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Cloning and characterization of a novel immunogenic protein 3 (NIP3) from *Brugia malayi* by immuno screening of a phage-display cDNA expression library

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Abstract

Iterative screening of a phage display cDNA expression library of the third-stage larvae (L3) of *Brugia malayi* with sera from putatively immune individuals (endemic normal, EN) identified a novel clone with insert showing significant homology to *Onchocerca volvulus* novel immunogenic protein-3 (Ov-NIP3) gene and *Caenorhabditis elegans* NIP3-like protein and hence the gene was designated *Brugia malayi* NIP3-like protein (BmNIP3). EST database analysis showed that ESTs from several gastrointestinal nematodes such as *Ancylostoma caninum*, *Teladorsagia circumcincta*, *Haemonchus contortus* and *Strongyloides stercoralis* has BmNIP3 homologues, but the gene has not been described from these parasites. Sequence analyses showed that BmNIP3 has three potential mucin-type O-glycosylation sites and several serine/threonine phosphorylation sites. As expected, BmNIP3 protein isolated from the parasite was serine/threonine phosphorylated. Further analyses showed that BmNIP3 is differentially transcribed, with highest level of expression present in the larval (L3 and L4) stages. Mice immunized with rBmNIP3 developed strong antibody responses predominantly of the IgG1 and IgG2a subtype. A similar analyses of the sera samples from EN individuals showed that they also carry high levels of IgG1 and IgG2 antibodies against BmNIP3, whereas, chronically infected patients carry largely IgG3 antibodies and MF individuals carry high levels of IgG1 antibodies against BmNIP3. This study thus describes a novel protein from *B. malayi* that appears to be highly immunogenic in both humans and mice.

Keywords

Brugia malayi; NIP3; EN; Phage-display; Biopanning

Introduction

Lymphatic filariasis caused by the filarial parasites *Brugia malayi* and *Wuchereria bancrofti* is a debilitating disease affecting over 120 million people in the tropical and sub-tropical countries (Molyneux 2003). These two parasites are closely related and show significant antigenic overlap that the antibodies generated against *W. bancrofti* in infected or immune individuals show significant cross-reactivity with *B. malayi* antigens (Lalitha et al. 2002; Rao et al. 2000; Rathaur et al. 2003). In the endemic areas, some individuals are naturally immune to the disease (Endemic Normal, EN); whereas, some carry the infection and show acute symptoms (Microfilaremics, MF) and others exhibit morphological evidence of lymph edema in the dependable parts as the infection becomes chronic (Chronic Pathology, CP). Although the nature of protective immune responses is highly debated over several years, (Peralta et al. 1999; Ravindran et al. 2000) the consensus is that the host immune responses play a major role in determining clinical manifestations of various groups (Helmy et al. 2000; Frank et al. 1996). In this respect the “EN” group, which resides in the endemic area and are constantly

exposed to the infection without showing any symptoms of parasitemia (Helmy et al. 2000; Frank et al. 1996) are probably the most attractive group since they carry circulating antibodies that may be host-protective. Therefore, there has been considerable interest in identifying the parasite antigens that generated the host protective antibodies in EN individuals.

The technique of displaying peptides or proteins on the surface of bacteriophages was first described by Smith (1985). A major advantage of this technique is that the protein displayed on the surface of phage is physically linked to the genetic material that codes for it. Therefore, the gene that codes for the displayed protein can be easily cloned from the phages. Recently, we used this technique to identify potential vaccine candidates of *B. malayi* (Gnanasekar et al. 2004). The phage-display screening is a simple, efficient and sensitive method. Phages displaying even one to five molecules of the protein/peptide on the surface can be successfully used for screening, identifying and cloning the genes of interest (Crameri et al. 1994). Phage display based screening is now routinely used for isolation of specific antibodies against targeted antigens and to identify linear epitopes of a protein or larger antigenic determinants of infectious agents (Folgori et al. 1994; Germaschewski and Murray 1996). Another area that is rapidly developing is the screening of phage-display cDNA libraries of cancer cells using sera from cancer patients to identify potential vaccine antigens (Somers et al. 2002). These reports suggest that phage-display screening technique has enormous potential as a tool in identifying candidate antigens that are important in vaccine development or as drug targets.

