

Characterisation of Sm20, a 20-kilodalton calcium-binding protein of *Schistosoma mansoni*

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A cDNA clone encoding part of a 20-kDa antigen of *Schistosoma mansoni* (Sm20) has been isolated. The amino acid sequence of this antigen, as predicted from the sequence of the cDNA, has significant homology to the family of calcium binding proteins which include calmodulin, troponin C and the light chain of myosin. Although we have been unable to show any immunological cross-reactivity between Sm20 and calmodulins from a range of other species, we have verified that Sm20 is a functional calcium binding protein. Sm20 is encoded by a small multigene family and is expressed in schistosomula and adult worms but not in eggs. The 20-kDa nascent polypeptide appears to be post-translationally modified to give a 38-kDa species. Sm20 is present in preparations of tegumental membranes and is easily removed from intact schistosomula by detergent treatment, suggesting that it is associated with the tegument. However, the cloned portion does not appear to be exposed on the surface.

Key words: Calcium; Calmodulin; *Schistosoma mansoni*; Tegument

Introduction

The intracellular calcium ion concentration of eukaryotic cells is closely regulated. In response to extracellular signals, the intracellular level of Ca^{2+} transiently increases and functions as a second messenger to induce a wide variety of calcium mediated physiological responses. The Ca^{2+} signal is transduced by a variety of calcium-binding proteins, which in turn transmit the signal to specific sub-cellular compartments. Many of these

calcium-dependent responses are mediated by calmodulin, an ubiquitous intracellular calcium binding protein. In addition, control of specific calcium dependent processes, such as muscle contraction, is mediated by specialised calcium binding proteins such as troponin C and the light chain of myosin.

In schistosomes the regulation of calcium levels is also important. Electron probe X-ray analysis of adult female and adult male worms [1] shows that females contain more calcium. This is most likely due to the calcareous corpuscles found in stage 4 vitelline cells of the female reproductive tract and must involve a specific mechanism for calcium accumulation in these cells. The secretory bodies in the pre-acetabular glands of cercariae also contain high levels of calcium [2]. This is thought to inhibit the proteases also found in the gland until penetration of the skin requires their activation. In addition, calcium is involved in the regulation of muscle contraction in schistosomes but can be disrupted by praziquantel or oxamniquine in a calcium-dependent manner [3,4].

Consequently, a number of calcium binding

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Abbreviations: GST, glutathione *S*-transferase; ORF, open reading frame; PBS, phosphate-buffered saline; SSC, saline sodium citrate.

proteins should exist in schistosomes which mediate the above processes. A calmodulin-related antigen has been purified and characterised from adult worms of *Schistosoma mansoni* by classical biochemical techniques. [5]. It is a 19-kDa peptide representing 0.5% of the total protein in adult worms as judged by radioimmunoassay with an antiserum against rat calmodulin. An 8-kDa calcium binding protein expressed only in cercariae [6] has also been identified. This paper describes the cloning and characterisation of a 20-kDa calcium binding protein which is associated with the tegumental membrane and has homology with the calmodulin family of calcium-binding proteins.

Materials and Methods

Parasite material. A Puerto Rican strain of *S. mansoni* was maintained in BALB/c mice and in the snail *Biomphalaria glabrata*. Adult worms were recovered by perfusion of the hepatic portal system [7]. After washing in saline, the parasites were frozen and stored in liquid nitrogen. For some experiments adult worms were derived from cercariae obtained from infected snails collected from endemic areas of Kenya.

Indirect immunofluorescence antibody assay. This was performed on live intact schistosomula as previously described [8].

Production of antisera. Antisera raised in rabbits against purified schistosomulum tegumental membranes (DT3, DT7, DT8) [9] and total bacterial extracts of λ gt11 lysogens [10] were prepared as previously described.

