Highlights

- *S. mansoni* has 13 TAL proteins with differing properties
- All SmTAL proteins dimerise
- SmTAL1, 2, 8 and 12 probably bind calcium via their second EF-hands
- SmTAL7, 9, 10 and 13 probably bind calcium via their first EF-hands
- There is no evidence for ion binding by SmTAL3 and 5

1 **The tegumental allergen-like proteins of *Schistosoma mansoni*: a**
2 **biochemical study of SmTAL4-TAL13**

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Abstract

Schistosoma mansoni, like other trematodes, expresses a number of unusual calcium binding proteins which consist of an EF-hand domain joined to a dynein light chain-like (DLC-like) domain by a flexible linker. These proteins have been implicated in host immune responses and drug binding. Three members of this protein family from *S. mansoni* (SmTAL1, SmTAL2 and SmTAL3) have been well characterised biochemically. Here we characterise the remaining family members from this species (SmTAL4-13). All of these proteins form homodimers and all except SmTAL5 bind to calcium and manganese ions. SmTAL9, 10 and 11 also bind to magnesium ions. The antischistosomal drug, praziquantel interacts with SmTAL4, 5 and 8. Some family members also bind to calmodulin antagonists such as chlorpromazine and trifluoperazine. Molecular modelling suggests that all ten proteins adopt similar overall folds with the EF-hand and DLC-like domains folding discretely. Bioinformatics analyses suggest that the proteins may fall into **two main** categories: (i) those which bind calcium ions reversibly at the second EF-hand and may play a role in signalling (SmTAL1, 2, 8 and 12) **and** (ii) those which bind calcium ions at the first EF-hand and may play either signalling or structural roles (SmTAL7, 9, 10 and 13). **The remaining proteins include** those which do not bind calcium ions (SmTAL3 and 5) **and three other proteins** (SmTAL4, 6 and 11). **The roles of these proteins are less clear, but they may** also have structural roles.

Keywords: schistosomiasis; EF-hand; dynein light chain; calcium binding protein; praziquantel; tegumental allergen protein

Introduction

Schistosoma mansoni and related blood flukes from the same genus cause a substantial burden of disease on humanity. It is estimated that 207 million people are infected by *S. mansoni* and every year it accounts for up to 280,000 deaths, second only to malaria among parasitic diseases [1-3]. The infection can be treated by the drug praziquantel [4]. This is generally well tolerated and effective [5, 6]. In the absence of a vaccine, mass drug administration projects are using praziquantel to break the cycle of infection in large populations [7, 8]. Interestingly, despite widespread use of the drug, reports of resistance to it are rare [6, 9]. However, *bone fide* resistance has been generated in the laboratory and it is assumed that it will eventually emerge in clinically relevant populations of the parasite [10]. Praziquantel's molecular target and mechanism of action are unknown [6, 11-13]. However, it is well-established that one of its effects is to disrupt calcium ion homeostasis resulting in uncontrolled influx of the ion and subsequent paralysis of the organism [4]. Therefore, calcium-regulatory systems in *S. mansoni* are of interest since they may provide clues about praziquantel's mode of action or identify potential novel targets which could be antagonised by new anti-schistosomal drugs.

In trematodes, there is a family of unusual calcium binding proteins which consist of an N-terminal EF-hand containing domain and a C-terminal dynein light chain-like (DLC-like) domain [14, 15]. Typically, trematodes have multiple isoforms of these proteins and their functions are not known. In *S. mansoni* there are 13 known members of the tegumental allergen (TAL) family of proteins, referred to as SmTAL1 etc [16]. These proteins have been linked to IgE-mediated immune responses in the host [17-19]. Partial protection from infection resulted from immunisation of mice with SmTAL1 [20]. Immunisation of mice with the *Schistosoma japonicum* protein SjTP22.4 (equivalent to SmTAL11) was synergistic with PZQ in killing the worm [21]. One family member (SmTAL3) has been identified as part of a complex which also includes a component of the microtubule motor, dynein [22]. In the liver flukes *Fasciola hepatica* and *Fasciola gigantica* four members of the family have

been identified and characterised [23-30]. It seems likely that further family members will be revealed in these species once their genomes are fully annotated. The four family members from *F. hepatica* characterised so far differ in their ion and drug binding properties, but all are predicted to have similar structures and all are able to dimerise. Family members have also been identified in the Chinese liver fluke *Clonorchis sinensis* and the carcinogenic liver fluke *Opisthorchis viverrini* [31-33].

As likely calcium-signalling proteins which appear to be unique to helminths, these proteins are of interest as potential drug targets. They are also of fundamental interest given that no similar proteins have been reported in other taxa. Although the protein family has high levels of sequence and predicted structural similarity, substantial variation in their biochemical properties has been observed. For example, while the majority of family members bind to calcium ions, some (e.g. SmTAL3) do not [34, 35]. However, the *in vivo* roles for the proteins and the need for large numbers of family members in each species remain largely unclear.

We have previously characterised the biochemical properties of SmTAL1 (Smp_045200.1; Sm22.6), SmTAL2 (Smp_086480.1; Sm21.7) and SmTAL3 (Smp_086530.1; Sm20.8) [34]. Here, we extend that work by reporting the characterisation of the remaining, known SmTAL proteins, namely: SmTAL4 (Smp_169190.1), SmTAL5 (Smp_195090.1), SmTAL6 (Smp_072620.1), SmTAL7 (Smp_042140.1), SmTAL8 (Smp_086470.1; Sm21.6), SmTAL9 (Smp_077310.1) SmTAL10 (Smp_074460.1), SmTAL11 (Smp_169200.1), SmTAL12 (Smp_045010.1) and SmTAL13 (Smp_042150.1). We investigated their ion binding properties, calmodulin antagonist interactions, oligomerisation properties and predicted three-dimensional structures. Based on these data, and associated bioinformatics analyses, we propose some functional classifications for this group of proteins.

