Isolation and characterization of recombinant Brugian parasitic transglutaminase

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An attempt has been made to identify and characterize a putative chemotherapeutic target antigen for human lymphatic filariasis by recombinant DNA techniques. Identification of transglutaminase (TGA), an enzyme vital for the growth and survival of the parasites, has opened a new drug target for developing potent therapeutic agents against the parasites. TGA gene from Brugia malayi adult female parasite (BmTGA) has been subcloned, expressed and purified as a 47 kDa fusion protein with N-terminal histidine tag. Since BmTGA lacks homology to mammalian TGAs, it is a promising drug target for developing chemotherapeutic agent and candidate vaccine against human lymphatic filariasis.

Keywords: adjuvants, filaricidals, immunoprophylaxis, molting, nematodes, protein disulphide isomerase, transglutaminase, vaccine

IPC Code: Int. Cl.8 C12N9/64

Introduction

Lymphatic filariasis is caused by infection with nematode parasites, Wuchereria bancrofti, Brugia malayi and B. timori, which are transmitted through blood-feeding mosquitoes. It is estimated that around 130 million people worldwide are infected with filarial parasites. It is a chronic parasitic disease prevalent in many tropical and sub-tropical countries. Persons suffering from this disease show symptoms like fever, chyuria (characterized by milky secretion in urine), hydrocele (accumulation of lymph) and elephantiasis (swelling) of extremities, along with other abnormalities of the lymphatic system.

The drugs available to date, for the treatment of this disease are Diethylcarbamazine and Ivermectin. These drugs have their own side-effects, such as nausea and dizziness, and lead to acquired drug resistance in parasites. Moreover, these two drugs are effective only against the microfilariae and cannot inhibit L3 and adult larval stages of the parasite. There is a need to develop a safe and effective drug for the eradication of lymphatic filariasis. Further, the non-availability of any suitable vaccine for filariasis due to limited parasitic material and lack of suitable animal models demands the identification of candidate vaccines for immunoprophylaxis. Hence, there is also a need to develop suitable molecules that can be used as drug targets, as well as vaccine candidates for elimination of human lymphatic filariasis.

In order to design a new drug, one must have a clear idea about the different biochemical pathways operating in the parasite and certain enzymes which are critical for the survival of the parasite, so that blocking that particular enzyme or pathway leads to instantaneous death of the parasite. The metabolic pathways in filarial parasites, such as glucose and folate metabolism, tubulin polymerization, neurotransmission and receptor functions, electron-transport pathway and energy-generating pathways, as well as some enzymes such as protein kinase, are used as putative target sites by antifilarial drugs. Transglutaminase (TGA) is one such enzyme which is vital for the growth and survival of the parasite.

Transglutaminases (TGAs, EC: 2.3.2.13) are a family of enzymes that catalyse the post-translational modification of proteins through the exchange of primary amines for ammonia at the γ-carboxyamide group of glutamine residue. Peptide bound lysine residues or polyamines serve as the primary amines to form either ε-(γ-glutamyl)lysine or (γ-glutamyl)polyamine between or within the proteins. The resulting bonds are covalent, stable and resistant to chemical, enzymatic and histological degradation. Such linkages are present in the exoskeleton or cuticle of the parasites. During the development from one larval stage into another, the cuticle is renewed and this process is termed molting. When the new cuticle is formed TGA plays a very important role in the formation of the ε-(γ-glutamyl)lysine crosslinks.
resulting in the stabilization of the exoskeleton. There are ample evidences indicating the significance of this enzyme in the filarial parasites. The inhibitors of TGA enzyme have been found to inhibit the molting of L3 to L4 stage larvae in *Onchocerca volvulus* and *Dirofilaria immitis*. Thus, TGA catalyzed reactions appear to be critical for the growth, development and maturation of nematode because of its role in cuticle biosynthesis. Lack of homology to mammalian TGA makes it a target for developing an effective chemotherapeutic drug that is not toxic to the host. Such a drug would specifically inhibit the parasite growth and development. Due to the presence of this enzyme in all stages and its major role in the molting process, we have chosen to use TGA as a drug target. An attempt is made to produce the recombinant protein in large-scale, which can be used for determining the three-dimensional structure by X-ray crystallography, so as to resolve the active site and develop enzyme-specific inhibitors. At the same time, the immunoprophylactic efficacy of this enzyme as a vaccine candidate could also be evaluated.

