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A novel small heat shock protein 12.6 (HSP12.6) from *Brugia malayi* functions as a human IL-10 receptor binding protein

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Abstract

Phage display cDNA expression library of the third stage larvae (L3) of *Brugia malayi* was screened for identifying target(s) that bound to the human interleukin-10 receptor (huIL10R). This iterative screening identified an insert that showed significant homology to *C. elegans* HSP12.6. The gene was designated *Brugia malayi* HSP12.6 (BmHSP12.6) and has orthologues in several gastrointestinal nematode genome (*Ancylostoma caninum*, *Ascaris lumbricoides* and *Ascaris suum*) but the gene or gene product has not been studied further in these parasites. Structural analyses of BmHSP12.6 showed that it has a highly conserved alpha-crystallin central domain that is characteristic of other small heat shock proteins (HSPs). BmHSP12.6 has a short N-terminal domain and an unusually small C-terminal domain flanking the crystallin domain suggesting that this protein belongs to a novel class of small HSPs. BmHSP12.6 appears to be differentially transcribed with highest expression in the vertebrate stages of the parasite (L4, adult and mf) compared to its mosquito vector stage (L3). More importantly recombinant BmHSP12.6 bound to huIL10R in a dose dependent fashion and inhibited the binding of human IL-10 (huIL10) to huIL10R *in vitro*. rBmHSP12.6 also enhanced the growth and proliferation of MC/9 mast cells *in vitro* similar to huIL10. This study thus describes a novel small HSP from *B. malayi* that has the capacity to bind to huIL10R, block binding of huIL10 to huIL10R and function similar to huIL10.

Keywords

Brugia malayi; HSP12.6; IL; 10; phage; display; biopanning

1. Introduction

Lymphatic filariasis is a mosquito borne disease caused by the parasites *Wuchereria bancrofti* and *Brugia malayi* infecting over 120 million people in the tropics with an additional one billion people at the risk of developing this disease [1]. These filarial parasites successfully infect humans and live in the lymphatics by successfully evading host immune responses [2,

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3]. Many hypotheses have been put forward for the parasite's successful establishment and longevity in the mammalian host [4], among them the most important are the production of parasite-encoded products that mimic host regulatory factors [5,6]. Putative host immune evasion genes including the cystatins [7], serpins [8], cytokine homologues such as transforming growth factor- β (TGF- β) [9], the macrophage migration inhibitory factor (MIF) gene families [10], and IL-16 (from the genome studies) have been identified from *B. malayi*. Majority of these putatively regulatory molecules (particularly the cytokine homologues) were identified based on *in silico* deduced sequence homology

Immune down-regulation is the hallmark of chronic lymphatic filarial infection, reflected in severely impaired antigen-specific proliferative responses and decreased interferon- γ production [11]. This impaired proliferative characteristic seen in the infected individuals is regulated, in part, by interleukin-10 (IL-10) [12]. Although there appeared to be no parasite-encoded IL-10 like molecule in the *Brugia* genome, possible existence of IL-10-like molecule was suggested by Maizels and Yazdanbakhsh [13] in helminth parasites. In this study, we screened a phage display cDNA expression library of the third stage infective larvae (L3) of *B. malayi* with human interleukin 10 receptor (huIL10R) and identified a *Brugia*-encoded molecule, BmHSP12.6 that bound to huIL10R.

2. Materials and Methods

2.1 Construction of T7BmL3 phage display expression system

B. malayi L3 cDNA was cloned into T7 select 1-1 phage display vector as described previously [14]. 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) and size fractionated to obtain PCR products of >300 bp length, using chroma spin columns (Clontech, Palo Alto, CA). 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). The library was *in vitro* packaged, titered and amplified as per the manufacturer's instructions. Size distribution of the inserts was verified by PCR amplification of randomly selected phage clones.

2.2 Biopanning

The strategy used for biopanning the T7 BmL3 phage display library to identify human IL-10R (R&D cytokines, Minneapolis, MN) binding phage clones was similar to those described previously [14] with slight modification. Briefly, 96 well plates (Pierce Biotechnology, Rockford, IL) were coated with 100 ng of huIL10R/well for 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 1hr at 37°C. 100 μ l of T7select BmL3 library (1×10^{11} pfu/ml) was added to wells coated with huIL10R and incubated for 1hr at room temperature. The unbound phages were discarded by washing the wells 5 times with PBST. The bound phages were then eluted with 200 μ l of T7 elution buffer (TBS containing 1%SDS) and amplified by infecting *E. coli* host BLT5403. The amplified phages were then subjected to another three rounds of panning as above to enrich the clones that bind to huIL10R.