Materials and Methods

Bioinformatics and molecular modelling

SmTAL proteins were modelled using Phyre2 in the intensive mode [36, 37]. These initial models were energy minimised using YASARA [38]. Where our analyses suggested that the proteins bound calcium ions at the second EF-hand (SmTAL8 and SmTAL12; see Results), the models were aligned to the EF-hand from Reps1 (PDB: 1FI6 [39]) which was a highly ranked template for the majority of the models and contained a bound calcium ion. Where our analyses suggested that the first EF-hand binds calcium ions (SmTAL7, 9, 10 and 13; see Results), the models were aligned to human CaBP7 (PDB: 2LV7 [40]), another highly ranked template. A new structure was generated by saving the SmTAL protein together with the calcium ion in the appropriate EF-hand and then this structure was minimised using YASARA. The final, minimised models are provided as supplementary information to this paper. Ramachadran plots were calculated using RAMPAGE (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) [41].

To investigate relationships between SmTAL proteins we constructed phylogenetic trees based on Kraemer et al's analysis of S100 proteins [42]. Multiple sequence alignments were performed in MEGA, version 6.06 [43, 44], using MUSCLE with the default parameters [45, 46]. Trees were optimised for maximum likelihood analysis, and the substitution method with the lowest Bayesian information criterion (BIC) score was chosen for the final tree. Neighbour-joining analyses were also carried out using MEGA. In both cases, evolutionary distances were estimated using the JTT method [47]. Both trees were tested with 1000 bootstrap replications [48], and rooted using several representatives of another EF-hand protein, calmodulin (CaM) as an outgroup.

Expression and purification of SmTAL proteins

DNA sequences coding for SmTAL4-SmTAL13 were amplified by PCR. SmTAL4, 5 and 8 coding sequences were amplified from plasmids kindly provided by Dr Colin Fitzsimmons (University of Cambridge, UK) [16]. The remaining coding sequences were amplified from *S. mansoni* cDNA provided by the Schistosomiasis Resource Center for distribution by BEI Resources, NIAID, NIH

(Manassas, VA, USA; <https://www.beiresources.org/>) [49]. SmTAL7, 8 10 and 12 coding sequences were amplified from adult *S. mansoni* cDNA (Strain PR-1, NR-48633, Lot 62506671), SmTAL6 and 9 from miracidia cDNA (Strain PR-1, NR-48631, Lot 62506669) and SmTAL13 from cercariae cDNA (Strain PR-1, NR-48632, Lot 62506670). Primers were designed to facilitate insertion of the amplicons into pET43 Ek/LIC (Merck, Nottingham, UK) using ligation independent cloning and following the manufacturer's protocol. This vector introduces bases coding for the amino acid sequence MAHHHHHVDDDDK at the 5'-end of the amplicon facilitating the purification of the recombinant protein by metal ion affinity chromatography. Correct insertion of the amplicons was checked by PCR and sequencing of the complete gene sequences (GATC Biotech, London, UK).

All SmTAL proteins were expressed in, and purified from, *Escherichia coli*. SmTAL5 and SmTAL9 were expressed in *E. coli* Rosetta(DE3) (Merck, Nottingham, UK) and the remaining SmTAL proteins in *E. coli* HMS174(DE3) (Merck). Single, recombinant colonies containing expression vectors for SmTAL4 and SmTAL6 were picked and cultured overnight, shaking at 37 °C in 5 ml of LB (Miller) broth supplemented with 100 µgml⁻¹ ampicillin. This culture was diluted into 1 l of LB(Miller) broth supplemented with 100 µgml⁻¹ ampicillin and grown, shaking at 37 °C for 3-4 h. After this time protein expression was induced by the addition of 0.3 g IPTG and the culture was grown, shaking at 37 °C for a further 4-5 h. SmTAL7, SmTAL8, SmTAL10, SmTAL11, SmTAL12 and SmTAL13 were expressed using the same protocol except that 0.4 g IPTG was used to induce expression. For SmTAL5, a recombinant colony was picked and cultured in 5 ml LB (Miller) supplemented with 100 µgml⁻¹ ampicillin and 34 µgml⁻¹ chloramphenicol overnight shaking at 30 °C. This culture was diluted into 1 l of LB (Miller) supplemented with 100 µgml⁻¹ ampicillin and 34 µgml⁻¹ chloramphenicol and grown shaking at 30 °C for 8-9 h before induction with 0.3 g IPTG. The temperature of the culture was reduced to 16 °C and the cells grown overnight. For SmTAL9, a single recombinant colony was cultured in 5 ml LB (Miller) supplemented with 100 µgml⁻¹ ampicillin and 34 µgml⁻¹ chloramphenicol for 9 h shaking at 37 °C. This culture was diluted into 100 ml LB(Miller) supplemented with 100 µgml⁻¹ ampicillin and 34 µgml⁻¹ chloramphenicol and grown overnight shaking at 37 °C before being

diluted into 1 l of LB(Miller) supplemented with 100 μgml^{-1} ampicillin and 34 μgml^{-1} chloramphenicol and grown for a further 9 h shaking at 37 °C. Protein expression was then induced by the addition of 0.25 g IPTG, the temperature of the culture **was** reduced to 16 °C and the cells grown overnight. In all cases, cells were harvested by centrifugation at 4200 g for 20 min. Cell pellets were resuspended in approximately 25 ml buffer R (50 mM Hepes-OH, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol) and frozen at -80 °C until required.

