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Identification and cloning of a novel tetraspanin (TSP) homologue from *Brugia malayi*†

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Abstract

This is the first report of a tetraspanin (TSP)-like molecule in the lymphatic filarial parasites. Expressed sequence tag (EST) database search for TSP like molecules in the filarial genome resulted in three significant EST hits (two partial ESTs from *Brugia malayi* and one full length EST from *Wuchereria bancrofti*). The full length gene cloned from *B. malayi* showed significant similarity to *Caenorhabditis elegans* TSP and human TSP and hence the gene was named *B. malayi* TSP (BmTSP). Subsequent Genbank analysis with the predicted ORF of BmTSP showed additional homologous genes reported from *Schistosoma mansoni* and *Taenia solium* parasites. Structural analyses showed that BmTSP has four transmembrane domains and other conserved domains such as CCG and two other critical cysteine residues present within the large extracellular loop similar to other reported TSPs. In addition, putative post-translational modifications such as *N*-glycosylation, protein kinase c phosphorylation, casein kinase II phosphorylation and *N*-myristoylation sites have been found in BmTSP sequence. Further, PCR analyses showed that BmTSP is differentially transcribed, with highest level of expression being present in the adult stages followed by L3 and mf stages. This study thus describes a novel TSP cloned from *B. malayi*, its putative functions in cuticle biogenesis and role in protective immunity.

Keywords

Brugia malayi; *TSP*; *cuticle*; *protective immunity*

Introduction

Lymphatic filariasis is a mosquito borne disease caused by the parasitic worms *Wuchereria bancrofti*, *Brugia malayi* and *B. timori.* This debilitating disease is endemic in Asia, Africa, the Pacific, and Latin America, with an estimated 120 million people at risk (Michael and Bundy 1997). Adult worms of the parasite live inside lymphatic vessels and are responsible for major pathology associated with this infection. Diethylcarbamazine (DEC) is the drug of choice for this disease and decreasing the infection rate by 60–70% can have significant medical, social, and economic benefits (Ottesen 2000). Currently, there are no vaccines available for this infection. Mass drug treatment in lieu of controlling this infection has been unsatisfactory and unsuccessful besides increasing the risk of drug resistance. An increase in the number of new cases in endemic countries suggests the need for a more effective drug or

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alternate control strategy to reduce the incidence of this infection (Wang et al. 1997). In this regard, a vaccine based control strategy seems to be a viable approach for controlling lymphatic filariasis (Gregory et al. 2000).

Published evidences support the notion that development of a vaccine against this disease is feasible (Li et al. 1993; Gregory et al. 2000; Gnanasekar et al. 2004; Dabir et al. 2006). For example, protective immunity can be achieved by vaccinating animals with irradiated (Oothuman et al. 1979) or chemically abbreviated larvae (Grieve et al. 1988). However, this method of vaccination is potentially limited because of safety issues in humans. Recent developments in recombinant DNA technology have circumvented these problems. Great deal of efforts have been made to identify candidate vaccine antigens by immunoscreening expression libraries of the parasite (Gnanasekar et al. 2004), differential screening of abundantly expressed mRNAs (Gregory et al. 1997) or by expressed sequence tag (EST) approach (Lizotte-Waniewski et al. 2000). Recombinant proteins isolated through these strategies have been shown to confer varying degrees of protection in animal models (Li et al. 1993). However, the levels of protection achieved with these antigens are unsatisfactory except those reported for ALT family of proteins (Gregory et al. 2000; Gnanasekar et al. 2004). Hence, there is a need to identify more candidate antigens for prophylactic use. Cuticular antigens of nematodes have been suggested as potential targets for protective immunity (Selkirk et al. 1989). In this study, we report the identification of a novel putative surface expressed cuticular tetraspanin (TSP) from *B. malayi*.

