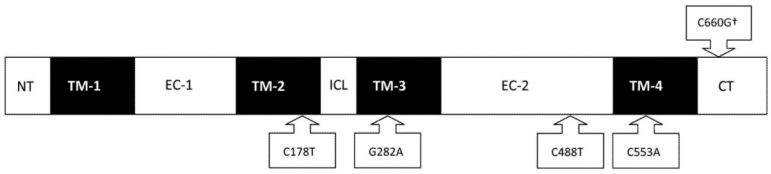

Fig. 1.

Schistosoma mansoni tetraspanin-2 protein (Sm-TSP-2) domains and their associated non-synonymous mutations. This box diagram shows the relative positions of transmembrane domains 1-4 (TM-1–TM-4), the intracellular loop (ICL) and extracellular domains 1 and 2 (EC-1, EC-2). NT and CT indicate the amino- and carboxy-terminal domains of the protein, respectively. The nature and position of non-synonymous mutations are shown in the arrowed boxes. (†) C660G was observed in all *S. mansoni tsp2* cDNA and gene sequences sampled. The original designation of a C at nucleotide position 660 is likely an error in the original published transcript sequence. This change leads to the truncation of the published CT sequence by 20 amino acids. This diagram is not to scale.

Table 1

Genetic diversity statistics for the schistosomes used in this study as determined from 11 microsatellite loci

	Microsatellite Loci Accession Numbers											Total
	AF325695	**AF325698**	**AF202965**	**AF202966**	**AF202968**	**L46951**	**M85305**	**R95529**	**AI067617**	**AI395184**	**BF936409**	
n_a[a]	11	16	6	3	5	15	9	10	3	4	7	89
Pop	11.2	18.4	6.5	2.0	3.5	15.1	13.1	12.1	6.8	5.2	7.6	101.5
H_o	0.650	0.700	0.550	0.100	0.550	0.950	0.800	0.900	0.500	0.450	0.800	0.650
H_e	0.891	0.876	0.765	0.099	0.635	0.928	0.849	0.833	0.528	0.454	0.744	0.891
F_{is}	0.276	0.205	0.287	−0.013	0.136	−0.024	0.059	−0.082	0.055	0.0087	−0.078	0.088

n_a, number of alleles for each locus; H_O, observed heterozygosityfor each locus; H_e, expected heterozygosity for each locus; F_{is}, inbreeding coefficient for each locus.

[a]The number of alleles is compared with rarified allelic richness of the schistosome population (Pop) from a previous study (Steinauer et al., 2009).

Table 2

Polymorphism of the tetraspanin-2 (*tsp2*) gene in *Schistosoma mansoni* field isolates

Allele	Mutation						
	T75C[a] G→G	C178T L→F	G282A M→I	T312C A→A	C488T S→L	A504G Q→Q	C553A L→I
1	-	-	-	-	-	-	-
2	-	-	√	-	-	-	√
3	-	-	√	√	-	√	-
4	√	-	√	-	-	-	√
5	-	√	√	√	-	√	-
6	-	-	-	-	√	-	-

[a]Number refers to nucleotide in GenBank accession no. **AF521091**

Table 3

Tetraspanin-2 (*tsp2*) alleles present in individual *Schistosoma mansoni* field isolates

Worm	Alleles	Worm	Alleles
A	1,1	K	1,3
B	1,1	L	1,6
C	1,1	M	2,3
D	3,3	N	2,3
E	3,3	O	2,3
F	1,2	P	2,3
G	1,1	Q	2,3
H	1,2	R	2,3
I	1,3	S	2,4
J	1,3	T	2,5