All the SmTAL proteins were purified by cobalt affinity chromatography essentially as previously described [29]. Briefly, cells were thawed, disrupted by sonication and the suspension clarified by centrifugation (22,000 g for 20 min). The cell extract was passed over a 1 ml cobalt agarose column (His-Select, Sigma, Poole, UK), which had been equilibrated in buffer W (buffer R, except 500 mM NaCl). The column was washed in 20 ml of buffer A and SmTAL proteins eluted with two 2 ml aliquots of buffer E (buffer A supplemented with 250 mM imidazole). Protein containing fractions were identified and dialysed overnight at 4 °C against buffer D (buffer R supplemented with 1 mM DTT). Purified proteins were frozen in aliquots of 20-100 μl at -80 °C until required. Once thawed, proteins were not refrozen.

Native gel electrophoresis

SmTAL4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 were resolved by discontinuous gel electrophoresis under non-denaturing conditions to detect ion binding. Proteins (SmTAL4, 52 μM ; SmTAL5 and 10, 15 μM ; SmTAL6 60 μM ; SmTAL8, 40 μM ; SmTAL12 and 13, 20 μM ; all others 10 μM) were incubated at 37 °C for 30 min in the presence of either 2 mM EGTA or 2 mM EGTA/4 mM divalent cation in a total volume of 10 μl . Following incubation, 10 μl of native gel loading buffer (120 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 1% (w/v) DTT) was added. The proteins were electrophoresed on 15 % (w/v) polyacrylamide gels (buffer: 25 mM Tris, 160 mM glycine, pH 8.8) at

20 mA (constant current) for 4 h on ice, except for SmTAL10 (1.5 h) and SmTAL13 (3 h). Proteins were visualised using Coomassie blue stain.

Analytical methods

Protein concentrations were determined by the method of Bradford using BSA as a standard [50].

Differential scanning fluorimetry (DSF) was carried out as previously described [14, 29].

Protein-protein crosslinking was carried out using *bis*(sulfosuccinimidyl)suberate (BS³), a reagent which links exposed lysine residues [51]. SmTAL proteins (20 µM) were incubated in the presence of 2 mM EGTA or 2 mM EGTA/4mM calcium chloride for 45 min at 37 °C. BS³ (50-500 µM) was then added and the reaction allowed to proceed for 60 min at 37 °C before being terminated by the addition of SDS loading buffer (120 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 1% (w/v) DTT, 4% (w/v) SDS) and heating to 95 °C for 3 min. Reactions were analysed by SDS-PAGE.

Results

Cloning, expression and purification of SmTAL4-13

In the case of SmTAL4-12, the DNA sequence amplified from *S. mansoni* cDNA was identical in sequence to those deposited in GenBank. However, a sequence variation was detected in SmTAL13 in which codon 26 (GAG encoding threonine) is substituted for GAA (alanine). This sequence (submitted to GenBank with the accession number **KX951466**) was detected in several different amplicons, suggesting that it represents real variation in the genome rather than a PCR amplification error. All ten SmTAL proteins could be expressed in, and purified from, *E. coli* cells with yields in the milligram per litre of bacterial culture range (Supplementary Figure S1). The proteins were all released into the soluble fraction on sonication.

188

189 *SmTAL proteins have different divalent ion binding properties*

190 The mobility of all 10 SmTAL proteins was measured in native gel electrophoresis (Figure 1). A shift
191 in the mobility of the protein in the presence of calcium ions compared to the absence of calcium
192 (ensured by the addition of the specific calcium chelator EGTA) indicates a change in conformation
193 resulting from ion binding. The mobility SmTAL6, SmTAL7, SmTAL8 and SmTAL12 differed in the
194 presence of calcium chloride suggesting that these proteins are able to bind the ion (Figure 1). For
195 all of these four proteins, the mobility of the untreated, recombinant protein was less than the EGTA
196 treated protein (Figure 1). This suggests that these proteins are purified largely in the calcium ion-
197 bound form. Therefore, for all proteins, tests with other ions were carried in the presence of EGTA
198 (to remove any bound calcium ions) and a molar excess of the ion. The four proteins which were
199 shown to bind calcium ions in this assay also bound to manganese ions (Figure 1). SmTAL6 and
200 SmTAL7 also bind to **cadmium and nickel**. In addition, SmTAL6 interacted with lead ions (Figure 1).
201 In these assays, no interaction with any of the ten proteins was detected with magnesium, iron (II) or
202 potassium ions. In a number of cases the presence of ion blurred the band in electrophoresis or
203 even made it disappear entirely. Examples include cadmium with SmTAL10, cobalt (II) with SmTAL4,
204 SmTAL5, SmTAL6, SmTAL9, SmTAL10 and SmTAL11, copper (II) with SmTAL10, nickel with SmTAL4,
205 SmTAL9, SmTAL10 and SmTAL11, zinc with SmTAL10 and lead with SmTAL4, SmTAL10 and SmTAL13.
206 This is consistent with the ion causing denaturation of the protein so that it no longer runs as a
207 discrete band. In this assay, SmTAL4, SmTAL5, SmTAL9, SmTAL10, SmTAL11 and SmTAL13 were not
208 shown to interact specifically with any ion.

