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Purification of a recombinant *Schistosoma japonicum* antigen homologous to the 22-kDa membrane-associated antigen of *S. mansoni*, a putative vaccine candidate against schistosomiasis

(cDNA cloning; antigen, 22.6-kDa; calcium-binding domain; parasitic disease; EF-hand motif; human blood fluke)

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SUMMARY

We describe the cDNA cloning, overproduction and purification of a 22.6-kDa antigen from the human blood fluke *Schistosoma japonicum*. A 777-bp cDNA (C32) was isolated from a *S. japonicum* λ ZAPII cDNA expression library immuno-screened with hyperimmune rabbit serum (HRS) raised against soluble adult *S. japonicum* proteins. The open reading frame of C32 encodes a protein of 191 amino acids (aa) which exhibits 71% identity to a 22.6-kDa membrane-associated antigen of *S. mansoni*, a putative vaccine candidate for schistosomiasis. We have identified a sequence motif known as an EF-hand calcium-binding domain in both the *S. japonicum* and *S. mansoni* aa sequences, suggesting that the 22.6-kDa antigens are able to bind Ca^{2+} . Further, we have, for the first time, obtained the 22.6-kDa antigen in purified, non-denatured, recombinant form, and in sufficient quantity to assess the protective value of the molecule in vaccination/challenge experiments. This was achieved by synthesizing the schistosome antigen with a short polyhistidine tag fused to the N-terminus which was then used for subsequent affinity purification. The recombinant protein was purified under non-denaturing conditions using nickel-chelate affinity chromatography.

INTRODUCTION

Schistosomiasis (Bilharzia) is a major parasitic disease affecting humans with an estimated 200 million people

currently infected and a further 500–600 million exposed to infection. The most important schistosome species infecting humans are *Schistosoma mansoni*, which causes intestinal schistosomiasis in Africa, Latin America and the Caribbean; *S. haematobium*, responsible for urinary schistosomiasis in Africa and the Middle East, and *S. japonicum*, the cause of a very debilitating form of intestinal schistosomiasis in Asia, predominantly in the Philippines and China. A variety of measures have been adopted in an integrated approach to control of schistosomiasis but the mainstay at present is repeated mass or targeted chemotherapy. The potential for the parasites in the field to develop resistance to the available drugs, the problems of reinfection following treatment with the requirement for subsequent retreatment and the cost involved in diagnosis and delivery of treatment, indicate

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Abbreviations: A, absorbance (1 cm); aa, amino acid(s); β Gal, β -galactosidase; bp, base pair(s); C32, clone 32; d, deoxyribo; HRS, hyperimmune rabbit serum; IPTG, isopropyl- β -D-galactopyranoside; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); NTA, nitrilo-triacetic acid; ORF, open reading frame; p, plasmid; PAGE, polyacrylamide-gel electrophoresis; pfu, plaque-forming unit(s); S., *Schistosoma*; SDS, sodium dodecyl sulfate.

that, although chemotherapy is a useful interim control measure, alternative approaches are desirable in the longer term. One such alternative is the development of anti-schistosome vaccines but, despite much interest and some encouraging developments recently (reviewed in Butterworth, 1992; McManus et al., 1993), no practical vaccines are available at the present time for human use.

To date, most research on anti-schistosome vaccine candidates has been carried out on *S. mansoni*. One such vaccine candidate is a 22.6-kDa membrane-associated antigen. This antigen is recognised by IgG antibodies in sera from mice protectively vaccinated with purified adult *S. mansoni* tegumental membranes, and has been shown to be present in nine schistosome species, including *S. mansoni* and *S. japonicum* (Jeffs et al., 1991). An antigen of corresponding size is also recognised by sera from *S. mansoni*-infected humans in Brazil (Jeffs et al., 1991). To date however, a purified recombinant molecule, available in sufficient quantity for vaccination/challenge experiments to be undertaken, has not yet been reported. Here we describe the cloning, overproduction and purification of the recombinant *S. japonicum* 22.6-kDa antigen.

