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A new member of the transmembrane 4 superfamily (TM4SF) of proteins from schistosomes, expressed by larval and adult *Schistosoma japonicum* [1]

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Abstract

The transmembrane 4 superfamily (TM4SF) comprises an assemblage of surface antigens from mammalian cells and from the human blood flukes. Member proteins of the TM4SF are characterized by the presence of four hydrophobic domains, which are presumed to be membrane-spanning, and specific conserved motifs. The Sm23 group of TM4SF, which includes Sm23, Sj23, and Sh23 from blood flukes, shows potential as immunodiagnostic and vaccine target antigens for use in controlling human schistosomiasis. Here we describe a cDNA from miracidia and adult *Schistosoma japonicum* parasites which apparently encodes a new member of the TM4SF. The deduced polypeptide, termed Sj25/TM4, has substantial amino acid homology to Sm23 from *Schistosoma mansoni* although it is not a species homologue of Sm23. Sj25/TM4 is predicted to span the cell membrane four times, with its NH_2- and COOH-termini embedded in the cytoplasm, and to have two extracellular hydrophilic loops, one of which may be N-glycosylated. This topology is characteristic of TM4SF proteins; in addition, Sj25/TM4 contains the sequence motifs conserved in the TM4SF. Southern hybridization analysis demonstrated that Sj25/TM4 and Sj23 are encoded by genes at separate loci and, further, showed interstrain variation at the locus encoding Sj25/TM4 in Chinese and Philippine isolates of *S. japonicum.* © 1997 Elsevier Science B.V.

Keywords: TM4SF; Schistosome; Sm23; Hydrophobicity; Transmembrane

The transmembrane 4 superfamily (TM4SF) of proteins comprises a group of cell-surface proteins that are characterized by the presence of four hydrophobic domains, which are presumed to be membrane-spanning. The family comprises about 20 members, so far, many of which including CD37, CD63 and TAPA-1 occur on leukocytes or tumor cells. Although the precise function of any member of the TM4SF has yet to be elucidated, these kinds of proteins may mediate signal transduction events at the cell surface involved with cell development, activation, growth, and motility [1–4]. Whereas many of the TM4SF antigens so far described have been associated with mammalian blood cells, the first member to be reported, Sm23, is a surface protein

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[1] Sequences described here have been deposited in the GenBank with accession numbers U77941 (adult protein) and AA185728 (miracidial protein).

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from the human blood fluke *Schistosoma mansoni* [5]. Sm23 is considered a potential candidate as a vaccine target in human schistosomiasis [6] and its T and B cell epitopes have been mapped [7]. In addition to its four putative transmembrane domains, Sm23 may also be attached to the schistosome surface by a glycosylphosphatidylinositol membrane anchor [8]. Homologues of Sm23 have been reported from two other schistosome parasites, *Schistosoma japonicum* [9] and *Schistosoma haematobium* [10].

Here we present the deduced amino acid sequence of what appears to be a novel member of the TM4SF. This antigen, which we term Sj25/TM4, is expressed by larval and adult stages of the Asian schistosome *S. japonicum*. Although it is not closely related to Sm23 at the primary sequence level, it nonetheless shares a number of physical characteristics with Sm23 and its species homologues Sj23 and Sh23, and with other TM4SF proteins. In particular, it shows strong similarity in the four domains predicted to span the cell membrane, shows a TM4SF-like hydropathicity profile, is of similar size, and exhibits the potential glycosylation sites and spacing of cysteine residues predicted for Sm23 and other TM4SF proteins.