In the present study, we displayed a cDNA library of the L3 stages of *B. malayi* on the surface of T7 bacteriophages and screened this library with serum from EN individuals. This approach identified a novel antigen that showed significant homology to an immunogenic protein from another filarid parasite. This manuscript describes cloning and characterization of this novel protein from *B. malayi*.

Materials and methods

Collection of sera samples

Sera samples used in this study were collected from individuals residing in Chennai, India, a region endemic for lymphatic filariasis. The Institutional review board at the College of Medicine at Rockford and the Center for Biotechnology, Anna University approved the protocols. After obtaining proper consent, blood samples were collected, serum separated and stored at -80°C .

Serum samples were classified into three major groups (EN, CP or MF) based on the detection of circulating parasites, antigens or by evaluating the clinical symptoms of the disease. Circulating microfilariae in the blood samples were identified as described previously (Gnanasekar et al. 2004) and the circulating filarial antigens were detected using a commercially available Og4C3 kit (Lalitha et al. 1998) and a WbSXP-based ELISA (Rao et al. 2000). Individuals with no circulating antigen or microfilariae were considered as EN, whereas, subjects with circulating microfilariae and/or circulating antigen as detected by Og4C3/WbSXP ELISA were considered as MF. Subjects showing lymphedema and other visual clinical symptoms of filariasis were classified into CP. Sera samples from non-endemic normal (NEN) individuals were a kind gift from Dr. Thomas B. Nutman, NIH, USA.

Construction of T7 BmL3 phage-display expression system

B. malayi L3 cDNA was cloned into T7 select 1–1 phage-display vector as described previously (Gnanasekar et al. 2004). Briefly, *B. malayi* L3 cDNA library constructed in Uni-ZAP XR vector was PCR amplified with T3 and T7 promoter primers. PCR products were purified using Qiaquick PCR purification method (Qiagen, Valencia, CA, USA) and size fractionated to

obtain PCR products of >300 bp length, using chroma spin columns (Clontech, Palo Alto, CA, USA). The PCR products were digested with *Eco* RI and *Hind* III enzymes and ligated to similarly digested phage display vector T7Select 1–1 cloning system (Novagen, Madison, WI, USA). The library was in vitro packaged, titrated and amplified as per the manufacturer's instructions. Size distribution of the inserts was verified by PCR amplification of randomly selected phage clones.

Biopanning

The strategy used for biopanning the T7 BmL3 phage display library to select EN-specific clones was similar to those described previously (Gnanasekar et al. 2004) with slight modification. Briefly, 96-well plates (Pierce Biotechnology, Rockford, IL) were coated with 1:100 diluted pooled EN serum sample (from 10 individuals) overnight at 4°C. After washing the wells with phosphate-buffered saline containing 0.1% tween-20 (PBST), non-specific sites were blocked with 5% BSA for 1 h at 37°C. Hundred microliters of T7select BmL3 library (1×10^{11} pfu/ml) was added to wells coated with EN sera and incubated for 1 hr at room temperature. The unbound phages were discarded by washing the wells five times with PBST. The bound phages were then eluted with 200 µl of T7 elution buffer (TBS containing 1%SDS) and amplified by infecting *Escherichia coli* host BLT5403. The amplified phages were then subjected to another three rounds of panning as above to enrich the clones that bind to EN sera.

Sequence analyses

The final EN-specific clones obtained after four rounds of biopanning were plated and single pure plaques were obtained. The gene inserts in these plaques were amplified by PCR as described previously (Gnanasekar et al. 2004). Briefly, the inserts were amplified using vector-specific primers, T7SelectUP and T7SelectDown. The PCR products obtained were purified using Qiaquick columns (Qiagen) and sequenced on both strands at the DNA core facility of the University of Illinois Chicago. Sequences were analyzed using a software program at the GenBank (<http://www.ncbi.nlm.nih.gov>) site and multiple sequence analysis was performed by Clustal W program. One of the PCR products showed significant homology to novel immunogenic protein (NIP)3-like gene of *C. elegans* and NIP-3 gene of *O. volvulus* (Ov-NIP-3). Therefore, the newly identified protein was designated *Brugia malayi* NIP3-like protein (BmNIP3).