Sera raised against the size-fractionated, detergent-soluble proteins stripped from intact schistosomula (Rb 16 to 20) were prepared as follows: 300 000 18-h mechanically prepared schistosomula were incubated at 4°C for 20 min in 1% deoxycholate. Denuded bodies were removed by centrifugation and SDS added to the supernatant to a final concentration of 0.1%. This material was boiled for 10 min then concentrated to 100 μ l by ultrafiltration on a Diaflo YM10 membrane (Amicon Corporation). Fractionation by gel filtration was achieved on a 7.5 $\times$ 600 mm TSK-G4000SW HPLC column (LKB) equilibrated in

0.1% SDS in PBS, pH 7.2, 0.5-ml fractions were collected and analysed by SDS-PAGE and silver staining. Adjacent fractions were pooled, and 5 apparently non-overlapping 1-ml pools were used to immunise rabbits (0.2 ml per injection). The immunisation procedures were as described previously [10].

Antiserum against bovine brain calmodulin raised in goats was purchased from Sigma.

Expression of Sm20 in pGEX. The 260 bp fragment of A1.11 was subcloned into the *Eco*RI site of the expression vector pGEX1 [11]. The fusion protein (Sm20GST) and the carrier protein alone (GST) were purified as described [11].

In vitro translation, immunoprecipitation and Western blotting. These were performed as previously described [10]. Gel samples of parasite material for SDS PAGE analysis were prepared as follows: 1 g of adult worms was homogenised in 1 ml of PBS, then centrifuged at 12 000 $\times$ g for 10 min. SDS gel sample buffer [12] was added to the resultant supernatant. 35 000 cercariae, 60 000 schistosomula or 300 000 eggs were solubilised by boiling in 1 ml of SDS sample buffer.

Calcium shift experiment. Calmodulin from bovine brain, human erythrocytes, *Neurospora crassa* and spinach were purchased from Sigma. Samples were resuspended in SDS gel sample buffer containing either 25 mM CaCl_2 or 2.5 mM EGTA and run on SDS-PAGE gels [12] containing 25 mM CaCl_2 or 2.5 mM EDTA [5].

Calcium binding assay. Proteins were western blotted as above then the nitrocellulose filter was incubated with ^{45}Ca , washed and autoradiographed as described [13].

cDNA library and production of lysogens. A cDNA library derived from mRNA of adult worms was cloned into λ gt11 [10]. Lysogens were produced from individual phage as previously described [10].

DNA sequencing. This was according to the dideoxy chain terminator method of Sanger [14] with the modifications required for the enzyme

Sequenase (United States Biochemical Corporation).

Northern and Southern blotting. Genomic DNA was prepared by the sarkosyl method. 1 g of adult worms was ground to a fine paste in liquid nitrogen, then 2 ml 0.5 M EDTA, 0.15% Sarkosyl, 100 $\mu\text{g ml}^{-1}$ proteinase K were added. The mixture was incubated at 50°C for 4 h, phenol/chloroform-extracted twice, and dialysed extensively against 50 mM Tris/10 mM EDTA/10 mM NaCl, pH 8. RNase A was added to a final concentration of 40 $\mu\text{g ml}^{-1}$ and the mixture incubated at 37°C for 1 h. Following three phenol/chloroform extractions, the preparation was dialysed against 10 mM Tris/1 mM EDTA, pH 8.

DNA was restricted under standard conditions [15] and electrophoresed on 0.8% agarose gels in TEA buffer (40 mM Tris, 16 mM EDTA, 2 mM Na-acetate, pH 8.1). The DNA was visualised by staining with 1 $\mu\text{g ml}^{-1}$ ethidium bromide, then transferred to nitrocellulose by the method of Southern [16]. RNA was separated on 1.0% agarose gels containing 2.2 M formaldehyde [17] and transferred to nitrocellulose as above.

Radiolabelling of DNA and hybridisation. Probes were prepared by the random priming method [18], but using the Klenow fragment of DNA polymerase I. Hybridisations were at 42°C in 50% formamide; washing was at high stringency (0.1 $\times$ SSC/0.1% SDS, 53°C).