EXPERIMENTAL AND DISCUSSION

(a) Cloning of the *S. japonicum* 22.6-kDa antigen and cDNA analysis

A *S. japonicum* (Chinese mainland origin) cDNA library was immuno-screened with HRS raised against soluble adult *S. japonicum* proteins. Thirty-six positively immunoreacting clones were identified in the first round of immuno-screening from a total of 6.5×10^5 pfu screened. These clones were then subjected to a second

round of immunoscreening using HRS. Twenty-nine clones were confirmed as positive following the second round of immunoscreening and plaque purification. Plasmids (pBluescriptII SK+), were recovered by automatic excision, partially sequenced from both ends, and the resulting nucleotide sequences used to search for homology in the GenBank database. One clone, C32, showed substantial homology with a *S. mansoni* gene, A12, reported to encode a 22.6-kDa antigen that is recognised by sera from mice protectively vaccinated with adult *S. mansoni* tegumental membranes, and by sera from Brazilian patients infected with *S. mansoni* (Jeffs et al., 1991). Clone A12 shows only a few nt differences to a previously reported *S. mansoni* clone designated I(H)A (Stein and David, 1986). The full nt sequence of clone C32 was obtained and is shown in Fig. 1. The 777-bp cDNA of C32 contains an ORF beginning with the initiation codon ATG at position 10 and ending with the termination codon TAA at position 583. The nt sequence of C32 shares 79% identity with that of the *S. mansoni* 22.6-kDa antigen (Jeffs et al., 1991), when the coding regions are aligned. However, the identity is significantly less beyond the coding regions. To maintain maximal identity, a 3-bp gap (one codon) was introduced near the start of the sequence. No other gaps or deletions were required to obtain maximal identity throughout the coding region. Northern blotting was performed to determine the size of the mRNA transcript encoding the *S. japonicum* 22.6-kDa antigen. The cDNA insert of C32 was radiolabelled and used to probe poly(A)⁺ mRNA isolated from *S. japonicum* adult worms. The probe hybridized with a single band of approx. 950 bp (data not shown), indicating that clone C32 encoded most, but not all, of the mRNA transcript synthesized by the parasite.

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1  CTTGTTATTATGGCAACTACTGAGTACAGATTAAAGTTTAAATGGAACAATTTATTAGAGCATTTCATAGAAATAGATAAAGATAAATGAATTGATTGATAAACAAGAACTAACGAAATAT
1  M A T T E Y R L S L M E Q F I R A F I E I D K D N N E L I D K Q E L T K Y
121 TGTCAACAGAATCAAAATGGATATGAAACAAATAGATCCATGGATTGCAAGGTTTGATACTGATAAAGATGGTAAAGTCAGTTTAGAGGAGTTTTCGTCGGATTCCGCTCAAAGGTTTGG
38 C Q Q N Q M D M K Q I D P W I A R F D T D K D G K V S L E E F C R G F G L K V W
241 GAAGTCCGTCGTGAAAAGGAAGATTAAAGAGAGACAAGGAAGGCAAGTATCCACACTTCCACTGGATATTCAAATATTCGCGGCAACCATGTCAAAGCTAAGCAATATAACATATGT
78 E V R R E K E E L K R D K E G K V S T L P L D I Q I I A A T M S K A K Q Y N I C
361 TGTAATTTAAAGAACTTCTTGATAAACTAGTCGAAGTGGTATGAAGTAAGAGCGTTGGCAATGACTTAAAGCATTTTGGATTCTGAATATGGCCGTGTATGGCAAGTGATTATA
118 C K F K E L L D K T S R T G D E V R A L A N D L K A F L D S E Y G R V W Q V I I
481 CTAAGTGGTTTCATATTGGATGAATTTTTCACATGAACCATTTTATCAATGCAGTTTAAAGTACAGTAATATGTTTGTCTATTGTGGCGAACACCGTCTTCGTAAGTTTAAATGAATAGT
158 L T G S Y W M N F S H E P F L S M Q F K Y S N Y V C L L W R T P S S * 191
601 ACAATGAATATACATTCATACACAATAAAGCGACAGTAGTCTAATCTACTATTCAATGCAATAAATAGACAATTTTACATAACTTTGAATTTGTTCAATGATTAACTACTAGT
721 CTTTAACTAACTCAAATCTAATCATTTTGTGTTATGAATGACAAAACAGTACATTATT