Materials and methods. Schistosomes, cDNA libraries, genomic DNA, Southern hybridizations. Two cDNA libraries were constructed with mRNA extracted from miracidia, purified as described previously [11], and from mixed sex adults of a Chinese (Anhui) strain of *S. japonicum* by standard methods, using the lambda phage vector UNIZAP XR (Stratagene, La Jolla, CA). Both libraries were directional, with an *Eco*RI site at the 5′-end of the cloned insert. Genomic DNA was obtained from mixed sex adults of both Philippine (Sorsogon) and Chinese (Anhui) strains of *S. japonicum*, and from mouse blood and rabbit blood, using a kit from Qiagen (Chatsworth, CA). Southern hybridizations were carried out using inserts of recombinant plasmids as probes. Purification and [α-^{32}P]dCTP labelling of probes and hybridization and washing of filters were carried out as described previously [12]. Probes were stripped from filters by two washes in 0.1% SDS at 95EC.

Expressed sequence tags, plasmid DNA. Expressed sequence tags (ESTs) from the schistosome cDNA libraries were obtained as follows, as part of the World Health Organization-sponsored *Schistosoma* genome initiative [13,14]. Randomly selected plaques were isolated from lawns of phage-infected *Escherichia coli* (XL1 Blue strain) cultured on LB-agar containing 12.5 μg/ml tetracycline, 50 μg/ml X-gal, and 50 μg/ml IPTG. Phage were eluted from the plaques into 100 mM Tris, pH 7.5, 200 mM NaCl, and the supernatant employed as the template for polymerase chain reactions employing pBluescript-specific primers (Forward, 5′-GTTTTCCCAGTCACGAC and Reverse, 5′-GGAAACAGCTATGACCATG). PCR products greater than ~400 bp in length were subsequently employed as the templates for cycle sequencing using the T3-specific oligonucleotide 5′-ATTAACCCTCACTAAAGGGA as the primer in order to determine the sequence of the 5′- end of the clone. Clones of interest were excised into pBluescript SK (-) constructs using the ExAssist helper phage and *E. coli* (BB4 strain) cells according to the manufacturer's instructions (Stratagene). Midipreps of recombinant plasmids were prepared from bacterial cultures using Qiagen 100 columns (Qiagen). The nucleotide sequence of inserts of plasmids was determined using universal forward and reverse primers, and gene-specific primers, the Taq DyeDeoxy Terminator Cycle Sequencing System (Applied Biosystems, Foster City, CA [ABI]) and an automated DNA sequencer (ABI, model 377).

Sequence and motif analyses. Analyses of nucleotide and deduced amino acid sequences were undertaken using the GCG Package software (University of Wisconsin). Sequences of clones of interest were used to search public databases for blocks of related sequence, using the 'blocks' server at http://www.blocks.fhcrc.org4. Comparisons of related proteins were undertaken using the Fasta, PileUp, Prettyplot, PEPWINDOW programs (GCG). Gene identifications were accomplished by comparisons using the BLAST algorithm [15]. Hydropathicity indices were determined by the method of Kyte and Doolittle [16]. Functional sites and motifs on target proteins were located using ScanProsite at the ExPASy World Wide Web (WWW) molecular biology server from the Geneva University Hospital and the University of Geneva (http://expasy.hcuge.ch/sprot/scnpsite.html) [17]. Protein topologies were predicted with TMpred, a program to predict transmembrane regions and their orientation [18]. Molecular characterization of novel proteins was aided by the Statistical Analysis of

Protein Sequence (SAPS) algorithm of Brendel et al. [19].

Results and discussion. A new schistosome antigen related to Sm23. Several hundred ESTs from our *S. japonicum* cDNA libraries have been lodged in dbEST (Fan et al., unpublished). Of these, one EST (GenBank accession No. AA241414) from the adult cDNA library encoded Sj23, which has previously been shown to be an immunodiagnostic antigen for schistosomiasis japonica [9] and a member of the TM4SF [1]. Two other ESTs, one each from the miracidial (GenBank accession No. AA185728) and adult (GenBank accession No. U77941) cDNA libraries, encoded a novel protein that, although related to Sj23/Sm23/Sh23 and the TM4SF, were clearly distinct from the Sm23 group of species homologues. The sequences of the latter two ESTs were identical, indicating that the protein that they encode is expressed in miracidial (larval) and adult stages of the schistosome (and perhaps other life cycle stages as well).