The present study was undertaken to subclone, express and characterize the TGA gene of *Brugia malayi* adult female parasite present in pTrc expression system (pTrcHisB). The clone in pTrcHisB showed feeble expression and high amount of protein degradation and could not meet the demands of large-scale production of recombinant protein for structural studies. Hence, an expression system which can produce more protein easily was selected and the TGA gene was subcloned, expressed, purified and characterized using T7 expression system.

**Materials and Methods**

**Reagents and Chemicals**

Chemicals of analytical grade and biotinamido-pentylamine,N,N’dimethylcasein, Guinea pig liver TGA and streptavidin-alkaline phosphatase were purchased from Sigma Chemical Company, St. Louis, MO, USA. Restriction enzymes, T4 DNA ligase were obtained from New England Biolabs, MA, USA. Hybond N+ nylon membrane and standard protein marker were from Amersham International, Birmingham, UK. Chemicals from Himedia, Mumbai, India were used for media preparations.

**Subcloning, Expression and Purification of Recombinant BmTGA Protein**

The *B. malayi* TGA from a previously obtained clone in pTrcHisB vector (pTrcBmA) was subcloned into pRSETB vector (Invitrogen) at the *PstI* site. The pTrcHisB-TGA was restriction-digested with *PstI*, purified the insert by gel-elution and then ligated into *PstI* digested pRSETB vector. After transformation of the ligation mixture into DH5α cells, colonies were screened for the presence of insert by lysate-PCR, using gene-specific primers and by restriction digestion of the plasmid extracted from the colonies. Once the presence of the insert was confirmed, the recombinant clone was transformed into *Escherichia coli* strain BL21(DE3), for the expression of *B. malayi* TGA recombinant protein (rBmA). The expression was checked on an SDS-PAGE and the protein was purified by immobilised metal affinity chromatography (IMAC). After transformation, single colony was inoculated into 3 mL Luria Bertani broth supplemented with ampicillin at a concentration of 100 µg/mL (LBA), and grown at 37°C overnight. This culture was then transferred into 100 mL LBA, and grown at 37°C till the cells attained an optical density of 0.6-0.7. Then 1 mL of the culture was taken and stored as uninduced control, while the remaining culture was induced with Isopropyl-thio-galactoside (IPTG) to a final concentration of 1mM and grown for 3 h at 37°C. Samples of 1 mL culture were taken every hour and checked for expression on an SDS-PAGE. Final hour culture were pelleted and solubilised in binding buffer (20 mM Tris, 50 mM sodium dihydrogen phosphate, 100 mM sodium chloride, pH 8.0). The cells were disrupted by sonication and centrifuged to yield the supernatant, which was further used for purification by IMAC, under non-denaturing conditions. The protein was eluted using an imidazole gradient and purified rBmA was dialysed against PBS to eliminate the imidazole. The IMAC purified recombinant six-histidine BmA was further confirmed by doing a Western blot with anti-histidine antibody as well as with anti-BmA. Briefly, the following protocol was followed. After electrophoresis, the SDS-PAGE gel was incubated for 10 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS). Nitrocellulose membrane (NCM), cut to the exact size of separating gel, was also incubated for 10 min in transfer buffer. Without trapping air bubbles, the
NCM was overlaid on the gel and sandwiched between Whatman no. 1 filter papers. Electrophoretic transfer was carried out at 120 mA using semiphore blotting apparatus. After transfer, the NCM was stained with Ponceau S (0.2% Ponceau S, Sigma, USA in 0.3% trichloro acetic acid and 0.3% sulfosalicylic acid) to ensure the transfer of the proteins. Membrane was washed in phosphate buffered saline (PBS) and blocked in 5% skimmed milk powder in PBS for 2 h at 37°C.