209 In differential scanning fluorimetry assays, all SmTAL proteins tested except SmTAL5 showed a
210 statistically significant change in thermal stability (as reflected by the “melting temperature”, T_m) in
211 the presence of calcium ions (Table 1). The same proteins showed a change in thermal stability with

manganese ions. Three of the proteins (SmTAL9, SmTAL10 and SmTAL11) also showed significant changes in the presence of magnesium ions (Table 1).

Some SmTAL proteins bind to praziquantel and to calmodulin antagonists

DSF was also used to detect interactions between SmTAL proteins and the calmodulin antagonists CPZ, W7, TFP and thiamylal. Since there are previous reports showing that the antischistosomal drug PZQ interacts with the calmodulin-like protein myosin regulatory light chain and with SmTAL1, we also tested this drug [12, 34]. CPZ affected the T_m of SmTAL5, SmTAL9, SmTAL12 and SmTAL13 (Table 2). W7 affected just SmTAL9, SmTAL12 and SmTAL13, whereas TFP interacted with the majority of the proteins (i.e. SmTAL4, SmTAL5, SmTAL8, SmTAL9, SmTAL12 and SmTAL13). PZQ interacted with SmTAL4, SmTAL5 and SmTAL8. In the case of SmTAL6, SmTAL7, SmTAL10 and SmTAL11, no interaction was detected with any of the drugs used in these experiments.

All the SmTAL proteins homodimerise

In protein-protein crosslinking experiments, a new band corresponding to approximately twice the molecular mass of SmTALs was seen in all cases (Figure 2). For some proteins (notably SmTAL9), this band was present even in the absence of crosslinker, suggesting a very high affinity interaction which was not completely dissociated under the conditions of SDS-PAGE. This effect was also seen in some of the purification gels (Supplementary Figure S1) where, in general, the protein concentrations were higher. In some cases, notably SmTAL10, higher molecular mass species were also detected following crosslinking (Figure 2). It is, therefore, possible that some of the proteins exist in higher order oligomers or aggregates in addition to dimers.

The SmTAL family proteins are predicted to have a broadly similar fold

236 Molecular modelling of the 10 SmTAL proteins revealed considerable similarities (Figure 3). Each
 237 had a compact, N-terminal domain with two EF-hand structures present and a C-terminal DLC-like
 238 domain largely composed of β -sheet structures. These two domains are joined by a linker, which is
 239 predicted to lack any defined secondary structure and is, most likely, quite flexible. The mobility in
 240 this part of the proteins means that the two domains are likely to adopt a range of orientations with
 241 respect to each other. There is also some variation in the length of the linker which is also likely to
 242 affect the relative orientations of the two globular domains. The most extreme case of this is
 243 SmTAL8 which has a 31 residue linker resulting in considerable separation between the two domain
 244 (Figure 3). In the case of SmTAL13 where our sequence differs from that previously reported, we
 245 made models corresponding to both sequences. The N-terminal domains (residue 1-76) of these
 246 models align well with a root means square deviation (rmsd) of 0.826 Å over 995 equivalent atoms,
 247 as do the C-terminal domains (residues 106-176; rmsd of 1.307 Å over 878 equivalent atoms).
 248 Although the overall fold of the EF-hand domain is well conserved between the ten proteins, the
 249 sequence in the two EF-hands varies considerably. A typical EF-hand provides six ligands from the
 250 protein to coordinate the ion, which are arranged approximately at right angles to each other.
 251 Consequently, they are known as the X, Y, Z, -X, -Y and -Z groups [52]. Detailed bioinformatics
 252 studies have identified the residues most commonly found at these positions. Aspartate is favoured
 253 at X, Y, Z and -X although considerable variation is possible at -X. At the -Z position, glutamate is
 254 favoured, although aspartate is used in a minority of cases. The -Y position is unique among the six
 255 in that the backbone carbonyl, not side chain groups are used for coordination. Threonine is the
 256 preferred residue at this position, but a wide variety of alternative residues are possible. Between
 257 the Z and -Y residues a glycine is almost always found to facilitate the tight turn which enables the
 258 EF-hand to wrap around the ion [52]. In previous work on FhCaBP2, the second EF-hand was shown
 259 to be the main site of interaction with calcium and manganese ions. This EF-hand largely conforms
 260 to the consensus, with a lysine at position -Y [26].

261 The first EF-hand deviates from the consensus in almost all of the 10 SmTAL proteins studied here.
262 Only SmTAL10 and SmTAL13 have the central glycine residue required to create the tight turn in the
263 middle of the motif. In general, the lack of the glycine appears not to affect the fold of the EF-hand
264 greatly. This could result from the use of homology modelling which estimates structures by
265 comparison to the closest available experimentally determined ones. This might be expected to
266 “force” residues into modelled structures which are not, in reality, favoured. Examination of the
267 Ramachandran plots for the first EF- hands for all ten proteins showed that, for SmTAL4, SmTAL6,
268 SmTAL9, SmTAL10 and SmTAL13 all the residues were in the “favoured” regions of the plot. For the
269 remainder of the proteins, some residues (typically the residue on the turn or those adjacent to it)
270 were in the “allowed” or “outlier” regions of the Ramachandran plot (data not shown). This suggests
271 that the EF-hand motif may be distorted from the ideal conformation in SmTAL5, SmTAL7, SmTAL8,
272 SmTAL11 and SmTAL12.