Construction of BmNIP3 expression vector

The open reading frame (ORF) of BmNIP3 was cloned in T7 expression vector, pRSET A (Invitrogen, Lajolla, CA, USA). The forward PCR primer corresponded to the beginning of ORF of BmNIP3 with the addition of an upstream in-frame *Bam*HI restriction site, (5'-CGCGGATCCATGATACTATCACTAGTG-3'). The reverse primer corresponded to the 3' end of BmNIP3 ORF flanked by an *Eco*RI restriction site (5'-CCGGAATTCTCA TACTTTGACAGTTGT-3'). PCR parameters were 95°C of denaturation for 30 s, 55°C of primer annealing for 30 s, 72°C of primer extension for 30 s and the cycle was repeated 30 times. A final extension of 5 min was performed at 72°C before storing the samples at 4°C. PCR products obtained were digested with *Bam* HI and *Eco* RI enzymes and ligated to similarly digested T7 expression vector pRSET A. Insert DNA was sequenced to ensure authenticity of the cloned nucleotide sequence on both the strands.

Expression and purification of BmNIP3

Recombinant construct of BmNIP3 in T7 expression vector was maintained in XL-1 Blue strains (stratagene). For expression, the recombinant plasmid was transformed into BL21(DE3) containing pLysS (Invitrogen) to minimize toxicity due to the protein. When OD₆₀₀ of the cultures reached 0.6, 1 mM of IPTG (Isopropyl thio-β-D-galacto pyranoside) was added to the

cultures to induce gene expression and incubated for an additional 3 h. Total proteins were separated in a 12% SDS-PAGE gels and the presence of histidine tagged protein was confirmed using a penta-His antibody (Qiagen). Subsequently, the histidine-tagged recombinant proteins were purified using an immobilized cobalt metal affinity column chromatography (Clontech) as per the manufacturer's recommendations. The purity of the recombinant protein was subsequently determined by separating the protein in a 12% SDS-PAGE and staining with coomassie Brilliant blue R250.

Immunization of mice with recombinant BmNIP3 (rBmNIP3)

Male Balb/c mice weighing 10–15 g purchased from (Charles River Laboratories, Wilmington, MA, USA) were used in these experiments. All animals were treated in accordance with an approved institutional protocol. Mice were immunized subcutaneously with 5 µg of rBmNIP3 in Gerbu adjuvant (Biotechnik GmbH, Gaiberg, Germany). Three booster injections were given at 2-weeks-interval using the same antigen dose. After the final booster dose, mice were bled, sera separated, and stored at –20°C. Antibody titer in the sera was determined using an ELISA and antibody reactivity was confirmed in a western blot analysis. Isotype of the antibody generated was determined using a mouse antibody isotyping kit purchased from Pierce Biotechnology using the manufacturer recommended procedures.

Preparation of soluble antigens of *B. malayi*

Different life cycle stages of *B. malayi* (L3, adult and Mf) were obtained from Dr. J.W. McCall, Filariasis Repository Research Service, Athens GA, through an NIAID supply contract (AI#02642). To prepare soluble extracts, parasites were homogenized in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl pH 7.4) in the presence of protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and insoluble material was pelleted by centrifugation at 10,000 g for 15 min at 4°C. The soluble antigenic fraction in the supernatant was collected and protein estimated using aBCAkit (Pierce Biotechnology).

Stage-specific expression of BmNIP3

BmNIP3 gene was PCR-amplified from the cDNA of various life cycle stages of *B. malayi* using insert specific primers 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* actin (BmActin) gene.

Levels of expressed BmNIP3 protein was also evaluated in the soluble antigen preparations of *B. malayi* using an immunoblot analysis. Briefly, *B. malayi* worm homogenates were resolved on 12% SDS-PAGE and transferred onto nitrocellulose membranes. Parasite antigens in the membrane were then probed with mouse anti-BmNIP3 (1:500 dilution) for 1 h at room temperature. After washing the membrane five times with tris buffered saline (TBS) containing 0.05% tween-20, HRP labeled goat anti-mouse antibody (Pierce Biotechnology) was added at 1:5,000 dilution and color developed by enhanced chemiluminescence method (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Purified rBmNIP3 was used as a positive control.