The NCM was washed in wash buffer [PBS with 0.05% Tween-20 (PBST)] thrice, followed by washing thrice in PBS alone and then incubated with primary antibody. In case of anti-histidine antibody, the primary incubation was 2 h at 37°C, while in case of anti-BmTGA antisera, the NCM was incubated with different dilutions of the primary at 4°C overnight. After washing in the wash buffer the membrane was incubated for 1 h with anti-mouse secondary antibody (1:20,000) conjugated with alkaline phosphatase. After secondary incubation, the blot was washed and incubated for 10 min in substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2). The colour development was carried out using 66 µL of nitroblue tetrazolium (NBT) and 33 µL of 5-bromo-5-chloro-indolyl-phosphate (pNPP) at a concentration of 1 mg/mL in substrate buffer (NaHCO3, Na2CO3 and MgCl2.6H2O, pH 8.6) was added to the wells and colour developed. The reaction was arrested after 20 min by addition of 100 µL of 3 N NaOH solution per well. The absorbance was measured at 405 nm in microplate reader.

**Enzyme Linked ImmunoSorbent Assay (ELISA)**

To determine the titre of the antiserum for recombinant pRSETB-BmTGA, which was raised in mice, we performed an ELISA. Briefly, the following protocol was followed. Purified recombinant BmTGA protein was coated at 100 ng/well concentration onto 96 well-plate in coating buffer (NaHCO3/Na2CO3, 0.067 M, pH 9.6) in 100 µL of volume and incubated overnight at 4°C. Antigen blank (coating buffer alone without antigen) and antibody blank (antigen without antibody) were also maintained. The plates were washed 4 times with PBS containing 0.1% Tween-20 (PBST). The unbound or non-specific sites were washed two times with 350 µL of 0.1 M Tris-HCl, pH 8.5; to obtain a total volume of 200 µL per well. After washing the microtitre plate for 2 h at 37°C, the liquid was discarded and the reaction stopped by washing twice with 350 µL of EDTA (200 mM), followed by 2 washes with 350 µL of 0.1 M Tris-HCl, pH 8.5. The streptavidin-alkaline phosphatase (0.25 mg/mL) was diluted to 1:150 with nonfat dry milk (0.5% in 0.1 M Tris-HCl, pH 8.5) for 30 min and washed twice with 350 µL of 0.1 M Tris-HCl, pH 8.5. Reagents were added to each well as follows: 5 mM CaCl2, 10 mM dithiothreitol, 0.5 mM 5-biotinamido pentylamine, rBmTGA and 0.1 mM Tris-HCl, pH 8.5; to obtain a total volume of 200 µL per well. After incubating the microtitre plate for 2 h at 37°C, the liquid was discarded and the reaction stopped by washing thrice in PBS (0.1% TritonX-100, followed by four 350 µL washes with 0.1 M Tris-HCl, pH 8.5. Then 200 µL of 0.1M Tris-HCl, pH 8.5 and 50 µL of phosphate substrate, p-nitro phenylphosphate (1mg/mL) were added to each well. A kinetic measurement of absorbance at 405 nm was determined at 30 sec intervals for a period of 30 min using a Vmax Kinetic Microplate Reader (Labmate). TGA activity is expressed as units of optical density (mOD/min). All reactions were performed in duplicate. Guinea pig liver TGA (50 ng/mL) was used as a positive control.

**Antiseras to rBmTGA**

Mice were immunized with 1µg of rBmTGA purified by IMAC. First immunization was given with
complete Freund’s adjuvant (CFA), while second dose was given with Incomplete Freund’s Adjuvant (IFA). Mice were bled from retro-orbital plexus, blood was collected, centrifuged, serum separated and stored at 4°C. The antibody titre was checked by ELISA and Western Blotting. Thereafter, immunization and bleeding were performed at alternate weeks with subsequent doses of the recombinant protein being given in 0.9% saline. The boosters were given till the antibody titre was about 1:10000 as determined by ELISA. At this stage the animals were bled and then finally sacrificed by cervical dislocation.