273 In general, the second EF-hands in the ten SmTAL proteins are closer to the consensus. All but
274 SmTAL6, SmTAL8 and SmTAL12 have the central, conserved glycine residue. Only SmTAL4’s second
275 EF-hand has a residue in the outlier region of the Ramachandran plot (Phe-55); SmTAL11 and
276 SmTAL12 have one residue in the allowed region (Thr-50 and Lys-52 respectively). All the remaining
277 proteins’ second EF-hands have all the residues in the favoured region. However, the residue at the
278 –Z position only conforms to the consensus (Glu) in the case of SmTAL8 and SmTAL12, suggesting
279 that these two proteins interact with calcium ions through the second EF-hand. The other proteins
280 have a range of residues which are all unable to provide oxygen atoms in their side chains for
281 coordination of the ion. While this may suggest that these EF-hands are unable to bind calcium, the
282 experimental evidence presented here clearly demonstrates that the majority of these proteins do
283 so. Therefore, the ion must either bind elsewhere (most likely the first EF-hand) or the EF-hand
284 must function differently to “classical” ones.

285

Bioinformatics analyses suggest functional groupings for this protein family

To assist understanding of the different biochemical properties of these proteins we conducted a phylogenetic analysis of all 13 SmTAL proteins, together with known family members from other trematodes using calmodulins as an out-group. Both maximum likelihood and neighbour-joining analyses gave similar results which revealed two clades (Figure 4). One clade includes SmTAL1, 2, 8 and 12. It also includes all four *F. hepatica* CaBPs (and their *F. gigantica* homologues). FhCaBP2 has been shown to interact with calcium primarily through its second EF-hand and sequence similarities at the EF-hands suggests that the same will be true for the other three *F. hepatica* CaBPs [14, 26]. In the four SmTAL proteins in this clade, the second EF-hand conforms to the consensus at the –Z position (Glu) suggesting that they also bind calcium at this site. Typically these SmTAL proteins are also associated with larger changes in thermal stability, suggesting greater conformational change on binding (Table 1 and [34]). This suggests a role for these proteins in calcium-mediated cellular signalling.

The second clade includes all the remaining SmTAL proteins (SmTAL3, 4, 5, 6, 7, 9, 10, 11, 13) alongside similar proteins from other trematodes (Figure 4). Four of these proteins (SmTAL7, 9, 10, 13) lack the critical glutamate in their second EF-hand sequences and are likely to bind divalent ions mainly at the first EF-hand. This conclusion is supported by the presence of the *C. sinensis* protein CsTAL3 (Cs20.8) in this clade. X-ray crystallographic studies have clearly demonstrated that this protein interacts through the first EF-hand [53]. However the biochemical properties of SmTAL proteins from this clade are diverse. Thus, this group may contain proteins with a mainly structural role and those which are involved in calcium signalling using the first EF-hand.

The remaining proteins (SmTAL3, 4, 5, 6 and 11) include proteins with no detectable divalent ion binding activity (SmTAL3 and SmTAL5) with some which have been shown to bind ions (SmTAL4, 6 and 11) (Figure 1, Table 1, Table 3 [34]). SmTAL3 has been shown to form part of a high molecular mass complex [22]. This protein, and also SmTAL5, readily form higher order oligomers in

crosslinking experiments (Figure 2). This suggests that at least some of these proteins may have structural, rather than signalling, roles.

Discussion

Despite similarity in sequences and predicted domain organisation there is known to be considerable variation in the biochemical properties of this protein family [14]. Therefore, it was not surprising to discover diversity in this group of ten SmTAL proteins. The results from binding experiments described in this paper and our previous work are summarised in Table 3 [34]. Native gel electrophoresis demonstrated different ion binding properties for the ten proteins. In this assay calcium ion binding was not detected for six of the proteins. However, it should be noted that this assay will only detect ion binding where the interaction is long-lived under the conditions of the experiment and results in a conformational change which alters the electrophoretic mobility of the protein. Therefore, while the presence of a shift is strong evidence of ion binding, the absence of a shift does not necessarily indicate that there is no affinity for that ion. In DSF, calcium binding was detected for all of the proteins, except SmTAL5. Therefore, we conclude that SmTAL5, like SmTAL3, has no significant calcium binding activity [34].

The location of the calcium binding site(s) in the remaining SmTAL proteins is not always clear. While the known members of the FhCaBP family appear to bind divalent ions primarily through the second EF-hand, this seems unlikely to be the case for all SmTAL proteins. Based on our structural predictions and bioinformatics analyses, we hypothesise that SmTAL1, 2, 8 and 12 all interact through the second EF-hand. These four proteins are grouped in the same clade as the FhCaBP family and, except for SmTAL8, have a second EF-hand that conforms well to the consensus sequence. SmTAL10 and 13 are likely to bind through the first EF-hand. For these two proteins, the second EF-hand lacks the glycine residue required for the tight turn, but this residue is present in the first EF-hand. They also both appear in the second clade in our bioinformatics analysis. It is

tempting to extend this argument to the other two SmTAL proteins in this clade (SmTAL7 and 9). While the *F. hepatica* proteins FhCaBP1, FhCaBP2, FhCaBP3 and FhCaBP4 all appear to interact primarily through the second EF-hand, there is experimental evidence for members of this protein family interacting through the first EF-hand [23, 25, 26, 29]. The crystal structure of the EF-hand domain from the *C. sinensis* protein, CsTAL3 has been solved and clearly demonstrates that this protein interacts through the first EF-hand [53]. X-ray scattering data on the full-length protein in solution suggested that it undergoes conformational changes on binding to calcium ions, becoming more extended in response to ion binding. This suggests that binding at the first EF-hand can also elicit the kind of conformational changes involved in signalling [53]. The situation remaining SmTAL proteins for which there is evidence of calcium binding (SmTAL4, 6 and 11) is less clear. These protein sequences were not clearly grouped in our bioinformatics analysis. Further experimental work will be required to confirm the locations of divalent cation binding sites in the SmTAL protein family.