Immunoprecipitation and detection of phosphorylation and O-β-glycosylation of BmNIP3

Native BmNIP3 was immunoprecipitated using Seize X protein A immunoprecipitation kit obtained from Pierce Biotechnology (Cat.No.45215). Briefly, 100 µg of mouse antiNIP3 antibodies or pre-immune serum were first allowed to bind to immobilized protein A columns for 15 min and washed with binding/wash buffer for 5 times in a microcentrifuge at 3,500 rpm for 1 min. The bound antibodies were then cross-linked to protein A using DSS cross linker. The unbound antibodies were eluted using immuno pure elution buffer. The cross-linked

antibodies were then used to immunoprecipitate BmNIP3 from L3 homogenate. Briefly, 100 µg of soluble antigens were incubated with the cross-linked antibodies for 1 h at room temperature. The unbound antigens were removed by washing five times with wash buffer. The bound antigens were eluted with 200 µl of elution buffer. Presence of serine/threonine phosphorylation and *O*-β-glycosylation in the eluted antigen was detected using an immunoblot analysis. Briefly, the immunoprecipitated antigen was resolved on 12% SDS-PAGE and transferred to nitrocellulose membranes and non-specific binding sites were blocked with 3% skimmed milk for 1 h at room temperature. The blots were either probed with a mouse monoclonal phosphoserine/threonine antibody (BD Transduction laboratories; used at 1:1,000 dilution) or with a mouse anti-*O*-GlcNAc monoclonal antibody (Pierce Biotechnology; used at 1:5,000 dilution) for 1 h at room temperature. After washing the membrane five times with TBS containing 0.05% tween20, HRP labeled goat anti-mouse antibody (Pierce Biotechnology) was added at 1:5,000 dilution and color developed by ECL (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Analysis of BmNIP3 antibodies in human sera

Presence of antiBmNIP3 IgG antibodies in the sera of patients with lymphatic filariasis was evaluated using an ELISA as described previously (Gnanasekar et al. 2004). Briefly, 1 µg of rBmNIP3 suspended in 100 µl of coating buffer (NaHCO₃/Na₂CO₃ 0.067 M pH 9.6) was added to each well of a 96-well plate and incubated overnight at 4°C. After incubation, the wells were washed five times with PBST and 100 µl of 5% BSA was added to block nonspecific sites by further incubation for 2 h at 37°C. Sera samples (diluted 1: 100) collected from EN (*n*=20), MF (*n*=20) or CP (*n*=20) were then added to the wells and incubated for 2 h at 37°C. Wells were washed five times with PBST and 100 µl of HRP-labeled goat anti-human IgG (Sigma, St. Louis, MO, USA) was added. After incubating the plates for one hour at 37°C, color was developed using OPD substrate (1 mg/ml) in substrate buffer. The reaction was stopped by adding 100 µl of 3N NaOH to the wells and absorbance measured at 405 nm using a microtitre plate reader (Dynatech Laboratories Inc., Chantilly, VA, USA).

Isotype specific ELISA

Since significantly higher levels of anti-BmNIP3 IgG antibodies were present in both infected and EN sera, further isotype (IgG1, IgG2, IgG3 and IgG4) analyses were performed on the anti-BmNIP3 IgG antibodies. The protocol for IgG isotype ELISA was similar to that described above. Briefly, monoclonal antibodies against human IgG1, IgG2, IgG3 and IgG4 purchased from Sigma were used to coat the wells of plates. Following incubation with sera samples, HRP labeled goat antihuman IgG and OPD substrate was sequentially added to detect the concentration of the respective isotype of antibodies in the sera.

Statistical analysis

ELISA data from various clinical groups were compared using a Kruskal-Wallis one-way analysis of variance on ranks using SigmaStat program (Jandel Scientific, San Rafael, CA, USA).

Results

Identification and sequence analysis of BmNIP3

The cDNA clone of BmNIP3 isolated by affinity panning was found to encode a partial length clone. Analysis of this partial clone using BLAST2 search available at <http://www.ebi.ac.uk> showed that there were 15 EST deposited from various life cycle stages of *B. malayi*. Seven of these ESTs were reported from the L3 stage, one from L4, five from adult stages and two from microfilaria stage. Forward and reverse primers were then designed based on the *B.*

malayi EST deposit AI771061 and the full length BmNIP3 gene encoding 139 amino acids was cloned.