The nucleotide sequence of clone 7A1 from the adult cDNA library contained a recombinant insert of 1005 bp which included a short 5′-untranslated region (-UTR), an open reading frame of 225 codons, including both ATG start and TAA stop codons, and a 3′-UTR of ~350 bp which included a possible polyadenylation signal AATGAAA and terminated in a polyA stretch (Fig. 1). The predicted molecular size of the polypeptide encoded by the insert of clone 7A1 was 25549 daltons. Based on its predicted size, we have named the encoded protein Sj25/TM4, for *Schistosoma japonicum* 25 kDa protein, of the transmembrane 4 superfamily (see below). Homology searches revealed that Sj25/TM4 was similar to the 23 kDa TM4SF antigen of *S. mansoni*, Sm23, and its *S. japonicum* and *S. haematobium* homologues Sj23 and Sh23 [5,9,10]. In particular, using Mail-BLAST, a match over Sj25/TM4 amino acid residues 161 to 216 was observed to Sj23 residues 157 to 212. This match had a BLAST score of 56 and included 26%

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attttacagattaaaagtttcctttcgtttactatacaatcatcaacagaacttgagatc   60
aatttattaaagataATGAAATTGTCATTTACGAAAGTATCCTTGACCAATATATTGATT  120
               M  K  L  S  F  T  K  V  S  L  T  N  I  L  I   - 15
CTATTCAATTGTTTATTTATTATATTCAGTATGATTGTTTTAACCTTTGGAGTTATTCCA  180
L  F  N  C  L  F  I  I  F  S  M  I  V  L  T  F  G  V  I  P   - 35
CAGATATATTTACTAAAATTTGCTAACATTCTACATGGTGTTAGACCATCCATCTTTCCA  240
Q  I  Y  L  L  K  F  A  N  I  L  H  G  V  R  P  S  I  F  P   - 55
ATAGTTTGTTTTACTGGTAGTTTTGTTATCATAGTTGCATGTGTTGGAATAATTGGATTG  300
I  V  C  F  T  G  S  F  V  I  I  V  A  C  V  G  I  I  G  L   - 75
ATGAAAGGCGGAAAATGTCTTCTCACTATGCATATTATCGCTTTAATCATTGCAACAATT  360
M  K  G  G  K  C  L  L  T  M  H  I  I  A  L  I  I  A  T  I   - 95
ATAGACATTTCAACGGCGACATTATCAGCTATCAAACAAAATGAGTTTTTAACGAAAGCT  420
I  D  I  S  T  A  T  L  S  A  I  K  Q  N  E  F  L  T  K  A   -115
GGACAGGTTTTAAATGATTCATCAAAACTTTACTATAAAAACCGTCTATATGCAACAGAA  480
G  Q  V  L  N  D  S  S  K  L  Y  Y  K  N  R  L  Y  A  T  E   -135
TTCGATTTGATGCATATCACTTTCAAATGTTGCAATGTAAAAAATGACTACTCTTTACTC  540
F  D  L  M  H  I  T  F  K  C  C  N  V  K  N  D  Y  S  L  L   -155
GGAACACTTCATTTAATACCAGAATCATGCACTCATGGAATCGAATTCTACAAACAGCAA  600
G  T  L  H  L  I  P  E  S  C  T  H  G  I  E  F  Y  K  Q  Q   -175
TGCAATGAACCATTAAATAAATATGTACGATATTATATTGACATATTGATATATCTGTGC  660
C  N  E  P  L  N  K  Y  V  R  Y  Y  I  D  I  L  I  Y  L  C   -195
TTTATATTTGGATTTATTAAACTCATCTACTCATTGTTTACATTTACACAACGACAACGA  720
F  I  F  G  F  I  K  L  I  Y  S  L  F  T  F  T  Q  R  Q  R   -215
ATATTCAGTGAGAAAACCCCTGTTGCATAAcacaagtttctcagtataaaaacaaataca  780
I  F  S  E  K  T  P  V  A  *                                  -224
caaacaacaaataatgaaaatatatcataaacgttacggttaattactttagaacacataaa  840
tattgattagtatccatgactagatgccattgtacatttactgataacaaaaatgtttat  900
gtattattaatcatgatgtaactttgtttttgcactacaaaattgttaccaatgtgataa  960
ttattgattatatttgaaagatattcaaaaaaaaaaaaaaaaaaa                1005
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Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA clone 7A1 encoding Sj25/TM4 from adult *S. japonicum*. Nucleotide residues are numbered above and amino acid residues below. Predicted start ATG and stop TAA codons are shown in bold face. Putative functional sites are underlined: NDSS (Asn-X-Ser/Thr), N-linked glycosylation; SSK, TFK, TOK and SEK, potential protein kinase C phosphorylation; TIID, TEFD, casein kinase II phosphorylation; RyyiDiliY, tyrosine kinase phosphorylation; and GVRPSI, GLMKGG, GQVLND, N-myristoylation.