Results

Cloning of a 20-kDa antigen. cDNA was synthesised from mRNA isolated from adult worms of *S. mansoni* and a library constructed in the expression vector $\lambda\text{gt}11$ [19]. The library was screened with a pool of sera raised against either purified schistosomulum tegumental membranes or soluble egg antigens, as previously described [10]. The fusion protein in $\lambda\text{gt}11$ of one clone, A1.11, was strongly recognised by three sera independently raised against purified tegumental membranes, but either weakly or not at all by two sera raised against soluble egg antigens. It was also strongly recognised by one (Rb 19) of a panel of sera (Rb 16–20) raised against individual size

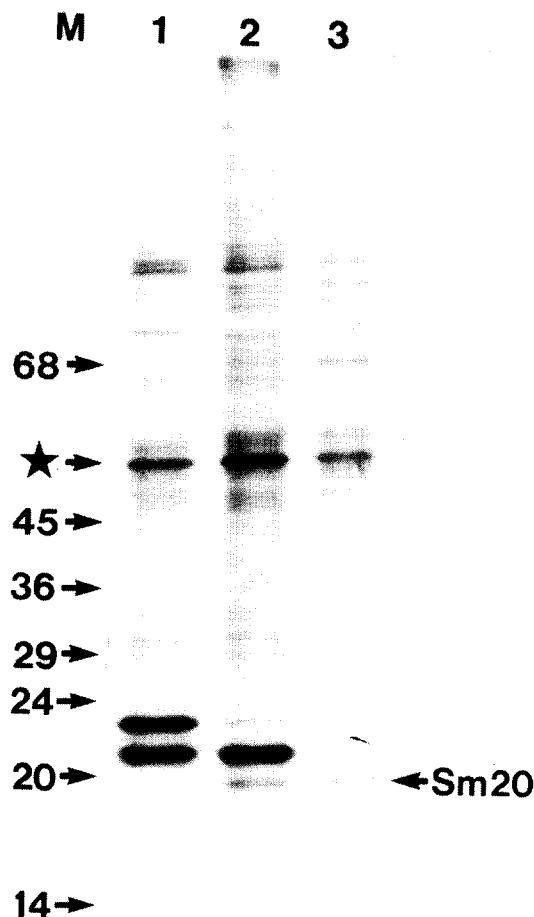


Fig. 1. The in vitro translation products of mRNA from adult worms of *S. mansoni* were immunoprecipitated with serum from: lane 1, Rb 20; lane 2, Rb 19; lane 3, a rabbit immunised with a lysogen of clone A1.11. M indicates the positions of marker proteins in kDa. * indicates the message-independent product of translation.

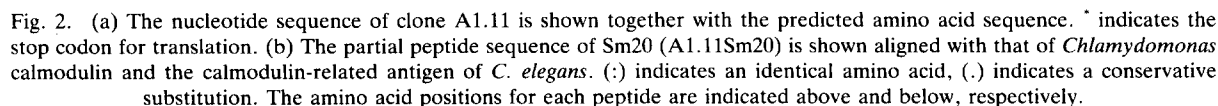
fractions of the material stripped from intact schistosomula by detergent. Rb19 immunoprecipitates three in vitro translation products of adult worm mRNA of approximately 24 kDa, 22 kDa and 20 kDa (Fig. 1, lane 2). The fusion protein was not recognised by a second serum from the series (Rb 20) which immunoprecipitates only the two in vitro translation products of 24 kDa and 22 kDa (Fig. 1, lane 1). This suggested that clone A1.11 encoded the 20-kDa antigen. This was confirmed as the serum raised against the fusion

Characterisation of clone A1.11. *EcoRI* digestion of the DNA from clone A1.11 in λ gt11 revealed the presence of two fragments of approximately 260 and 210 bp. These could represent either two independently cloned cDNAs tandemly ligated into the vector, or a single cDNA containing an internal *EcoRI* site. The fusion protein expressed by A1.11 is approximately 126 kDa. Discounting the 115-kDa portion contributing by β -galactosidase, this indicates that approximately 11 kDa of the fusion protein is contributed by the insert(s). In theory, the 260-bp fragment had a protein coding capacity of 10 kDa and the 210-bp

fragment could encode 8 kDa. Given the level of accuracy of measurement of protein sizes on SDS polyacrylamide gels, it was impossible to be certain whether one or both inserts were being expressed in the clone.