Subsequent Genbank analysis showed that BmNIP-3 sequences were 40% identical to the *C. elegans* NIP3 and 25% identical to the *O. volvulus* NIP3 (Fig. 1). In addition, WU-BLAST-2 analysis also showed that BmNIP3 gene is homologous to several ESTs deposited in the Genbank identified from various gastrointestinal nematodes such as *Ancylostoma caninum*, *Strongyloides stercoralis*, *Haemonchus contortus* and *Teladorsagia circumcincta*. CLUSTAL W alignment analysis of the predicted ORFs of these ESTs showed that BmNIP3 was 42% identical to *S. stercoralis*, 41% identical to *A. caninum*, *T. circumcincta* and 40% identical to *H. contortus* (Fig. 1b). Although most of these ESTs have complete ORFs, the respective genes have not been described or characterized. Hence in designating BmNIP3, we followed the *C. elegans* and *O. volvulus* NIP3 nomenclature. Interestingly, there is no known human or mammalian homolog for this protein. SOSUI signal beta version available at PROSITE data base analysis showed that all NIP3 proteins are secretory proteins. BmNIP3 has a signal peptidase cleavage site at aa18, CeNIP3 at aa16 aa, OvNIP3 at aa24, AcNIP3 at aa16, TcNIP3 at aa16, HcNIP3 at aa16 and SsNIP3 at aa28 (Fig. 1b). An important finding was the presence of three potential mucin type *O*-glycosylation sites in BmNIP3 sequence at the three contiguous threonine amino acid residues at position 134–136 (Fig. 1a) suggesting that BmNIP3 may be a glycosylated protein. Surprisingly, this glycosylation site appears to be absent in the NIP3 homologues of other parasites (Fig. 1b). PROSITE scan analysis showed that BmNIP3 has a potential tyrosine sulfation site at aa92–106 and several predicted phosphorylation sites. These include cAMP- and cGMP-dependent protein kinase phosphorylation site at aa131–134, protein kinase c phosphorylation sites at aa49–51, aa80–82, aa86–88 and aa136–138 and casein kinase II phosphorylation sites at aa15–18 and aa78–81.

Phosphorylation and glycosylation of NIP3

Since BmNIP3 sequence showed several phosphorylation and glycosylation sites, we analyzed whether the native BmNIP3 isolated from the parasite is serine/threonine phosphorylated and whether native BmNIP3 is glycosylated. Immunoblot analysis of the immunoprecipitated BmNIP3 with phosphoserine/threonine antibodies confirmed that BmNIP3 is a phosphorylated protein (Fig. 2) as suggested by the PROSITE scan analyses. The phosphorylated state of BmNIP3 in the parasite suggests that this protein may have an important role in the various cellular functions of the L3 stage parasite. A similar immunoblot analysis using *O*-βGlcNAc antibodies showed that BmNIP3 is probably not a β glycosylated (data not shown) protein. However, since the sequence analyses suggest that BmNIP3 has three potential mucin type *O*-glycosylation sites, presence of α glycosylation could not be ruled out as there are no commercially available *O*-αGlcNAc antibodies.

Expression of rBmNIP3

The rBmNIP3 cloned in pRSET A was expressed as histidine tagged fusion protein. The molecular mass of the recombinant fusion protein (with the histidine tag) was approximately 24 kDa (Fig. 3). SDS-PAGE analysis showed that expressed rBmNIP3 consisted of over 10% of the total *E. coli* proteins. Subsequently, the recombinant protein was purified using metal affinity column chromatography (Fig. 3) and size confirmed by immunoblotting using anti-His antibody (data not shown).

Stage-specific expression of BmNIP3 in different life-cycle stages of *B. malayi*

PCR amplification of BmNIP3 gene from cDNA libraries of different life cycle stages of *B. malayi* showed that probably the gene is differentially transcribed in different stages. Although, we did not use a quantitative RT-PCR in these studies, based on the intensity of normalized bands, BmNIP3 transcripts were predominantly seen in the early larval stages of the parasite

(L3 and L4) followed by adults and microfilaria (Fig. 4). These findings were consistent with the higher number of ESTs reported from L3 stages.