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Fig. 1. The nt sequence of the *S. japonicum* 22.6-kDa antigen and deduced aa sequence. **Methods:** An *S. japonicum* cDNA library was constructed in λ ZAPII using poly(A)⁺ RNA isolated from parasites of Chinese mainland origin. cDNA was ligated into the *EcoRI* site of λ ZAPII. The library was immuno-screened as described by Waine et al. (1993a), using HRS raised against soluble adult *S. japonicum* (Chinese mainland origin) proteins. HRS was pre-adsorbed against *E. coli* proteins before use in immunoscreening. After plaque purification, recombinant phagemids were recovered by the automatic excision procedure using helper phage, as described by the manufacturer (Clontech, USA). Recombinant cDNA plasmids were partially sequenced from both ends on an Applied Biosystems automated DNA sequencer using T7 and T3 dye-labelled primers and the resulting DNA sequences used to search for homology in the GenBank database. C32 was then fully sequenced by a combination of automated sequencing, and manual sequencing (Sanger et al., 1977) using primers designed to match the newly read sequence. The nt data reported in this paper has been submitted to GenBank with Accession number L08198.

(b) Protein analysis

The ORF of C32 encodes a polypeptide of 191 aa, as inferred from the nt sequence (Fig. 1). This is one aa longer than the *S. mansoni* homologue, due to the presence of an additional threonine residue at position three (Fig. 2). Alignment of the aa sequences (Fig. 2), reveals 71% absolute identity and 84% identity when conservative substitutions are taken into account. The predicted M_r of the protein encoded by C32 is 22 588. This was confirmed by in vitro transcription and translation of clone C32 using [^{35} S]methionine, which generated a single radioactive product of approx. 22.6-kDa (data not shown). A negative control reaction using non-recombinant pBluescriptII showed no radiolabelled protein at this position, confirming that the 22.6-kDa product of C32 was due exclusively to the insert and not to pBluescript vector sequences.

Although the biological function of the schistosome 22.6-kDa antigen is currently unknown, we have identified a sequence motif characteristic of a family of

proteins such as calmodulin, parvalbumin, and troponin C, which have a Ca^{2+} -binding domain known as the EF-hand (Tufty and Kretsinger, 1975). This type of domain consists of a 12-aa loop flanked on both sides by a 12-aa α -helical domain. The central loop binds Ca^{2+} via the six aa at positions 1, 3, 5, 7, 9 and 12, which donate oxygen for metal coordination. Using computer-assisted alignment, we identified an EF-hand Ca^{2+} -binding domain in both the *S. japonicum* and *S. mansoni* 22.6-kDa antigens. In *S. japonicum*, this domain is located between aa 46–75, while for the *S. mansoni* sequence, it is located between aa 45–74 (see Fig. 2). Table I shows that at the positions required for Ca^{2+} coordination (x, y, z, -x, -y, -z), both species have the required type of aa residues. While there is some deviation from the test sequence in the flanking sequences on either side of the binding loop, it is worth noting that the carp muscle Ca^{2+} -binding domain shown (Table I), also diverges from the test sequence in one of the helices flanking the loop. Furthermore, Reid et al. (1980), have shown that

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1 MATTEYRLSLMEQFIRAFIEIDKDNELIDKQELTKYQQNQMDMKQIDPWIAFDTDKDGKVSLE (Sj)
  || :|| :||:||||:|||| :||:||||| ||| :||| ||||| ||||| ||:|:|
1 MATETKLSQMEEFIRAFLEIDADSNEMIDKQELIKYCQKYLDMKLLIDPWIAFDTDKDNKISIE (Sm)

67 EFCRGFGLKVWEVRREKEELKRDKEGVSTPLDIIIAATMSKAKQYNICCKFKELLDKTSRTGD (Sj)
||||||| ||:||||:||||:|||| || :||:||||| |||:|||| ||| :| |||||
66 EFCRGFGLKVSEIRREKDELKKERDGKPKLPNIEIIAATMSKTKOYEICCOFKEYVDNTRTGN (Sm)
  -O++-+---O++-+-O+

133 EVRALANDLKAFLDSEYGRVWQVILTGSIWMNFSHEPFLSMQFKYSNYVCLLWRTPS* 191 (Sj)
  :||:| || :|| :|||||:|||||:|||||:||||| ||||| |||||
132 DMREVANKMKSLLDNTYGRVWQVLLTGSIWMNFSHEPFLSIQFKYNNYVCLWRTPSQ* 190 (Sm)

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Fig. 2. Comparison of the deduced aa sequences of the 22.6-kDa proteins from *S. japonicum* (Sj) and *S. mansoni* (Sm). The *S. mansoni* aa sequence is that reported in Jeffs et al. (1991). Identical residues are indicated by solid vertical lines. Similar residues are indicated by broken vertical lines. Stop codons are marked with an asterisk (*). The EF-hand Ca^{2+} -binding domain is underlined. Residues located in the highly charged region discussed in section b, are marked below by (-) for negatively charged residues; (+) for positively charged residues; or (○) for residues that are not charged.