The sequence of clone A1.11. The orientation of the 260-bp and the 210-bp fragments of A1.11 in λ gt11 were determined after subcloning of the *KpnI*-*SacI* fragment containing the inserts and some flanking λ gt11 sequence into M13 mp18 and mp19. Oligonucleotides complementary to the regions flanking the *EcoRI* site in the β -galactosidase gene were used to sequence the λ -schistosome DNA junctions of the clone. This showed that the 260-bp fragment is situated 5' to the 210-bp fragment and would be transcribed and translated first. It also confirmed the open reading frame used for the production of the β -galactosidase-Sm20 fusion protein.

The complete DNA sequence of both *Eco*RI fragments was determined with overlapping runs on both strands. The co-linearity of the two fragments was demonstrated by sequencing through



their junction using the *KpnI*–*SacI* fragment and the oligonucleotide primers described above. The complete nucleotide sequence is shown in Fig. 2a. It is very A+T-rich (65%) in agreement with other published genes from *S. mansoni*. The sequence revealed that the central *EcoRI* site had not been created by the ligation of two independently *EcoRI* linked fragments, but is a naturally occurring *EcoRI* site. Multiple translation termination signals occur in two reading frames but the third contains an open reading frame (ORF) of 90 amino acids. This ORF is in frame with the β -galactosidase gene, confirming that this is the ORF used to produce the fusion protein in λ gt11. The ORF is represented by the entire 260-bp fragment and 21 bp of the 210-bp fragment. A 3' untranslated region of 169 bp is present, but no poly(A)⁺ addition signal or poly(A) tail.

The amino acid sequence of Sm20 (Fig. 2a), predicted from the nucleic acid sequence, contains a high level of acidic amino acids (28%). When this sequence was used to search the Swissprot and PIR databases for homology to other proteins, significant homology was seen with calmodulin, troponin C, and the regulatory light chain of myosin. The greatest homology was seen with *Chlamydomonas* calmodulin. There was 45% identity, but, including conservative substitutions, an overall homology of 86% (Fig. 2b). *Chlamydomonas* calmodulin is unusual in that it has a 10-amino-acid extension at the C terminal when compared with calmodulins from other species. The portion of Sm20 cloned includes the third and fourth predicted calcium binding domains and the majority of the 2nd domain. Sm 20 shows only 35% identity with the calmodulin related antigen of *C. elegans* (Fig. 2b) but 42% homology with troponin C from chick skeletal muscle and 36% homology with myosin regulatory light chain from smooth muscle. There are no potential sites for N linked glycosylation.

Characterisation of the gene encoding Sm20. A Southern blot of genomic DNA digested with each of three different restriction enzymes (Fig. 3) was probed with the radiolabelled 260-bp fragment of A1.11. The number and pattern of the bands suggested that Sm20 is encoded by a small multigene family, probably with two or three members.