Immunoblot analysis on the soluble protein extracts of L3, microfilariae and adult parasite stages showed that the expression levels of BmNIP3 proteins was high in the infective stages of the parasite (L3) and was barely detectable in the adults and microfilariae (Fig. 5). Soluble extracts of L4 stages could not be analyzed in these studies due to non-availability of this sample to us. The molecular size of the native BmNIP3 in SDS-PAGE is around ~21 kDa, whereas the recombinant protein runs at ~24 kDa. This size discrepancy between native and rBmNIP3 may be that the rBmNIP3 has a 3-kDa His tag in addition to the signal sequences, which are normally cleaved off from native secreted BmNIP3. Nevertheless, these findings suggest that BmNIP3 may be a stagespecific protein expressed highly by the infective L3 stages of *B. malayi*.

Antibody responses in mice immunized with rBmNIP3

Subclass analyses of the anti-BmNIP3 antibodies in the sera of mouse immunized with rBmNIP3 show that the majority of the antibodies are of the IgG1, IgG2a isotype. Levels of IgA and IgM were undetectable (Fig. 6).

Anti-BmNIP3 antibodies in the sera of immune individuals and individuals with lymphatic filariasis

Although BmNIP3 clone was selected based on binding to the putative immune EN sera, we wanted to evaluate whether the infected patients carry antibodies against BmNIP3. The ELISA results presented in Fig. 7 show that significant ($P<0.01$) amounts of anti-BmNIP3 antibodies were present in the sera of EN, MF and CP individuals compared to sera from NEN individuals. Further analyses of the ELISA data suggest that EN individuals carry significantly ($P<0.05$) higher titers of anti-BmNIP3 antibodies compared to individuals in the MF or CP group.

Since anti-BmNIP3 IgG antibodies were significantly increased in all the group of subjects except NEN, further analyses were performed to determine the isotype of anti-BmNIP3 IgG antibodies. These analyses showed that the majority of the anti-BmNIP3 IgG antibodies in the sera of EN individuals were of IgG1 and IgG2 isotypes, whereas, antibodies in the sera of CP patients were of IgG3 isotype (Fig. 8). Individuals in the MF group had high levels of anti-BmNIP3 IgG1 antibodies. Anti-BmNIP3-IgG4 isotype of antibodies were not detectable in all three groups (EN, MF or CP) of subjects. Similarly, antibodies to BmNIP3 were absent or were not detectable in the sera of NEN subjects.

Discussion

Immunoscreening of a phage display cDNA expression library of *B. malayi* L3 with putative immune (EN) sera identified a novel antigen designated as BmNIP3, which showed significant homology to the previously described OvNIP3 and CeNIP3. The BmNIP3 gene appears to be differentially transcribed with highest expression present in the L3 stages. Both EN and infected individuals carry antibodies against BmNIP3 in their sera. However, a critical analysis on the isotype of IgG antibodies showed that anti-BmNIP3 antibodies were predominantly of the IgG1 and IgG2 isotype in the sera of EN individuals, whereas, chronically infected individuals carry predominantly IgG3 antibodies against BmNIP3.

Technique of displaying peptides or proteins on the surface of bacteriophages is a widely used approach to isolate genes of interest. Several proteins including peptide libraries can be displayed on the surface of bacteriophages. Such phage-displayed proteins or peptides are then screened using appropriate ligands to identify the protein(s) of interest. Since the proteins

displayed on the surface of phage is physically linked to the genetic material that codes for it, the gene coding for the displayed protein can be easily cloned from the phages. The uniqueness of the technique is that even few protein molecules displayed on the surface of bacteriophages could be used as bait to screen and subsequently clone the protein of interest with good efficiency. In this study, we displayed a library of *B. malayi* antigens as fusion to the capsid protein of T7 bacteriophage. This system has been already described previously (Gnanasekar et al. 2004). One of the major advantages of the T7 system is the high efficiency in vitro packaging of cDNA that allow construction of large-size libraries. However, there are some limitations to the T7 phage display system (Gnanasekar et al. 2004). Despite these limitations, we were able to display cDNA library of *B. malayi* L3 containing 3×10^8 independent clones on the surface of T7 phage. Over 90% of these clones had insert sizes above 500 bp. Given the size of the phage display library generated (~30,000), we predicted that most of the transcripts will be expressed based on their relative abundance in the L3 stage. This library was then screened using sera samples from EN individuals. These individuals live in the endemic area, carry antibodies against the parasites and are refractive to the infection. Therefore, EN individuals are considered immune to the infection.