Materials and methods

EST database analyses

TSP antigens expressed on the surface of several helminth parasites are shown to be promising vaccine targets because of their immunodominant nature (Hancock et al. 2006; Loukas et al. 2006; Tran et al. 2006). However, to date, there are no reports on the existence of a TSP-like molecule in the lymphatic filarial parasites. Therefore, in this study, we searched the EST databases of filarial parasites with *Schistosoma mansoni* TSP sequences for the presence of TSP homologues in the filarial genome at the BLAST server (www.ebi.ac.uk/blast2/parasites.html). This search revealed three ESTs, of which two were from *B. malayi* (EMBL accession # AA161593 and AW061610) and one from *W. bancrofti* (EMBL accession # CD455836). The reported ESTs from *B. malayi* were partial clones, whereas, the EST from *W. bancrofti* was a full length clone. We first decided to amplify the *B. malayi* TSP homologue as parasite materials and cDNA libraries were readily available in our laboratory. For PCR cloning, forward (5′ATGGTTCACGGCTGTGGTAAT3′) and reverse primers (5′TTAAGCATAATAGT ATGGTGTTTGATAGCG3′) were designed from *W. bancrofti* EST (CD455836). *B. malayi* adult cDNA library was used as the template and *B. malayi* TSP gene was then amplified by PCR using the following parameters: 95°C of initial denaturation for 30 s, following 45°C of primer annealing for 60 s, 72°C of primer extension for 60 s and 72°C of primer extension for 60 s. These conditions were repeated for 35 cycles and a final extension of 5 min was performed at 72°C before storing the samples at 4°C. PCR products were then cloned in TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and the DNA insert was sequenced on both strands at the University of Illinois core DNA sequencing facility to confirm the authenticity of the gene. Sequences were analyzed using a software program at the GenBank (www.ncbi.nlm.nih.gov) site and multiple sequence analysis was performed by Clustal W program. Insert analysis showed significant identity with *W. bancrofti* full length EST. Subsequent Genbank analyses showed that the sequence had significant similarity with *Caenorhabditis elegans* TSP (CeTSP) and *S. mansoni* TSP (SmTSP) and hence the newly identified gene was designated BmTSP.

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Stage-specific expression of BmTSP

BmTSP genes were then amplified from the cDNA libraries of various life cycle stages of *B. malayi* using insert specific primers by PCR and separated on a 1% agarose gel. After staining with ethidium bromide, band intensity was determined using NIH image software. PCR products were then normalized to the housekeeping *B. malayi* GAPDH (BmGAPDH) gene.

Results and discussion

Identification of a novel tetraspanin (TSP) gene from *B. malayi*

The predicted ORF of the sequence that we cloned from *B. malayi* was found to encode 229 amino acids which shared 99% identity with *W. bancrofti* EST (accession # CD455836). This high sequence identity is not surprising as genes cloned from *B. malayi* and *W. bancrofti* are shown to be highly conserved between these two species (Gnanasekar et al. 2002; Rathaur et al. 2003). Subsequent analyses of the amino acid sequences deposited in Genbank showed high homology with *Homo sapiens* CD81 (25% identity and 23% similarity), *H. sapiens* CD9 (23% identity and 25% similarity), *S. mansoni* TSP-2 (21% identity and 21% similarity), *Taenia solium* TSP (19% identity and 20% similarity) and *C. elegans* TSP-15 (12% identity and 23% similarity) antigens (Figure 1). Phylogenetic analyses showed that filarial derived TSPs are closely related but distantly separated from trematode and tape worm TSPs (Figure 2). In addition, PROSITE scan tool analyses showed that BmTSP has four different patterns of putative post translational modifications. These include two *N*-glycosylation sites at aa7–10 and aa158–161, three protein kinase c phosphorylation at aa9–11, aa91–93 and aa191–193, two casein kinase II phosphorylation sites at aa137–140 and aa191–194 and one *N*-myristoylation site at aa83–88.

TSPs are abundantly expressed transmembrane proteins ranging from 25 to 50 kDa which is present in almost all cells and tissues (Todres et al. 2000). To date 87 different TSPs have been identified from various species; 32 in mammals, 35 in flies and 20 in worms (Todres et al. 2000). In general, fewer studies have attempted to characterize TSPs compared to integrins, another membrane associated protein. Nevertheless, TSP is back in the lime light and is gaining significant momentum due to recent reports on its varied functions including its potential as a vaccine candidate (Hemler 2001; Loukas et al. 2006). Certain genetic mutations in the TSP gene are associated with mental retardation in humans and retinal dystrophy in mice (Hemler 2001). Similarly, TSP-15 gene knockout studies in *C. elegans* suggests that TSP are essential for epithelial integrity (Hemler 2001). Thus, some of the major cellular functions of TSP proteins include regulation of cell motility, cell morphology, invasion, fusion and cell signaling events.