It is reasonable to assume that all 13 TAL proteins arose from a single ancestral protein which almost certainly bound calcium ions. If this is the case, then SmTAL3 and SmTAL5 have lost the ability to bind calcium through evolution. This suggests that, while the majority of SmTAL proteins reversibly interact with calcium ions in a functionally important way, there are calcium-independent functions of some family members. Other than a single report that SmTAL3 forms part of a larger complex which includes dynein components, no functional information is available for the SmTAL family [22]. The requirement for 13 different family members is also an unresolved mystery. We hypothesise that the majority of the proteins function in calcium signalling processes and that their functions are likely to partially overlap. The two non-calcium binding SmTAL proteins most likely function as structural components of larger complexes. Given the parasite's requirement to live in two hosts (a warm-blooded mammal and a cold-blooded snail) as well in several free living forms, it is possible that different family members **fulfill** similar role(s) but in different life cycle stages. Consistent with this hypothesis, mRNA coding for the 13 SmTAL proteins show different expression profiles [16].

While some of the mRNA molecules are expressed in all life cycle stages (e.g. SmTAL2, 7, 8 and 12), others are only produced early or late in the cycle. SmTAL6 mRNA is produced only in eggs, miracidia and sporocysts. In contrast, SmTAL3 and 11 mRNA are mainly produced in adult worms [16]. Of course, mRNA expression does not always correlate perfectly with the presence of the corresponding proteins. Some proteins can have long half-lives and persist after the corresponding mRNA is degraded. Indeed some human calcium binding proteins can persist in cells for days or weeks [54, 55]. Nevertheless, these differences in mRNA expression strongly suggest that the levels of each SmTAL protein will vary across the life-cycle. We did not observe any obvious correlation between the thermal stability of the proteins and their life cycle stage – for example SmTAL6 (whose mRNA is produced in the eggs) has the highest melting temperature in the DSF assay.

Although we have been able to make some broad functional predictions about these proteins based on sequence analysis and our biochemical data, further experiments will be required to elucidate their roles. Based on our drug-binding experiments, it seems unlikely that it will be possible to identify compounds which selectively antagonise one family member. Therefore, genetic methods are more likely to be successful in obtaining worms which lack a functional form of one family member. RNAi methods are now well-established in *S. mansoni* although some caution would be required since the proteins may have long half-lives (see above) [56]. CRISPR/Cas9 gene editing techniques may also become established for *Schistosoma* spp and some enabling technologies have already been reported [57]. These methods may enable the selective knock-down or knock out of the individual family members and studies on the resulting phenotype to be undertaken. Classical biochemical techniques may also be useful. Affinity chromatography or pull-down experiments could be used to identify protein binding partners within cell extracts. The most commonly used forms of these assays tend to identify mainly soluble proteins. Given the SmTAL proteins' likely role in calcium signalling, it is possible that they interact with membrane bound or associated proteins (e.g. subunits of the voltage-gated calcium channels). Therefore, methods which enable the capture and identification of membrane proteins should also be used [58]. Such studies will be potentially

388 valuable since they will permit the inference of functional roles for the SmTAL family members and
389 may provide some clues into their different roles within the parasite.

390 The tantalising finding that praziquantel interacts with SmTAL1, 4, 5 and 8 suggests that these
391 proteins may be pharmacologically important. This further suggests that novel drugs might be
392 designed to antagonise the functions of family members. The protein kinase inhibitor, staurosporine
393 has been identified by *in silico* methods as a likely binding partner for *C. sinensis* protein CsTegu20.6.
394 This protein is a member of the same protein family as the SmTAL proteins [33]. Although this has
395 not been tested experimentally, it does suggest that there are druggable pockets in these proteins
396 which could be exploited in drug discovery. Overall, the work to date on this protein family suggests
397 that they perform diverse roles at various life cycle stages in trematodes. Further elucidation of
398 these roles is likely to increase our understanding of the basic biochemistry of these organisms and
399 may pave the way to novel treatments for the infections they cause.

400

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406

Figure legends

Figure 1: Ion binding of SmTAL proteins. Native gel electrophoresis (for conditions, see Materials and Methods) was used to assess binding to a range of divalent metal cations. A discrete shift in electrophoretic mobility (marked with an asterisk, *) indicates interaction with the ion.

Figure 2: Homodimerisation of SmTAL proteins. Protein-protein crosslinking was used to assess the ability of the SmTALs to dimerise. The appearance of additional band (**) at approximately twice the molecular mass of the native protein (*) indicates dimerization. In some cases (notably SmTAL5, 10 and 11) additional bands at higher molecular masses were detected suggesting oligomerisation or aggregation of these proteins also occurs. Note that SmTAL9 has a detectable amount of dimer present even in the absence of crosslinking agent. In each gel: M, molecular mass markers (116, 66, 45, 35, 25, 18, 14 kDa); U, untreated protein (20 μ M); numbers, protein treated with the corresponding concentration (in μ M) of BS³. EGTA indicates experiments done in 2 mM EGTA; Ca²⁺, experiments done in 2 mM EGTA/4 mM calcium chloride. For other experimental conditions, see Materials and Methods.

Figure 3: Predicted EF-hand structures from SmTALs. The overall fold of the SmTAL proteins was predicted by molecular modelling (see Materials and Methods). In each case, the EF-hand domain is shown on the left and the DLC-like domain on the right. Calcium ions are shown in the first or second EF-hand according to the predictions made in this paper. Where no calcium ion binding is predicted, or the position of the ion is uncertain, the structure is shown with no ions bound.