Screening and affinity panning of the phage-display expression library with EN sera identified a clone that encoded the full-length sequence of a novel protein, BmNIP3. Extensive sequence analysis showed that BmNIP3 is homologous to OvNIP3 and CeNIP3. Lizotte-Waniewski et al. (2000) were the first to identify and clone the NIP3 gene from *O. volvulus* after screening a cDNA expression library with immune sera. Based on the novelty and immunogenicity of this protein, they coined the term OvNIP3. This nomenclature was subsequently carried to the CeNIP3. In our studies, we also used an immunoscreening strategy to identify the BmNIP3. Therefore, as suggested by Lizotte-Waniewski et al. 2004, this protein may be a highly immunogenic protein. BmNIP3 has an N-terminal signal peptide sequence allowing this protein to be secreted by the larval stages and are thus readily available to the host immune system.

Both putatively immune EN individuals and subjects with lymphatic filarial infection show highly elevated levels of IgG in their sera. However, analyses of the isotype of anti-BmNIP3 IgG antibodies showed contrasting pattern of IgG responses. IgG1 and IgG2 was predominant in EN individuals, whereas, IgG3 was predominant in chronically infected patients and IgG1 was elevated in MF individuals against BmNIP3. The antibody isotype responses in individuals showing acute infection (MF) were more similar to EN individuals, except that they did not develop any anti-BmNIP3 IgG2 antibodies. Interestingly, mice immunized with NIP3 showed predominantly IgG1 and IgG2a similar to that of isotype profile seen in EN.

Previously it has been reported that antigens isolated from L3, mf and adult stages induce different isotype antibody profile in various clinical groups with lymphatic filariasis (Atmadja et al. 1995; Kurniawan et al. 1993; Kurniawan-Atmadja et al. 1998a, b). L3 surface antigens have been shown to induce predominantly Ig-G1 in EN, MF and CP individuals; IgG2 in MF and CP; IgG3 in CP and very low IgG4 in all these three groups (Kurniawan-Atmadja et al. 1998a). Our studies also showed that BmNIP3 is abundantly expressed in L3 stages, yet did not induce detectable IgG4 levels in any of the clinical groups that we tested. Additional studies aimed at characterizing and localizing BmNIP3 on L3 might help explain these differences.

Sequence analyses predicted the presence of potential tyrosin sulfation sites in BmNIP-3. Tyrosine sulfation is a post-translational modification that occurs in the transgolgi and influences the physiology of proteins by playing a major role in protein-protein interactions. The role of this tyrosine sulfation in BmNIP3 is not known at this time, but may be important for its extracellular secretion. Sequence analyses and specific staining with anti-phosphoserine/threonine antibodies confirmed that BmNIP3 is a serine/threonine phosphorylated protein. It

is well-established that phosphorylation of several key molecules is an important event during the differentiation process of L3 and may thus play a central role in larval development. Nevertheless, a role of phosphorylated BmNIP3 needs to be determined. Additional information revealed from sequence analyses of BmNIP3 showed that it has three mucin-type *O*-glycosylation sites, suggesting that BmNIP3 may be a glycosylated protein. However, specific staining for *O*- β glycosylation showed that BmNIP3 is not an *O*- β glycosylated protein. Unfortunately, we were unable to determine whether BmNIP3 is an *O*- α glycosylated protein due to non-availability of a commercial antibody. Additional studies are needed to confirm whether the protein is glycosylated or not. Nonetheless, studies by Mohanty et al. (2001) showed that glycoprotein antigens of the filarial parasites elicit a strong IgG2 response in immune host. Therefore, it is possible that the high levels of IgG2 antibodies generated in EN individuals might suggest that native BmNIP3 potentially exists in a glycosylated form. Furthermore, based on the ORF, the predicted molecular weight of BmNIP3 is ~15 kDa, whereas, the native BmNIP3 runs at ~21 kDa on SDS-PAGE. This increase in molecular size of the protein could be due to glycosylation and or other post-translational modifications.

Analyses of the proteins from various stages of the parasite showed that BmNIP3 is highly expressed in the L3 and L4 stages, whereas, BmNIP3 was absent or was barely detectable in other stages. This suggests that BmNIP3 may have an important role in the establishment of the infective larvae in the host. The presence of antibodies in the sera of acute or chronically infected individuals might suggest recent exposure to the infection, especially, given the low expression of BmNIP3 in the mf and adult stages. A true function for BmNIP3 remains to be identified. However, based on the results from *O. volvulus* studies and from the present studies it appears that NIP3 is a major immunodominant antigen of the larval filarid worms. Similarly, further studies are needed to determine the significance of the high levels of anti-BmNIP3 antibodies in the putatively immune EN individuals.