TABLE I

Comparison of EF-hand motifs for carp muscle Ca^{2+} -binding parvalbumin and the *S. japonicum* and *S. mansoni* 22.6-kDa antigens

	Test sequence for ^a		
	Helix EL--LL--L	Loop O-O-O-G-L-O--O	Helix L--LL--L
<i>S. japonicum</i> ^b	* * *	* * * * *	* *
	QIDPWIA RF	DTDKDGKVSLEE	F C R G F G L K
	* * *	* * * * *	* *
<i>S. mansoni</i> ^b	LIDPWIA RF	DTDKDNKISIEE	F C R G F G L K
	* * *	* * * * *	* * *
MCBP ^c	DVKKAFAII	DQDKSGFIEEDE	LKLFLQNF
		x y z -y -x -z	

^aIn the test sequence (Tufty and Kretsinger, 1975), O denotes oxygen containing residues (D,E,N,Q,S,T), L denotes hydrophobic residues (L,I,V,F,M), E denotes glutamic acid, G denotes glycine, (-) denotes any aa and x,y,z,-x,-y,-z refer to vertices of the Ca^{2+} coordination octahedron. Residues matching the test sequence are marked by asterisks. Computer assisted alignment was performed using an algorithm designed to identify EF-hand motifs (Genetics Computer Group, Madison, WI, USA).

^bThe *S. japonicum* and *S. mansoni* 22.6-kDa antigen sequences are indicated by *S. japonicum* and *S. mansoni*, respectively.

^cMCBP denotes one of the two Ca^{2+} -binding regions of carp muscle Ca^{2+} -binding parvalbumin (Tufty and Kretsinger, 1975). In MCBP, the oxygen ligand at the -y vertex is provided by the peptide group; hence any aa at this position may provide a peptide oxygen for Ca^{2+} coordination.

synthetic peptides lacking the helix on the N-terminal side can still bind Ca^{2+} , while other investigators have demonstrated metal binding to the loop sequence alone (Marsden et al., 1988; MacManus et al., 1990). Together, these experiments indicate that a complete helix-loop-helix unit is not required for binding of Ca^{2+} . Thus, it is possible that even though the schistosome flanking helices do not match the test pattern exactly, the loops (which do match exactly) can bind calcium. Given that the *S. mansoni* 22.6-kDa antigen is associated with the tegumental surface membrane, it is possible that the protein may play some role in binding and regulating the movement of Ca^{2+} across the membrane. Alternatively, the protein may have another biological role, but requires the coordination of Ca^{2+} for activation and/or normal functioning. One further unusual feature of this protein is the extremely high frequency of charged aa between positions 78–93 (77–92 for *S. mansoni*), where 13 of the 16 aa are positively or negatively charged (see Fig. 2). In this region, only conservative substitutions occur between the two species, with like-charge being substituted for like-charge. The biological significance of this highly concentrated region of charged aa is currently unknown, although it is interesting that this region is located adjacent to the EF-hand motif.

(c) Overproduction and purification of recombinant protein

For overproduction and purification of recombinant protein we used the QIAexpress system (Qiagen). In this system recombinant protein is synthesized with a polyhistidine tag fused to the N terminus, the presence of which allows proteins to be purified from a cleared bacterial lysate, based on the high affinity of Nickel-NTA resin for proteins carrying the tag. The cDNA of clone C32 was subcloned into the QIAexpress vector, pQE-10, so that the correct reading frame was maintained. Synthesis and purification of the recombinant protein were performed essentially as described in Waite et al. (1993b), using imidazole to elute the recombinant fusion protein from the resin under non-denaturing conditions. A protein of the predicted size, that was not present in non-recombinant pQE-10 induced under the same conditions, was synthesized in whole bacterial lysate at high levels (approx. 20 mg/l bacterial culture), indicating that this expression system is a highly efficient means of producing the schistosome 22.6-kDa membrane-associated antigen. The results of the purification procedure are shown in Fig. 3. The over-produced recombinant protein is clearly visible in extracts of whole bacterial lysate analysed by SDS-PAGE (lane 1). This protein is not present in the flow-through when the cleared lysate is passed over the affinity column (lane 2), indicating that it has bound suc-