**Results**

The pTrcBmTGA gene was subcloned into pRSETB vector and sequenced fully. After sequencing, it was found that there was a stop codon at position 1101 of the nucleotide sequence, the ORF being 1.10 kb. Hence, the gene coded for a protein of 367 amino acids (Fig. 1). The original gene (pTrcHisBmTGA) was also sequenced and it was found to have a stop codon at position 1101, the ORF being 1.10 kb. Based on this sequence the deduced molecular weight of this protein was 43 kDa. The apparent molecular weight of pRSETB-TGA was also observed as 47 kDa on the SDS-gel electrophoresis. pRSETB-TGA does not show any homology with known mammalian TGA sequences in the sequence database. The multiple sequence alignment of pRSETB-TGA with known human and other mammalian TGAs, such as human blood coagulation factor XIII, A1 polypeptide (F13A1) (accession no. NM000129), human tissue TGA (accession no. NM198951), human epidermal TGA (accession no. NM003245), rat prostate TGA (accession no. BC066665), human erythrocyte membrane band 4.2 (accession no. BC096093) and also *B. malayi* TGA (accession no. AY273895) was performed (Fig. 2) using ClustalW program.

The presence of the insert was confirmed by both lysate-PCR of the transformants using gene-specific primers as well as by restriction digestion of the clone with HindIII and *PstI*. Fig. 3 shows the PCR product of ~ 800 bp with pRSETB-BmTGA internal primers. The PCR was done with internal primers: forward primer position 195-215 of the sequence 5’ CGA TGA ATT CAG TGT TAG TGG 3’ and reverse primer position 992-1012 of the sequence 5’ CTG TTC TTC CGG TGG TTC CTC 3’ (Fig. 1). Therefore, the PCR product was ~ 800 bp (0.8 kb) in size. In Fig. 4, the linearised clone (~ 4.6 kb) and the release of insert (~ 1.6 kb) after double digestion are indicated.

**Expression, Purification and Characterisation of pRSETB-BmTGA**

The BmTGA was expressed as an N-terminal 6-histidine fusion protein in T7 expression system (pRSETB). The fusion protein was found to have a molecular weight of 47 kDa on the SDS-PAGE. Fig. 5 shows expression of pRSETB-BmTGA at different time points along with uninduced clone and induced vector controls. The expression level was monitored for up to 3 h after induction. Thereafter, the level of expression was saturated and there was no further increase in the amount of recombinant protein. Hence, for further experiments, the same condition was maintained. The expressed protein was purified by IMAC under non-denaturing conditions due to the enzymatic nature of the protein. The column was washed with an imidazole gradient of 25-100 mM. The fusion protein was finally eluted at an imidazole concentration of 150 mM. Fig. 6 shows the SDS-PAGE analysis of purified BmTGA, which is seen as a single band at 47 kDa. Antiserum was raised in mice against the purified recombinant protein. The final titre of the antiserum was 1:10,000 as determined by Western blot analysis and by ELISA (data not shown). The immunoreactivity of the recombinant protein was confirmed by anti-histidine antibody as well as antiserum raised against pRSETB-BmTGA in mice. Fig. 7 (a & b) shows the Western blot analysis of purified recombinant BmTGA with: a) anti-histidine antibody, and b) anti-BmTGA antiserum. The recombinant BmTGA also showed enzymatic activity as determined by the solid-phase microtitre plate assay. As seen in Fig. 8, the activity was calcium-dependent and inhibited by EDTA. The nature of inhibition was competitive. Negligible activity was observed in control without the protein. TGA from Guinea pig liver was used as a standard.

**Discussion**

An attempt was made to characterize a putative drug target for the development of specific inhibitors, which can also serve as a suitable vaccine candidate against human lymphatic filariasis. Previous reports
Fig. 1—Nucleotide sequence of pRSETB-TGA. The position of the forward and reverse primers are in boldface and underlined.
UMA et al: RECOMBINANT BRUGIAN PARASITIC TRANSGLUTAMINASE

have highlighted the significance of the enzyme TGA in the molting and survival of the filarial parasites. One strategy lies in large-scale production of this enzyme for structural studies, so as to develop active-site blocking agents. The other aspect is to analyze the immune response to this enzyme as a potential vaccine candidate. Previously, TGA gene from *B. malayi* adult female parasite was cloned in pTrcHisB vector and submitted to GenBank with accession no. AY273895.