Figure 4: Protein sequence analysis of the SmTAL family and related proteins. (a) Maximum likelihood and (b) neighbour-joining analyses were conducted as described in the Methods. Calmodulin (CaM) sequences were used as an outgroup. This figure displays the bootstrap consensus trees (1000 replicates). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Accession codes: FhCaBP1 (AML33332); FhCaBP2 (AJF23779); FhCaBP3 (AFX60920); FhCaBP4 (AFM84632); FgCaBP1

432 (AAZ20312); FgCaBP3 (AEX92828); FgCaBP4 (AEX92829); Cs GAA37705 (GAA37705); Cs GAA47752
 433 (GAA47752); Cs GAA56892 (GAA56892); Cs20.8 (ABC47326); Cs21.6 (AEI69651); Cs21.7 (GAA49984);
 434 Cs22.3 (ABK60085); OvT265 08981 (XP_009173195); OvT265 10763 (XP_009175494); Sh21.7
 435 (XP_012797374); Sh22.6 (BAF62289); Sh_teg_ant (SmTAL3) (XP_012797371); Sh_teg_ant (SmTAL8)
 436 (XP_012797375); Sh_teg_ant (SmTAL11) (XP_012797369); ShTAL4 (XP_012797370); ShTAL5
 437 (XP_012797368); ShTAL9 (XP_012797728); ShTAL12 (XP_012799748); ShTAL13 (XP_012795330);
 438 ShMS3 03822 (XP_012795331); ShMS3 04275 (XP_012795756); Sj20 (AAP06272); Sj22.6
 439 (AAB52407); Sj_teg_ant (CAX72713); Sj_EF_prot (CAX73132); SjTAL9 (CAX73272); SJCHGC00558
 440 (AAW26845); SJCHGC01853 (AAW25529); SJCHGC06339 (AAP06136); SJCHGC08815 (AAX27568);
 441 SJSCG09029 (AAW26125); **SmCaM1 (ADW78835), SmCaM2 (ADW78836); FhCaM (CAL91032);**
 442 **EmCaM (CDS37648); HmCaM (CDS28106); DjCaM (BAD88634); HsCaM (AAD45181).**
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Table 1: Thermal stability (T_m in °C) of SmTAL proteins in the presence and absence of selected divalent metal ions.

Protein	Untreated	EGTA	CaCl ₂	MnCl ₂	MgCl ₂
SmTAL4	59.1± 0.2 ^{ns}	58.7± 0.2	60.3± 0.4****	60.4± 0.1****	58.9± 0.1 ^{ns}
SmTAL5	47.6± 0.1 ^{ns}	47.9± 0.1	47.7± 0.4 ^{ns}	47.8± 0.2 ^{ns}	48.0± 0.3 ^{ns}
SmTAL6	73.9± 0.4 ^{ns}	74.1± 0.1	76.5± 0.3****	68.6± 0.1****	74.1± 0.3 ^{ns}
SmTAL7	64.8± 0.3*	63.9± 0.4	69.5± 0.3****	67.6± 0.1****	64.6± 0.3 ^{ns}
SmTAL8	58.7± 0.2****	61.4± 0.4	59.3± 0.3***	60.0± 0.7**	61.2± 0.3 ^{ns}
SmTAL9	68.6± 0.1 ^{ns}	68.8± 0.2	59.5± 0.0****	53.6± 0.2****	62.5± 0.0****
SmTAL10	67.6± 0.5****	64.5± 0.3	72.0± 0.2****	73.8± 0.3****	74.5± 0.0****
SmTAL11	49.8± 0.4****	47.2± 0.4	49.5± 0.0****	49.4± 0.1****	50.1± 0.1****
SmTAL12	69.7± 0.5**	66.8± 0.2	71.5± 0.2****	71.4± 0.1****	63.1± 1.4 ^{ns}
SmTAL13	63.2± 0.1 ^{ns}	63.2± 0.2	62.3± 0.3***	60.6± 0.2****	63.1± 0.1 ^{ns}

In all cases, the protein concentration was 10 µM buffered by Hepes-OH (20 mM; pH 7.4). Where present, EGTA was 1 mM. Ions were present at 2 mM with 1 mM EGTA. Significance was determined *versus* EGTA treatment group using ANOVA (n = 3; significance threshold of $p \leq 0.05$) and Tukey's post-hoc test (mean of each group tested against the mean of every other group). ^{ns}, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

Table 2: Thermal stability (T_m in °C) of SmTAL proteins in the presence and absence of selected drugs.