Structurally, TSP family of proteins has four transmembrane domains, a highly conserved CCG motif and two other critical cysteine residues which are present within the large extracellular loop. Structural analysis of the filarial encoded TSPs show that they also carry all these conserved features to be qualified as TSP family of proteins. SOSUI analysis showed that filarial TSPs has four transmembrane (Tm) domains (Tml aa8–30, Tm2 aa64–86, Tm3 aa96–118, Tm4 aa195–217), CCG motif at aa153–155 and two additional cysteine residues in the second extracellular loop at aa170 and 180. These structural features confirm that the BmTSP gene that we identified from *B. malayi* is indeed a new member of the TSP family. Interestingly, a protein namely DiL3M C4 also having four transmembrane domains have been reported from the dog filarial parasite *Dirofilaria immitis* (Tsuji et al. 2000). Although this antigen has the characteristic transmembrane domains of TSP, it lacks the conserved domains and is thus not included in the TSP family.

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Stage-specific expression of BmTSP in different life-cycle stages of *B. malayi*

TSP proteins of *C. elegans* (Moribe et al. 2004), *S. mansoni* (Tran et al. 2006) and *T. solium* (Hancock et al. 2006) are expressed exclusively on the surface of the parasite similar to the mammalian TSP proteins. Prediction of protein localization using PSORT program analyses suggest that BmTSP is also expressed on the surface. Further localization studies using anti-BmTSP antibodies will determine whether BmTSP are present on the parasite cuticle. Nevertheless, in the present study, we determined the expression of BmTSP mRNA in various life-cycle stages of the parasite. These studies showed that BmTSP is indeed expressed in all mammalian life-cycle stages of *B. malayi* with highest level being expressed in the adult stages followed by L3 and mf (Figure 3).

Analysis of *C. elegans* genome reveal the presence of 20 distinct TSP genes (Moribe et al. 2004). Although our EST database search did not result in any additional hits, at this time, we do not know whether *B. malayi* expresses other variants of TSP. RNA interference (RNAi) analyses on each of the 20 TSP genes of *C. elegans* showed that out of 20 TSP proteins only CeTSP-15 had any conclusive function (Moribe et al. 2004). *C. elegans* lacking the CeTSP-15 gene had impaired barrier function of the hypodermal membrane, degeneration of the hypodermis and blistered cuticle. Interestingly, BmTSP shares 23% sequence similarity with CeTSP-15. Although at present we do not know the putative function of BmTSP, we believe that BmTSP may have a possible role in cuticular biogenesis and maintenance of cuticle integrity similar to CeTSP-15.

The nematode cuticle is a complex extracellular structure, which provides many important basic functions such as protection from dehydration, abrasion and immune attack (Maizels et al. 1993). In addition to serving as a protective covering, the cuticle also has important roles in absorptive, enzymatic and secretory activities (Maizels et al. 1993). Several immunological studies reiterate the importance of cuticular and surface antigens as targets of protective immunity (Maizels et al. 1989; Selkirk et al. 1989; Selkirk and Blaxter 1990). However, little is known about the protective capabilities of these cuticular antigens. Recent studies by an Australian group of investigators have raised hopes that the surface expressed TSP of *S. mansoni* can be developed into a vaccine against schistosomiasis (Tran et al. 2006). Similarly, TSP from *T. solium* has also been shown to be a potent immunodominat antigen in the infected individuals (Hancock et al. 2006). Since parasite TSPs appear to play an important role in maintaining the extracellular integrity and these molecules are recognized by the host immune system suggests that BmTSP may be a potential target for vaccine or drug development against lymphatic filariasis.

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                     10        20        30        40        50        60
                      |         |         |         |         |         |
BmTSP       ------MVHGCGNRTLKFLFFTANLLTCAFGALLFGISLWAYHDENFLQKLEKVQEIHKE
WbTSP       ------MVHGCGNRTLKFLFFTANLLTCAFGALLFGISLWAYHDENFLQKLEKVQEIHKE
Hscd81      ------MGVEGCTKCIKYLLFVFNFVFWLAGGVILGVALWLRHDP----QTTNLLYLELG
Hscd9       ------MPVKGGTKCIKYLLFGFNFIFWLAGIAVLAIGLWLRFDS----QTKSIFEQETN
TsTSP       ------MGLSCGGNFLKFLVFFFNAIVFIGGGVIAAFGIYLLVET------K------KS
SmTSP2      ------MALGCGYKCLQCLLIIFNCGAFICGLGLIVVGALGLHSV-------VN-----H
CeTSP15     MGALGDSAYGARGRLIKFSYIVTALISILFSISCICYGIWLLARR------------SQY
                         . ::   :         .      .