The putative thioredoxin domains of BmTGA and pRSETB-TGA are in boldface and underlined. Regions of identity (*), strong similarity (:), and weak similarity (.) are indicated.

Fig. 2—Multiple sequence alignment of the deduced amino acid sequence of pRSETB-TGA with proteins of other species using Clustal W program. Hu factor XIII: Human coagulation factor XIII, A1 polypeptide (F13A1) (accession no. NM000129); Human TGA2: Human transglutaminase2 (accession no. NM198951); Human TGA3: Human epidermal transglutaminase3 (accession no. NM003245); Rat TGA4: Transglutaminase4 from rat (accession no. BC066665); Hu erythro TGA: Human erythrocyte membrane band 4.2 (accession no. BC096093); pRSETB-TGA: Transglutaminase subcloned in pRSETB vector; BmTGA: *B. malayi* transglutaminase (accession no. AY273895).
The clone was expressed as a 6-histidine tagged fusion protein. This recombinant protein was purified and the enzyme activity was observed. The immunoreactivity of the protein was shown by Western blot with anti-histidine vector (T7 expression system). The clone was expressed as a 6-histidine tagged fusion protein. This recombinant protein was purified and the enzyme activity was observed. The immunoreactivity of the protein was shown by Western blot with anti-histidine.
and anti-TGA antibody. The molecular weight of \textit{B. malayi} native enzyme was about 56 kDa. However, the pRSETB-TGA clone expressed a protein of only 47 kDa, but the level of expression was much higher than in the case of pTrcHisB clone and the amount of protein degradation was also considerably reduced.

Having a single thioredoxin motif (-CGHC-), the recombinant enzyme showed significant characteristic activity of cross-linking of biotinylated pentylamine to dimethylcasein as determined by the microtitre plate assay. Nucleotide sequence analysis of this clone against other sequences in the database showed that it does not have any homology to mammalian TGAs, but it showed 99% similarity with \textit{B. malayi} TGA (accession no. AY273895) and 85% similarity with TGA from \textit{Dirofilaria immitis} (accession no. AF008300). The amino-acid sequence has only one thioredoxin motif (Cys-Gly-His-Cys) as opposed to other nematode TGAs with two such motifs\textsuperscript{9,13}. However, this clone has significant homology to PDI and to PDI-related ERp60, a feature which is characteristic of all nematode TGAs. Since this pRSETB-TGA clone shows TGA activity, inspite of having only one thioredoxin domain, probably the cysteine and histidine residues present in the single thioredoxin motif may be involved in the cross-linking function as was reported for \textit{C. elegans} PDI-3\textsuperscript{17}. \textit{In silico} analysis of the antigenic propensity showed that there are about 14 antigenic determinants in the predicted amino acid sequence of pRSETB-BmTGA (data not shown). These antigenic determinants can impart the characteristics of a good vaccine candidate to the protein.

Thus, a putative drug target, TGA from the filarial parasite \textit{B. malayi} has been identified and characterized. It was cloned in pRSETB vector, expressed as a 47 kDa fusion protein with N-terminal histidine tag and purified using IMAC. It was further
characterised by its immunoreactivity with anti-histidine and antiBmTGA antibody, and by its enzymatic assay. Its lack of homology with mammalian TGAs makes it an ideal drug target. At the same time, the presence of different antigenic determinants in the sequence enhances the need to study the immunoprophylactic potential of the recombinant protein for human lymphatic filariasis.

Acknowledgement
We thank Drs Kapil Mehta, Chandrashekar and Eswaran Devarajan for providing pTrcHisB-TGA clone and their comments. The financial assistance of UGC-DRS programme and ICMR are acknowledged.

References