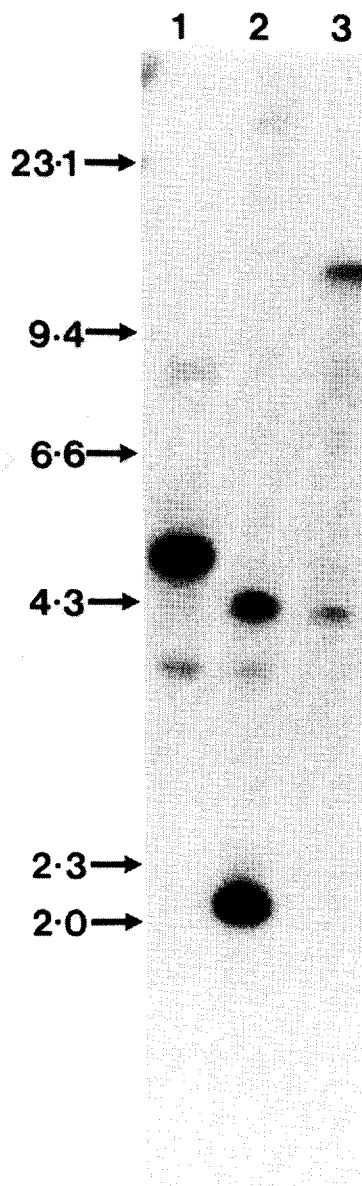


Fig. 3. Genomic DNA from *S. mansoni* digested with: lane 1, *EcoRI*; lane 2, *HindIII*; lane 3, *BamHI* was separated by agarose gel electrophoresis, Southern-blotted and probed with the 260-bp fragment of clone A1.11. The resultant autoradiograph is shown. Marker sizes are indicated in kb.

Expression of Sm20 during the life cycle of *S. mansoni*. The level of expression of the RNA encoding Sm20 was determined for different life cycle stages by Northern blot (Fig. 4). RNA encoding Sm20 was found in schistosomula (lane 2)

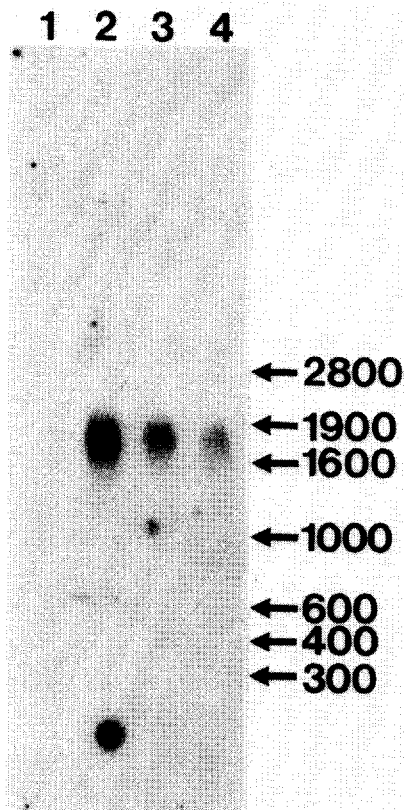


Fig. 4. A Northern blot of RNA isolated from: lane 1, eggs; lane 2, schistosomula; lane 3, adult worms of a laboratory strain; lane 4, adult worms derived from cercariae collected in Kenya was probed with the 260-bp fragment of clone A1.11. The resultant autoradiograph is shown. Marker sizes are indicated in bp.

and in adult worms, either derived from cercariae collected from endemic areas of Kenya (lane 4) or of the laboratory strain (lane 3). No RNA representing Sm20 was detected in eggs (lane 1). The size of the mRNA was estimated to be 1700 bp.

Western blots (Fig. 5) of extracts of mechanically transformed schistosomula cultured in vitro for 18 h (lane 4) or 48 h (lane 5) or adult worms (lane 6) probed with a serum raised against the lysogen of A1.11, revealed two antigens, of 20 and 38 kDa which were not recognised by a control serum raised against a lysogen of λ gt11 (lanes 1–3). This suggested that the 20-kDa primary translation product was post-translationally modified to give the 38-kDa species.