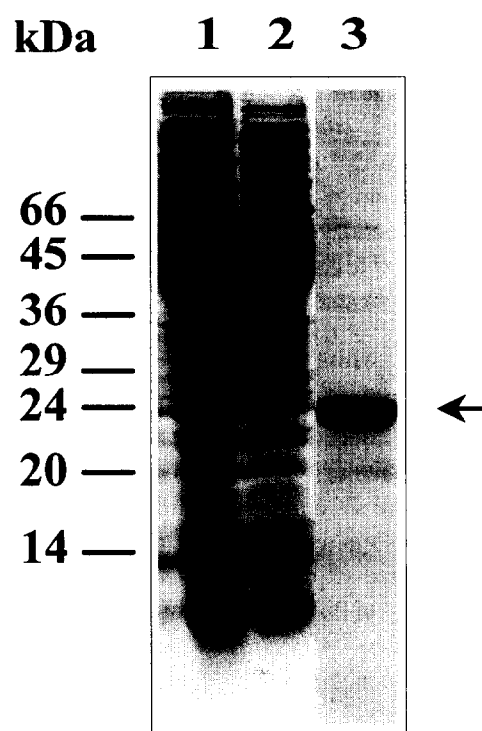


Fig. 3. Synthesis and purification of recombinant protein. Lane 1, whole bacterial lysate before purification showing the overexpressed recombinant protein (at the position marked by the arrow). Lane 2, the flow-through collected when the bacterial lysate was applied to the column. Note that the recombinant protein visible in lane 1 is not present in the flow-through. Lane 3 shows the purified recombinant protein eluted from the column by imidazole. **Methods:** The cDNA of clone C32 was subcloned into the pQE-10 expression vector using standard molecular cloning procedures (Sambrook et al., 1989). Briefly, PCR was used to generate an additional *Pst*I site outside the coding region of C32 using the primer 5'-TATATGGATCCTGCAGTGTATTATGGCAACTAC. After digestion with *Pst*I, the PCR products were purified using Prep-a-gene DNA purification matrix (Bio-Rad, Hercules, CA, USA) and ligated with *Pst*I digested pQE-10 vector treated with calf-intestinal alkaline phosphatase (Boehringer-Mannheim, Indianapolis, IN, USA). The ligation reaction was used to transform *E. coli* 'SURE' cells (Stratagene, La Jolla, CA, USA) by electroporation. Clones containing inserts in the correct orientation were identified by restriction enzyme analysis and DNA sequencing. Expression and purification were performed as described (Waite et al., 1993b).

cessfully to the column. Most of the other proteins in the lysate have clearly passed through the column without binding. After washing the column, the recombinant protein was then eluted under non-denaturing conditions using imidazole. The purified recombinant protein obtained after elution is shown in lane 3, purified to virtual homogeneity by the single column pass.

This report is the first to describe the purification of the recombinant 22.6-kDa membrane-associated antigen of schistosomes. Given that IgG antibodies to the homologous protein in *S. mansoni* are associated with protective immunity in mice, the purified, recombinant protein we have obtained from *S. japonicum* may prove important as a component of an anti-*S. japonicum* vaccine. Further,

recombinant antigens purified under non-denaturing conditions, should more closely resemble the natural parasite antigen than those isolated under denaturing conditions. With the availability of purified, non-denatured recombinant protein, we will now be able to undertake vaccination/challenge experiments to examine the protective value of this antigen.

(d) Conclusions

(1) A cDNA encoding the complete ORF of a *S. japonicum* 22.6-kDa antigen has been cloned and sequenced.

(2) The deduced aa sequence of this protein is highly homologous to, and one aa longer than, the 22.6-kDa membrane-associated antigen of *S. mansoni* (previously identified as a putative vaccine candidate for schistosomiasis).

(3) Both the *S. japonicum* and *S. mansoni* 22.6-kDa proteins contain a sequence motif characteristic of an EF-hand Ca^{2+} -binding domain.

(4) The *S. japonicum* 22.6-kDa protein has been synthesized at high levels in *E. coli* and the recombinant protein purified to virtual homogeneity under non-denaturing conditions. This will enable vaccination/challenge experiments to be undertaken and an assessment made of the protective value of the 22.6-kDa protein.

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