identity and 46% homology. Matches with similar scores and homologies were also obtained to CD53, CD63, and the tumor-associated antigen CO-029, which are also members of the TM4SF [20]. Based on these homology comparisons, and on the presence of other diagnostic motifs, as discussed below, Sj25/TM4 appeared to be related to the Sm23 group of schistosome TM4SF antigens. However, it did not appear to be a direct homologue of the Sm23 group.

Sj25 / TM4 has four transmembrane domains and other TM4SF characteristics. When the primary amino acid sequence of Sj25/TM4 was examined for the presence of functional sites, the following motifs were indicated: NDSS, N-linked glycosylation; GVRPSI, GLMKGG, GQVLND, N-myristoylation; SSK, TFK, TOK, SEK, protein kinase C phosphorylation; TIID, TEFD, casein kinase II phosphorylation; and RYYIDILIY, tyrosine kinase phosphorylation.

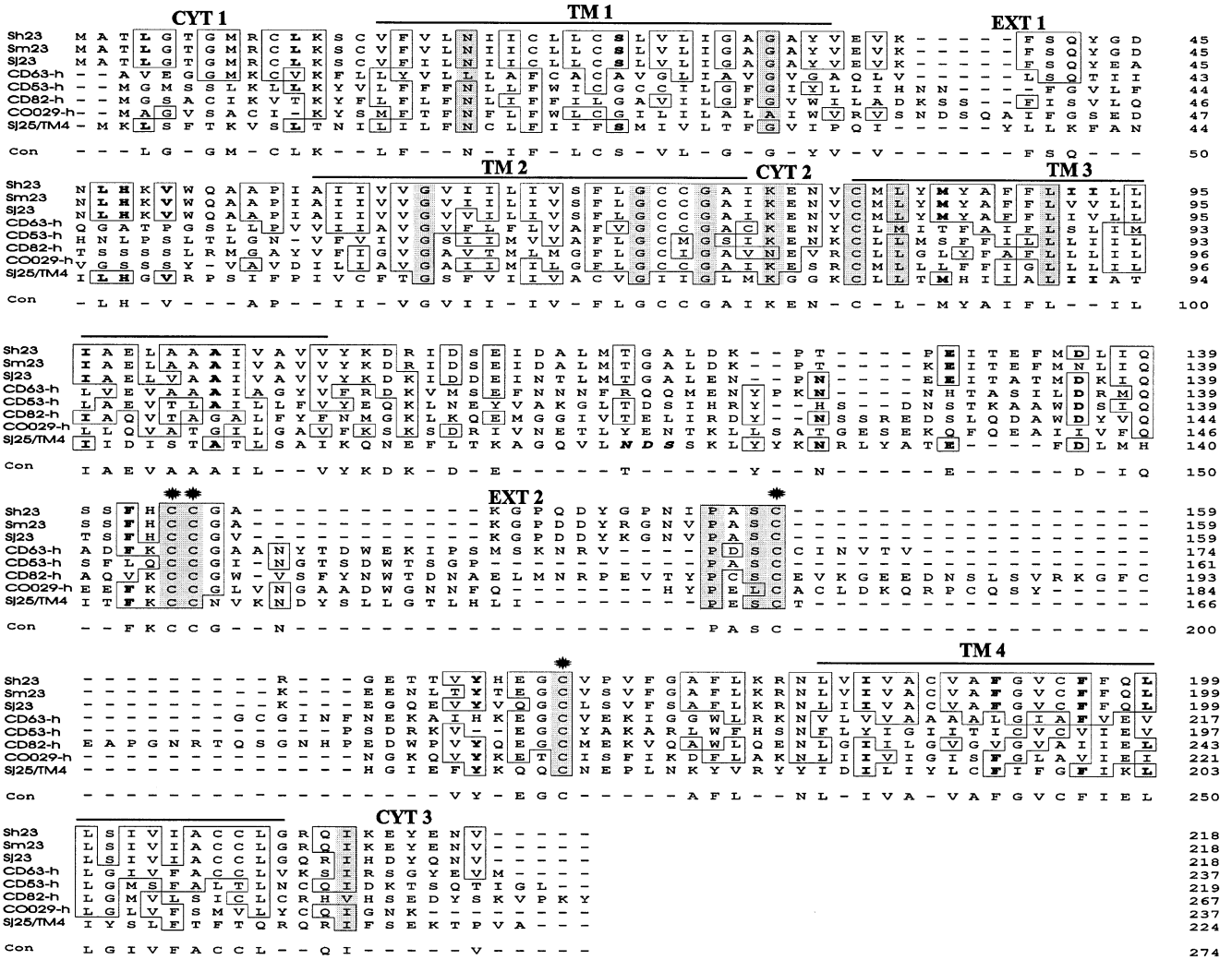

Fig. 2. Multiple sequence alignment of the deduced amino acid sequences of Sj25/TM4 with seven known members of the TM4SF. Amino acid numbering is at the right. Residues with homology to six other members are shaded. Identities in amino acid residues among 4 of 8 proteins are shown as the consensus sequences (Con). Residues conserved between Sj25/TM4 and the Sm23 group from schistosomes are in bold face type. The TM4SF-like domains of Sj25/TM4 are indicated as follows: CYT 1 to 3, cytoplasmic domains; TM 1 to 4, transmembrane domains; EXT I and 2, extracellular domains. Four conserved cysteine residues characteristic of the large extracellular domain of TM4SF are marked by asterisks, and the conserved PXSC motif of this domain is indicated by dark shading. The putative site of N-glycosylation of Sj25/TM4 is in bold italicized type. Database accession numbers for the proteins aligned here: Sh23, U23771; Sm23, P19331; Sj23, P27591; CD63, P08962; CD53, P19397; CD82, P27701; CO029, P19075; Sj25/TM4, U77941.