                     70        80        90       100       110       120
                      |         |         |         |         |         |
BmTSP       YSPELTQHQIGLWLLIIIGASIFLVGFLGCCGAICEST-KLLALFSIIVLILAILEVASI
WbTSP       YSPELTQHQIGLWLLIIIGASIFLVGFLGCCGAICEST-KLLALFSIIVLILAILEVASI
Hscd81      DKPAPNTFYVGIYILIAVGAVMMFVGFLGCYGAIQESQ-CLLGTFFTCLVILFACEVAAG
Hscd9       NNNS--SFYTGVYILIGAGALMMLVGFLGCCGAVQESQ-CMLGLFFGFLLVIFAIEIAAA
TsTSP       GGTVS--LVLPAFIT-AFGLLLFLIGFLGCFGACYNNT-CMLKTFAAIVGILLVAEIICA
SmTSP2      WSEIEPPLQSLIIFIIALGCFLFVLGALGMFGACMKNV-CLLTTYCILLSILMVAEIAAG
CeTSP15     AELVSPSLYVDVGRILVIISILSILNYLICFYAIFKEMRCFVTSCAVASIVIAVMLIIGG
                                 : .:. *    *  :.   ::        ::    :

                    130       140       150       160       170       180
                      |         |         |         |         |         |
BmTSP       VLIIAGKD-------QFKNALYNLLSKTGESEDE--MQHFKPIEDLFQCCGPTN------
WbTSP       VLIIAGKD-------QFKNALYNLLSKTGESEDE--MQHFKPIEDLFQCCGPTN------
Hscd81      IWGFVNKDQIAKDVKQFYDQALQQAVVDDDANNA--KAVVKTFHETLDCCGSSTLT----
Hscd9       IWGYSHKDEVIKEVQEFYKDTYNKLKTKDEPQ----RETLKAIHYALNCCG----L----
TsTSP       IVLLVYRHDFVRLVGKEMQEAIQELQSKRLSGSDPTLKALEELQAKLKCCGGVG------
SmTSP2      IFAIVEKPK----VKKHITSALKKLVDKYRND-EHVRKVFDEIQQKLHCCGADS------
CeTSP15     CIGLNFRDQLTHYTPLNLKMLTSLRELYGTHDMKGITESWDALQSNFKCCGVNGTDNAQI
                  :               .                 . :.  :.***

                    190       200       210       220       230       240
                      |         |         |         |         |         |
BmTSP       ---------ETMVRYIENGLCED----------------ELRNKPNCFAVISDHFDSSQ
WbTSP       ---------ETMVRYIENGLCED----------------ELRNKPNCFAVISDHFDSSQ
Hscd81      ---------ALTTSVLKNNLCPSGSNII------------SNLFKEDCHQKIDDLFSGKL
Hscd9       ---------AGGVEQFISDICPK-KDVL------------ETFTVKSCPDAIKEVFDNKF
TsTSP       ---------PSDWRVAPPSCCGKESGSC------------TSPYQTGCAEAMYNEMKDSA
SmTSP2      ---------PKDYGENPPTSCSKD----------------GVQFTEGCIKKVSDLSKAHL
CeTSP15     WKTSKWYMHQRAPKLLIPESCCIPSEIERCRSNPFDQDAPPPYYTSTCYEPLQNDLLHVM
                                *                          *   : :

                    250       260       270       280       290
                      |         |         |         |         |
BmTSP       KDIIKISVVLIIIDLLALFSTSMLYKAFRYQTPYYYA-----------------
WbTSP       KDIIKISVXLIIIDLLALFSTSMLYKAFRYQTPYYYA-----------------
Hscd81      YLIGIAAIVVAVIMIFEMILSMVLCCGIRNSSVY--------------------
Hscd9       HIIGAVGIGIAVVMIFGMIFSMILCCAIRRNREMV-------------------
TsTSP       LAFGIVIIVIGLIQIGAIICAACLAKKVSEYEKV--------------------
SmTSP2      NAIIVSVFLFALVQMICLVFAVCVLLAIKRGDDEYNDITKTLSEKINRKQPLKN
CeTSP15     NVASWLCITNAIVQIIPSVAGCWYSKLIRK------------------------
                   .   :: :   .        .
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Figure 1.

Multiple alignments (CLUSTAL) of the amino acid sequences of TSP family of proteins from *B. malayi* (BmTSP, accession no. EF397425), *W. bancrofti* (WbTSP, accession no. CD455836), *H. sapiens* (HsCD81, accession no. NP_004347), *H. sapiens* (HsCD9, accession no. NP_001760), *S. mansoni* (SmTSP-2, accession no. AF521091), *T. solium* (TsTSP, accession no. AY211879) and *C. elegans* (CeTSP-15, accession no. Q9XVM9). (★) Indicates identical amino acids, (:) indicates strongly similar amino acids and (.) indicates weakly similar amino acids.