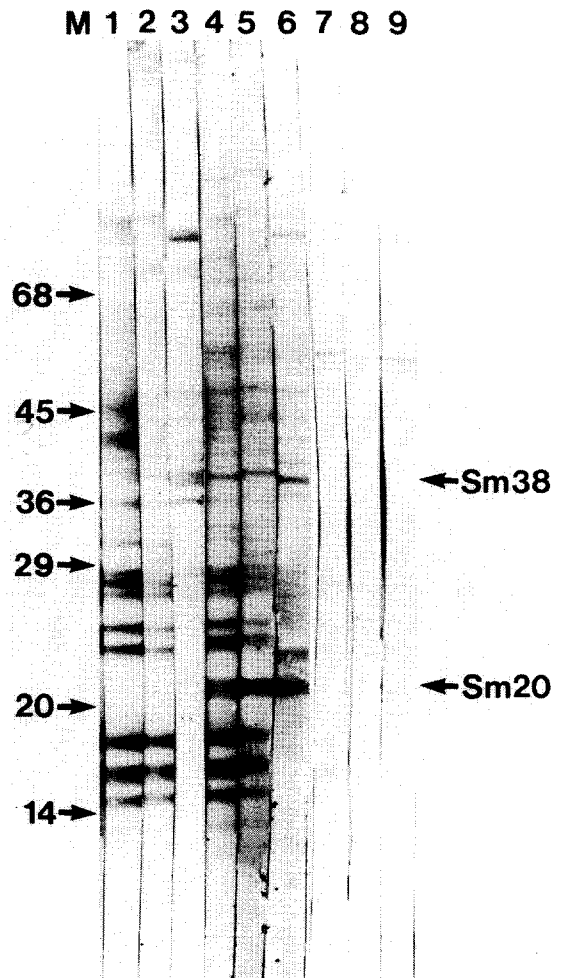


Fig. 5. A Western blot of 18-h schistosomula (lanes 1,4,7), 48-h schistosomula (lanes 2,5,8) and adult worms (lanes 3,6,9) probed with a control serum raised against a lysogen of λ gt11 (lanes 1–3), a serum raised against the lysogen of clone A1.11 (lanes 4–6) or the preimmune serum of the same rabbit (lanes 7–9). Marker sizes are indicated in kDa.

Is Sm20 schistosome calmodulin? To determine whether Sm20 was schistosome calmodulin a number of experiments were performed. An antiserum against bovine brain calmodulin was used to probe Western blots of either fusion proteins of Sm20 in a variety of vectors or extracts of different life cycle stages of *S. mansoni* but no antigens were recognised (data not shown). In addition, this serum did not immunoprecipitate an in vitro translation product of adult worm mRNA.

Western blots of purified calmodulin from bovine brain, human erythrocytes, *Neurospora crassa* or spinach were probed with the serum raised against the fusion protein of A1.11 in λ gt11, but none of these were recognised (data not shown).

Is Sm20 a calcium-binding protein? Most calcium-binding proteins exhibit a marked increase in mobility on SDS-PAGE when run in the presence of calcium rather than EDTA. This is exemplified by bovine brain calmodulin, where the apparent molecular weight decreases from 19 000

to 14 000 (Fig. 6 A and B). The in vitro translation product of Sm20 immunoprecipitated by Rb19 exhibits a shift of 1.7 kDa (Fig. 6 C and D). This is a specific change as most peptides immunoprecipitated by the serum DT8 show no significant change.

To directly test whether Sm20 was a calcium binding protein the ^{45}Ca isotope of calcium was used to probe Western blots of either the purified fusion protein of the 260-bp fragment of Sm20 with GST or GST alone (Fig. 7, lanes 1 and 3). The autoradiograph showed that the fusion protein of Sm20 (lane 2) did bind ^{45}Ca but that GST did not (lane 4).