These motifs are indicated on Fig. 1. The presence of the potential site for tyrosine kinase phopshorylation is noteworthy since a role in signal transduction has been considered in relation to other TM4SF proteins including Sm23 [1,21,22].

Wright and Tomlinson [1] define classical TM4SF molecules by the presence, inter alia, of four highly conserved hydrophobic domains (TM 1 to 4), which are thought to span the cell surface bilayer. The NH_2- and COOH-termini of these molecules are predicted to form short cytoplasmic domains (typically 5 to 14 amino acids in length, domains CYT 1 and CYT 3). Two extracellular domains are predicted; a short domain (EXT 1, 20 to 27 amino acids) between the transmembrane domains TM 1 and 2, and a larger domain (EXT 2, 75 to 130 amino acids) between TM 3 and 4. A very short cytoplasmic domain (CYT 2) may occur between TM 2 and 3. Fig. 2 presents an alignment of the deduced amino acid sequence of Sj25/TM4 with seven TM4SF antigens, including the Sm23/Sj23/Sh23 group. The positions and topology of the TM4SF domains of Sj25, predicted with TMpred [18], are also shown in Fig. 2. Three consecutive hydrophobic domains are clustered at the NH_2-terminus, followed by a single hydrophilic domain and a hydrophobic domain located at the COOH-terminus. The sizes of the putative domains of Sj25/TM4 fall within the TM4SF domain sizes predicted by Wright and Tomlinson [1], as follows: CYT 1, 13 amino acids, CYT 3, 11 residues, EXT 1, 21 residues, EXT 2, 81 residues, TM 1 to 4, 22, 24, 21 and 25 residues, respectively. Fig. 2 demonstrates the conservation of this domain structure in all the proteins shown.