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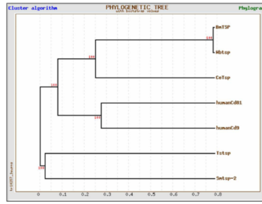

Figure 2.
Phylogenetic tree analysis of TSP family of proteins. The tree distances were generated according to the ClustalW algorithm, and the tree was constructed using TreeTop (Phylip program). Phylogenetic tree analysis shows that filarial TSPs are closely related with distantly separated from trematode (*S. mansoni*) and cestode (*T. solium*) parasites.

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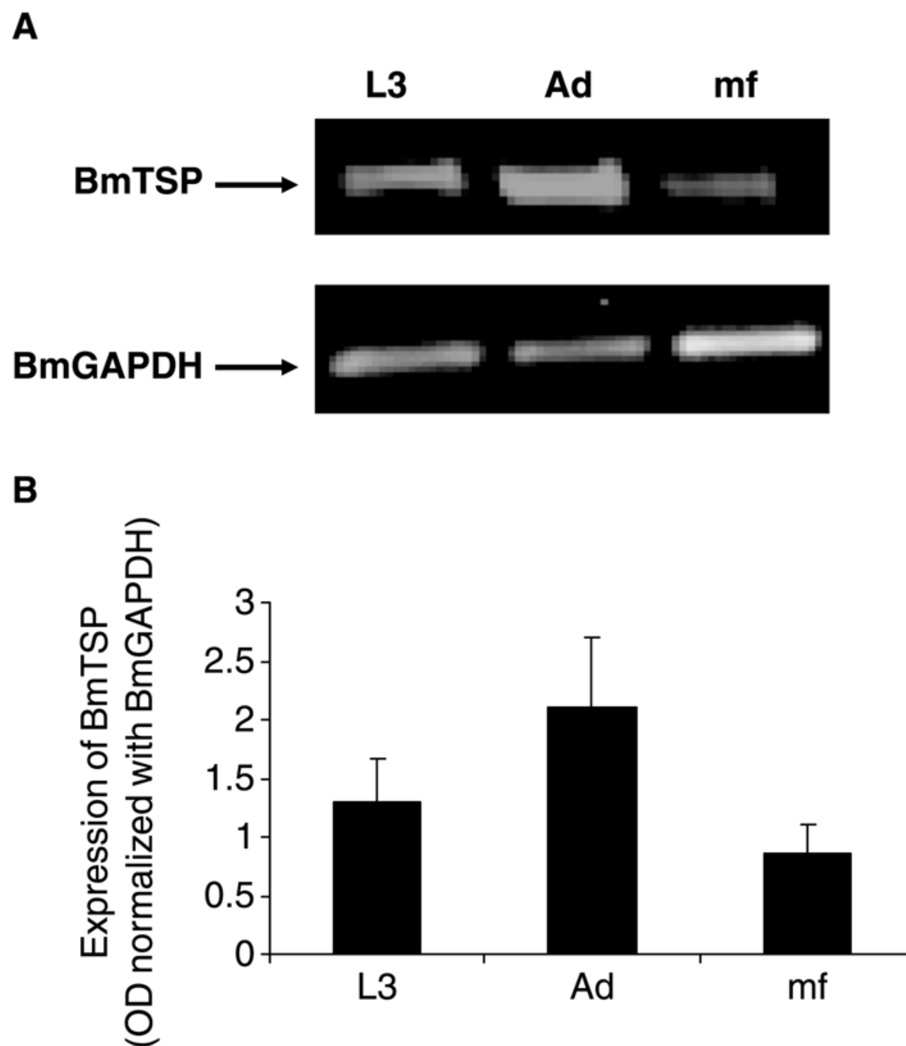

Figure 3.

Expression of BmTSP mRNA in various life cycle stages of *B. malayi*. A. BmTSP and BmGAPDH transcripts were amplified by PCR from the cDNA libraries of various life cycle stages (L3, adult and Mf) of *B. malayi* using primers specific for BmTSP and BmGAPDH. Expression of BmGAPDH was used as an internal house keeping control gene. PCR products were resolved on a 1% agarose gel and stained with ethidium bromide. B. Band intensity was normalized to BmGAPDH PCR products and values were calculated using NIH image software. Data represented in B is average of values from three similar experiments.