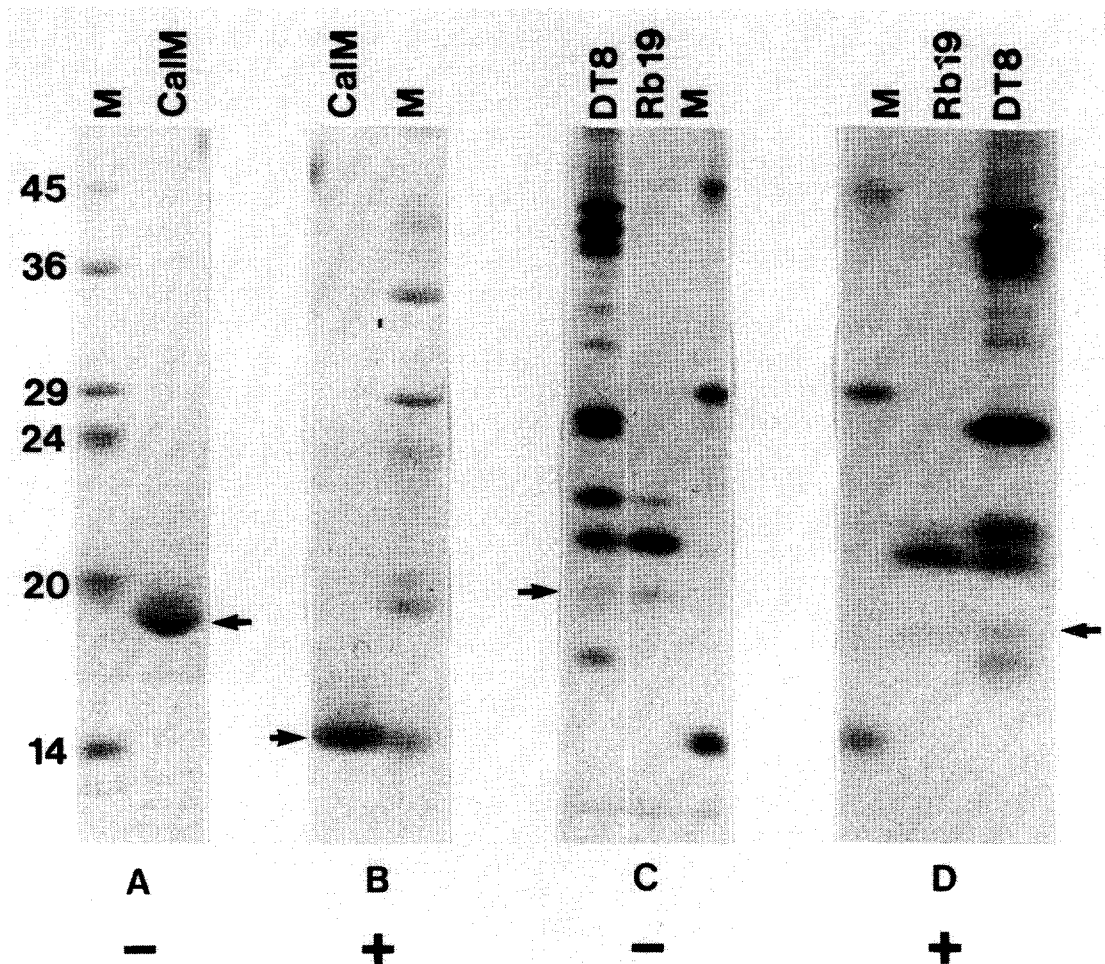


Fig. 6. Bovine brain calmodulin (CaM) and the in vitro translation products of adult worm mRNA immunoprecipitated by Rb19 or DT8 were run on SDS-polyacrylamide gels in the presence of EDTA (panels A and C) or CaCl_2 (panels B and D). Panels A and B show the polyacrylamide gels stained with Coomassie Blue. Panels C and D show the autoradiographs of the same gels. The sizes of molecular weight markers (M) are indicated in kDa. CaM (Panels A and B) and Sm20 (Panels C and D) are arrowed.