The homology between Sj25/TM4 and the TM4SF antigens is most marked in the hydrophobic domains TM 1 to 4 (Fig. 2). TM3 and TM4 are characterized by highly conserved, polar residues which are E or Q in almost all other TM4SF members [1]. By contrast, they are D and K in Sj25/TM4. The extracellular domains of TM4SF are less conserved than in the transmembrane hydrophobic regions but they have three conserved motifs CCG, PXSC, and EGC [1]. Sj25/TM4 has all four conserved Cys residues, in CCN, PESC and QQC motifs (Fig. 2), although CCN is divergent from the CCG motif of other TM4SF

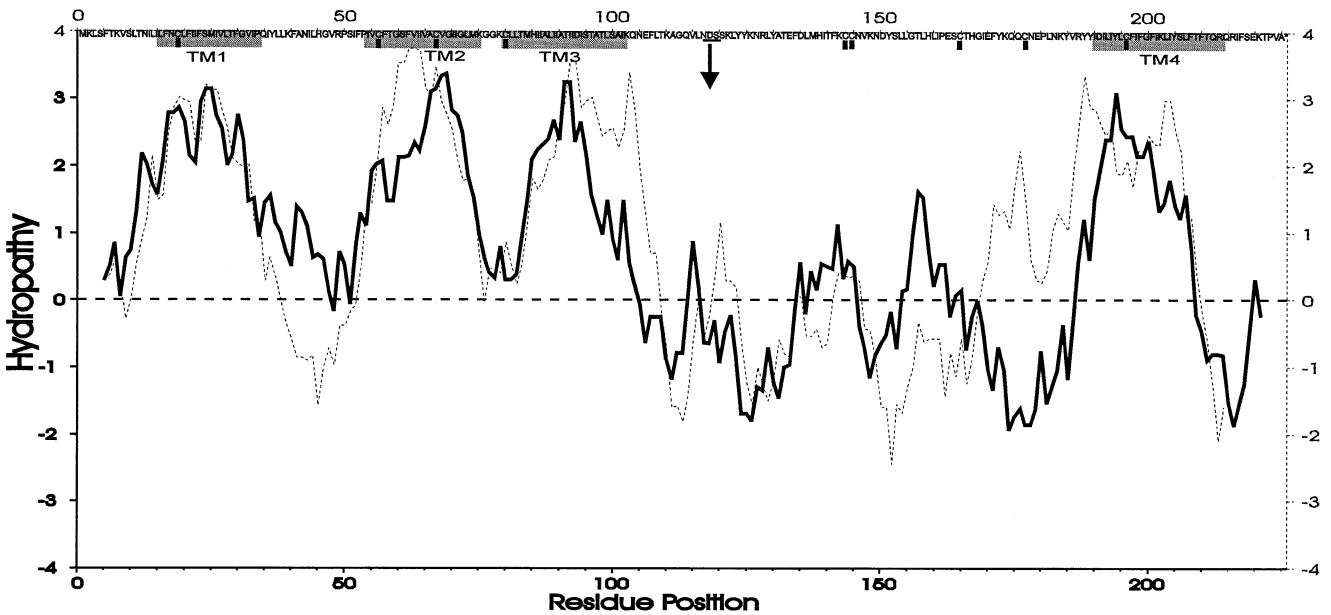

Fig. 3. Hydropathicity plots comparing Sj25/TM4 (solid line) and Sj23 (dotted line) in both hydrophobic and hydrophilic regions. The plots were produced with the PEPWINDOW (GCG) software. Hydropathy indices were determined by the method of Kyte and Doolittle [16]. Hydrophobic residues are positive. Cys residues are denoted by vertical bars and the arrow denotes the putative site of N-glycosylation. The positions of the hydrophobic transmembrane domains TM 1 to 4 of Sj25/TM4 as determined with TMpred [17] are indicated.