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Fig. 1.

a Deduced amino acid sequence of BmNIP3 open reading frame (ORF). The amino acid positions are numbered above the amino acid sequences. Potential mucin type *O*-glycosylation sites of BmNIP3 are marked as ggg on threonine amino acids. **b** Multiple alignments (CLUSTAL) of the amino acid sequences of NIP3 family of proteins from *B. malayi* (BmNIP3, accession no. AY464549), *O. volvulus* (OvNIP3, accession no. AAF64253), *C. elegans* (CeNIP3, accession no. NP_505622), *A. caninum* (AcNIP3, accession no. AW700605), *T. circumcincta* (TcNIP3, accession no. CB043547), *H. contortus* (HcNIP3, accession no. BF186681), and *S. stercoralis* (SsNIP3, accession no. BE223624). * Identical amino acids, (:) indicates strongly similar amino acids and (.) indicates weakly similar amino acids. Secretory signal sequences of NIP3 proteins are italicized

Fig. 2.

Detection of serine/threonine phosphorylation of NIP3 in *B. malayi*. Immobilized anti-BmNIP3 antibodies were incubated with soluble L3 antigens and the bound proteins were eluted and resolved on a 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane and probed with mouse anti-BmNIP3 antibodies and a mouse anti phosphoserine/threonine antibody. Reactive bands were detected using a chemiluminescent method after probing with HRP labeled goat anti-mouse IgG. Results show that native BmNIP3 is serine/threonine phosphorylated. Immunoprecipitation with mouse pre-immune serum served as negative controls. Data represent results from one of three similar experiments

Fig. 3.

Expression and purification of rBmNIP3. Cultures of *E. coli* containing pRSETA and BmNIP3 expression construct were induced with IPTG. Following induction, rBmNIP3 was purified from the cultures using a cobalt metal affinity chromatography column. Approximately 1 μ g of the purified protein was then separated in a 12% SDS-PAGE and stained with coomassie brilliant blue R250

Fig. 4.

Expression of BmNIP3 mRNA in various life cycle stages of *B. malayi*. **a** BmNIP3 transcript was PCR-amplified from the cDNA libraries of various life cycle stages (L3, L4, adult and Mf) of *B. malayi* using primers specific for BmNIP3. PCR products were resolved on a 1% agarose gel and stained with ethidium bromide. **b** Band intensity was normalized to BmActin amplified from the same samples using BmActin-specific primers and values were calculated using NIH image software. Data represent results from one of three similar experiments

Fig. 5.

Expression of BmNIP3 protein in various life cycle stage of *B. malayi*. Soluble protein extracts (10 µg/lane) from L3, adult (Ad) and microfilaria (Mf) stages of the parasite were resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane and probed with a mouse antiBmNIP3 antibody. HRP labeled goat anti-mouse IgG was used as the secondary antibody and the reactive bands were detected using a chemiluminescent substrate. Recombinant BmNIP3 was used as a positive control. Arrow indicates bands with strong immunoreactivity

Fig. 6. Subclass of anti-BmNIP3 antibodies in the sera of mice immunized with 5 µg of rBmNIP3. Subclass analysis was performed using a mouse antibody isotyping ELISA kit. Data presented is the mean ($\pm$ SD) value from 10 mice per group and represent one of two similar experiments

Fig. 7.

Anti-BmNIP3 IgG antibodies in the sera of human subjects. Recombinant BmNIP3 were coated onto the wells of a microtiter plates and the levels of anti-BmNIP3 IgG antibodies in the sera samples collected from various groups of human subjects (EN, MF, CP and NEN) were evaluated using an ELISA. Each spot on the scatter plot represent an individual, $n=20$. Data presented is representative of one of two similar experiments

Fig. 8. Isotype of anti-BmNIP3 IgG antibodies in the sera of various groups of human subjects (EN, MF, CP and NEN) were evaluated using an isotype-specific ELISA. Data presented is mean ($\pm$ SD) value from 20 patients in each group and represent one of two similar experiments