Protein	Untreated (+Calcium)	DMSO	PZQ	CPZ	W7	TFP	ThA
SmTAL4	60.7± 0.2 ^{ns}	60.7± 0.3	60.1± 0.1**	60.3± 0.2 ^{ns}	60.5± 0.0 ^{ns}	60.2± 0.2*	60.6± 0.1 ^{ns}
SmTAL5	47.5± 0.2 ^{ns}	47.7± 0.3	46.4± 0.7*	45.8± 0.3***	47.8± 0.3 ^{ns}	44.4± 0.4****	47.3± 0.4 ^{ns}
SmTAL6	76.7± 0.2 ^{ns}	76.4± 0.2	76.3± 0.2 ^{ns}	76.2± 0.2 ^{ns}	76.2± 0.2 ^{ns}	76.4± 0.1 ^{ns}	76.1± 0.1 ^{ns}
SmTAL7	69.6± 0.4 ^{ns}	69.5± 0.2	69.3± 0.2 ^{ns}	69.2± 0.0 ^{ns}	69.1± 0.3 ^{ns}	68.9± 0.2 ^{ns}	69.4± 0.3 ^{ns}
SmTAL8	58.8± 0.4 ^{ns}	58.6± 0.4	56.6± 0.1****	58.7± 0.4 ^{ns}	58.4± 0.2 ^{ns}	57.2± 0.3***	58.6± 0.2 ^{ns}
SmTAL9	59.5± 0.0 ^{ns}	59.8± 0.1	59.9± 0.1 ^{ns}	59.2± 0.2***	58.9± 0.1****	58.9± 0.1****	59.9± 0.1 ^{ns}
SmTAL10	72.1± 0.4 ^{ns}	71.6± 0.9	71.7± 0.3 ^{ns}	71.6± 0.1 ^{ns}	71.7± 0.3 ^{ns}	71.3± 0.4 ^{ns}	71.5± 0.9 ^{ns}
SmTAL11	49.3± 0.3 ^{ns}	49.2± 0.3	49.1± 0.1 ^{ns}	49.4± 0.2 ^{ns}	49.6± 0.2 ^{ns}	49.1± 0.1 ^{ns}	49.3± 0.2 ^{ns}
SmTAL12	71.4± 0.1 ^{ns}	71.1± 0.1	70.8± 0.2 ^{ns}	70.5± 0.0***	70.6± 0.1**	69.8± 0.2****	70.9± 0.1 ^{ns}
SmTAL13	62.4± 0.1 ^{ns}	62.3± 0.2	61.8± 0.4 ^{ns}	60.6± 0.2****	61.1± 0.1****	60.4± 0.1****	62.2± 0.1 ^{ns}

SmTAL proteins were present at 10 µM in the presence of EGTA (1 mM)/calcium chloride (2 mM) and buffered by Hepes-OH (20 mM; pH 7.4). All drugs were present at a concentration of 250 µM with DMSO at 1%(v/v). Significance was determined by comparison to the DMSO treated group using ANOVA (n = 3; significance threshold of $p \leq 0.05$) and Tukey's post-hoc test (mean of each group tested against the mean of every other group). ^{ns}, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. PZQ, praziquantel; CPZ, chlorpromazine; W7, *N*-(6-Aminohexyl)-1-chloro-naphthalene-5-sulfonamide; TFP, trifluoperazine; ThA, thiamylal.

Table 3: Summary of experimental results from binding experiments.

Protein	Ions bound (native gels)	Ions bound (DSF) ²	Drugs bound (DSF)	Phylogenetic grouping
SmTAL1 ¹	Ca ²⁺ , Mn ²⁺ , Ni ²⁺ , Sr ²⁺	Ca ²⁺	PZQ, CPZ, W7, TFP	
SmTAL2 ¹	Ca ²⁺ , Mn ²⁺ , Mg ²⁺	nd	nd	
SmTAL3 ¹	None	None	CPZ, W7, TFP	
SmTAL4	None	Ca ²⁺ , Mn ²⁺	PZQ, TFP	
SmTAL5	None	None	PZQ, CPZ, TFP	
SmTAL6	Ca ²⁺ , Mn ²⁺ , Ni ²⁺ , Pb ²⁺	Ca ²⁺ , Mn ²⁺	None	
SmTAL7	Ca ²⁺ , Mn ²⁺ , Ni ²⁺	Ca ²⁺ , Mn ²⁺	None	
SmTAL8	Ca ²⁺ , Mn ²⁺	Ca ²⁺ , Mn ²⁺	PZQ, TFP	
SmTAL9	None	Ca ²⁺ , Mn ²⁺ , Mg ²⁺	CPZ, W7, TFP	
SmTAL10	None	Ca ²⁺ , Mn ²⁺ , Mg ²⁺	None	
SmTAL11	None	Ca ²⁺ , Mn ²⁺ , Mg ²⁺	None	
SmTAL12	Ca ²⁺ , Mn ²⁺	Ca ²⁺ , Mn ²⁺	CPZ, W7, TFP	
SmTAL13	None	Ca ²⁺ , Mn ²⁺	CPZ, W7, TFP	

¹ Data from previous work of Thomas et al. Note that this study considered a slightly smaller range of ions in the native gel and DSF experiments. It was not possible to determine a T_m value for SmTAL2 and so ion or drug binding by DSF were not determined (nd).

² Only calcium (SmTAL1-3) or calcium, magnesium and manganese (SmTAL4-13) ions were considered in this experiment

None means “none of those ions or drugs tested”.

Figure 1

Figure 2

Figure 3

SmTAL4

SmTAL5

SmTAL6

SmTAL7

SmTAL8

SmTAL9

SmTAL10

SmTAL11

SmTAL12

SmTAL13

Figure 4
(a)

(b)

Supplementary Figure S1

[Click here to download Supplementary Material: Figure S1 purification.pptx](#)

SmTAL4 molecular model

[Click here to download Supplementary Material: SmTAL4-mini.pdb](#)

SmTAL5 molecular model

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SmTAL6 molecular model

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SmTAL8 molecular model

[Click here to download Supplementary Material: SmTAL8-Ca2-mini.pdb](#)

SmTAL11 molecular model

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SmTAL12 molecular model

[Click here to download Supplementary Material: SmTAL12-Ca2-mini.pdb](#)

SmTAL7 molecular model

[Click here to download Supplementary Material: SmTAL7-Ca1-mini.pdb](#)

SmTAL9 molecular model

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SmTAL10 molecular model

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SmTAL13 molecular model

[Click here to download Supplementary Material: SmTAL13-CT-Ca1-mini.pdb](#)