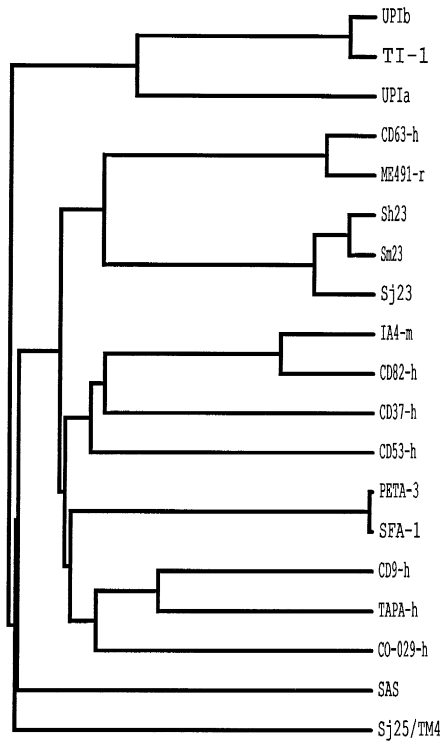

Fig. 4. Dendrogram of multiple pairwise alignments of deduced amino acid sequences of Sj25/TM4 and 18 members of the TM4SF to produce clusters of similar sequences. Similarities were obtained using the Fasta and PileUp programs. The database accession numbers for the TM4SF proteins compared are: UPIa, Z29475; TI-1, M64428; UPIb, Z29378; ME49, D21264; IA4, D14883; CD37, P11049; PETA-3, U14650; SFA-1, D29963; CD9, M61880; TAPA-1, M33680; SAS, U01160; Sh23, U23771; Sm23, P19331; Sj23, P27591; CD63, P08962; CD53, 19397; CD82, P27701; CO029, P19075; Sj25/TM4, U77941.

members [1]. The presence of all four conserved Cys residues of the extracellular domain indicated that Sj25/TM4 may have a similar disulfide bridging and conformation to the extracellular domain of classical TM4SF proteins. Sj25/TM4 has nine Cys residues (4%), whereas the Sm23 group has fifteen (7%). The spacing of these Cys residues in Sj25/TM4 was NH_2-18 amino acids-C-38-C-10-C-11-C-63-CC-18-C-10-C-18C-29-COOH. This spacing was not unusual, according to the SAPS algorithm [19]. The potential site of N-glycosylation in Sj25/TM4, NDSS, occurs in the large extracellular domain [1]. The predicted site of N-glycosylation of the Sm23 group occurs in the same domain [5,9].

A hydropathicity plot of Sj25/TM4 is presented in Fig. 3, alongside a similar plot for Sj23. The similarity in hydrophobicity of domains TM 1 to 4 and, indeed, in the general pattern of hydropathicity of Sj25/TM4 and Sj23, is striking. The location of the N-glycosylation site on the large hydrophilic loop, EXT 2, is indicated. These hydropathicity comparisons provide further support for the inclusion of Sj25/TM4 as a member of the TM4SF group.

A primary sequence alignment of eighteen TM4SF proteins with Sj25/TM4 in the form of a dendrogram is presented in Fig. 4. The dendrogram suggests that Sj25/TM4 is more closely related to Sm23 than to the UP1a, UP1b, and TI-1 proteins, although as far as membership of the TM4SF is concerned, Sj25/TM4 is not closely related to the Sm23 group. This analysis, however, consolidates the position of Sj25/TM4