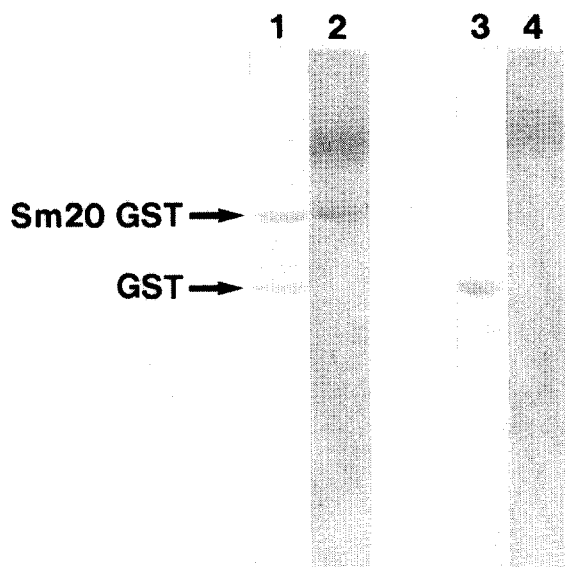


Fig. 7. A Western blot of the fusion protein of Sm20 in pGEX (Sm20GST) (lanes 1 and 2) and GST alone (lanes 3 and 4) was probed with ^{45}Ca . The resultant autoradiograph (lanes 2 and 4) and Sudan black-stained blots (lanes 1 and 3) are shown.

Localisation of Sm20. Sm20, or possibly an antigenically related peptide, is present in preparations of tegumental membranes of schistosomula and can be stripped from intact schistosomula by mild detergent treatment as sera against these preparations contain anti-Sm20 antibody. This suggests that Sm20 has a surface or tegumental membrane associated location. However, the serum raised against the fusion protein of Sm20 in $\lambda\text{gt}11$ does not give positive indirect immunofluorescence of the surface of schistosomula.

Discussion

We report here the cloning of a 20-kDa peptide antigen (Sm20) of *S. mansoni* which appears to be post-translationally modified to give a 38-kDa species. This modification is most likely to be either covalent dimerisation or glycosylation. However, the portion of Sm20 sequenced so far does not contain any potential sites for *N*-linked glycosylation. Sm20 is encoded by a small multi-gene family and is expressed in schistosomula and adult worms, but not in eggs. The predicted amino acid sequence of Sm20 shows that it has homol-

ogy to the family of calcium binding proteins which include calmodulin, troponin C and the light chain of myosin. However, no immunological cross-reactivity could be detected between Sm20 and calmodulins from a range of other species. This does not preclude Sm20 from being schistosome calmodulin, as the lack of cross-reactivity could be due to the extent of divergence between the species.

A calmodulin-related polypeptide has been identified from the nematode *Caenorhabditis elegans* [20], which is very divergent from other eukaryotic calmodulins. Sm20 is even more divergent from this group but shows even less homology to the *C. elegans* polypeptide. An 8-kDa calcium-binding protein has been cloned from *S. mansoni* [6]. This peptide is distinct from Sm20, both in its size and its cercarial specific expression.

A calmodulin-related protein has been purified from adult worms of *S. mansoni* [5]. It was shown to be a peptide of 19-kDa which was recognised by an antiserum raised against rat brain calmodulin (Amersham). This antiserum is no longer available but an antiserum against bovine brain calmodulin (Sigma) did not show any cross-reactivity with Sm20 or any other antigen in adult worms.

Sm20 does appear to be a calcium-binding protein as it exhibits a calcium dependent change in mobility on SDS-polyacrylamide gels. This change is not as large as that seen with bovine brain calmodulin but is similar to that reported for a calmodulin-like flagellar protein from *Chlamydomonas* [21]. The fusion protein of Sm20 with GST is able to bind the ^{45}Ca isotope of calcium confirming that Sm20 is a calcium-binding protein.

Sm20 can be removed from intact schistosomula by detergent treatment and is present in preparations of tegumental membranes. However, antibody against the cloned portion of Sm20 does not recognise epitopes present on the surface of intact schistosomula indicating that either they are not present on the surface or are masked, perhaps by the addition of carbohydrate.

Classically, calmodulin is an intracellular calcium-binding protein, but is often found complexed with the internal face of plasma membranes. The above data are consistent with such

a location for Sm20. However, this must be investigated in detail using antisera raised against the complete Sm20 antigen.

In conclusion, we have determined that Sm20 is a calcium-binding protein but cannot demonstrate any cross-reactivity with calmodulins of other species. The modification of Sm20 to give the 38-kDa species is not a characteristic of calmodulins, and may indicate a different role for Sm20 in schistosomes.

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