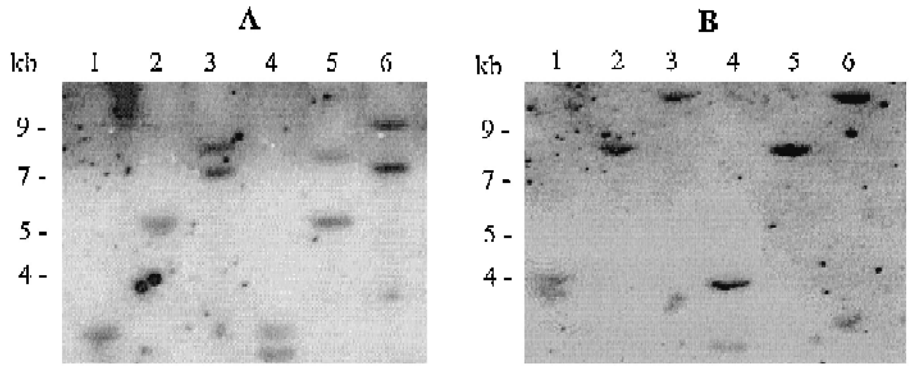

Fig. 5. Autoradiograph of Southern blot of endonuclease-digested, genomic DNAs from Chinese and Philippine strains of *S. japonicum* hybridized to the radiolabelled sequences encoding Sj25/TM4 (panel A). Panel B shows an autoradiograph of the same filter re-probed with labelled sequences encoding Sj23, after the labelled Sj25/TM4 sequences were removed. Lanes 1 to 3, Chinese strain DNA digested with *Eco*RI, *Xba*I and *Pst*I; lanes 4 to 6, Phillipine strain DNA digested with *Eco*RI, *Xba*I and *Pst*I.

within the TM4SF, given that UP1a, UP1b, and TI-1 are considered bona fide members [1].

Strain variation at the Sj25/TM4 locus. Southern blot analysis using three separate restriction enzymes (*Eco*RI, *Xba*I and *Pst*I) revealed a simple pattern of hybridization, which suggested that a single copy gene encodes Sj25/TM4 (Fig. 5, panel A). Restriction fragment length polymorphisms (RFLPs) were detected between the Chinese and Philippine stains of *S. japonicum* with each of these enzymes (Fig. 4, panel A), even though only *Eco*RI cleaved the Sj25/TM4 cDNA. After the filter was stripped of the Sj25/TM4-specific probe, and hybridized to a radiolabelled Sj23 probe, a pattern of hybridization distinct from that seen with Sj25/TM4 was obtained, verifying that Sj25/TM4 and Sj23 are encoded by separate genes (Fig. 5, panel B). As with Sj25/TM4, RFLPs were evident which distinguished the Chinese and Philippine strains of *S. japonicum*. The RFLPs evident in Fig. 5 are of interest because previous reports have not been able to readily differentiate these two strains using other sequences as probes [23]. Hybridization of labelled Sj25/TM4 to a Southern blot of endonuclease-digested, genomic DNAs from *S. japonicum*, mouse, and rabbit showed that the sequence was present only in the schistosome DNA (data not shown), demonstrating that clone 7A1 encoding Sj25/TM4 was of *S. japonicum* origin and not a host gene contaminant as has been previously reported in other cDNA libraries from schistosomes [14].

Sj25/TM4, a new member of the TM4SF from the Asian blood fluke. In summary, we report here a new member of the TM4SF expressed by miracidia and adult *S. japonicum* parasites. It will now be of interest to determine the site(s) of expression of Sj25/TM4, given that it is expressed in, at least, miracidia and adult worms, and to determine whether a homologue exists in other species of schistosomes. (An EST from *S. mansoni*, GenBank no. N21921, shares some homology with Sj25/TM4.) It will be of interest also to examine the molecular association between Sj25/TM4 and Sj23 within the same cells and tissues of the schistosome and, given the prominence of Sm23 as a vaccine antigen candidate [6], to examine the role of Sj25/TM4 in the immune response of humans and experimental animals to *S. japonicum* infection. As with other members of the TM4SF, the cellular function of Sj25/TM4 also awaits elucidation.

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