2.3 Sequence analyses

The final huIL10R 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 [14]. 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 (www.ncbi.nlm.nih.gov) site and multiple sequence analysis was performed using Clustal W.

2.4 Stage-specific expression of BmHSP12.6

BmHSP12.6 and BmGAPDH genes were amplified from the cDNA 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.

2.5 Construction of BmHSP12.6 expression vector

Primers designed based on the *B. malayi* EST AW160068 allowed us to identify and characterize the full length BmHSP12.6 gene encoding 113 amino acids was cloned from *B. malayi* L4 cDNA library. To characterize this protein, full length BmHSP12.6 was cloned in prokaryotic expression vector pRSET A, using insert specific primers (forward primer 5' CGCGGATCCATGGAAGAAAAGGTAGTG3' containing *Bam* HI site and reverse primer 5'CCGGAATTCTCATGCTTTCTTTTGGCAGC3' containing *Eco*R I site). Primers were synthesized at IDT DNA technologies. Using insert specific primers, HSP12.6 gene was amplified by PCR from *B. malayi* L3 cDNA library. PCR parameters were 95°C of denaturation for 30s, 55°C of primer annealing for 30s, 72°C of primer extension for 30s and the cycle was repeated for 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.

2.6 Expression and purification of rBmHSP12.6

Recombinant construct of BmHSP12.6 in the T7 expression vector was maintained in XL-1 blue strain (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, one mM of IPTG (Isopropyl thio-β-D-galacto pyranoside) was added to the cultures to induce gene expression and incubated for an additional 3h. Total proteins were separated in a 12% SDS-PAGE 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 (data not shown). Another recombinant protein rBmNIP3, similarly expressed and purified was used as a control protein in our experiments. Both rBmHSP12.6 and rBmNIP3 proteins were passed through polymyxin B columns (Thermo Fisher, Rockford, IL) to remove contaminating LPS if any.

2.7 ELISA to determine rBmHSP12.6 binding to huIL10R

The binding of rBmHSP12.6 to huIL10R was tested at various concentrations starting from 0.5 µg/ml to 10 µg/ml. Briefly, the binding assay was performed as follows. Wells coated with huIL10R were blocked with 5% BSA followed by addition of varying concentration of rBmHSP12.6 or a control protein rBmNIP3 and incubated for 2h at room temperature. Following incubation, mouse monoclonal penta-His antibodies conjugated to HRP (1:5000) were added and incubated for 1h at room temperature. Following washing, the binding of rBmHSP12.6 to huIL10R was detected by development of color on addition of OPD substrate (Sigma, St. Louis, MO). Color developed was measured at 405nm.

2.8 Competitive binding assay

The ability of rBmHSP12.6 to compete with huIL10 for binding to huIL10R was determined by a competitive ELISA. Briefly, wells coated with huIL10R were blocked with 5% BSA

followed by addition of varying concentration of rBmHSP12.6 starting from 0.1 ug/ml to 10 ug/ml and incubated for 2h at room temperature. Similarly, a control protein, rBmNIP3 was also included in this experiment for comparison. Following incubation, 100 ng/ml of recombinant human IL-10 (rhuIL10) was added and incubated for 1h at room temperature. After incubation, wells were washed and biotinylated goat anti huIL10 antibody was added and incubated for 1h at room temperature. Following washing, streptavidin HRP (1:10000) was added and color developed using OPD substrate (Sigma). Color developed was measured at 405nm.

2.9 MC/9 mast cell proliferation assay

A mast cell proliferation assay was performed as described previously by Thompson-Snipes *et al* [15] to determine whether rBmHSP12.6 possess huIL10-like activity. The murine mast cell line, MC/9 purchased from ATCC, Manassas, VA was maintained in RPMI 1640 (ATCC) supplemented with 10% FBS, 0.05 mM 2-ME, and 10% Rat T-STIM (Becton Dickinson, San Jose, CA) at 37°C in 5% CO₂ environment. About 5×10^3 mast cells were suspended in RPMI containing IL-3 (10ng/ml), IL-4 (10ng/ml) and polymyxin B (10 µg/ml) and plated on a flat bottomed 96 well tissue culture plates and stimulated with 10 µg/ml of rBmHSP12.6 or rBmNIP3 (10 µg/ml, a non-specific recombinant protein prepared from *B. malayi* L3 cDNA using similar procedures as rBmHSP12.6). rhuIL10 (100 ng/ml) stimulated cells served as positive control. Mast cells were also cultured in the presence of a combination of rBmHSP12.6 and rhuIL10 or a combination of rBmHSP12.6 and shuIL10Ra. After 72h of culture, cell viability was determined using a kit purchased from Dojindo Molecular Technologies Inc., Gaithersburg, MA.

2.10 Statistics

Data were compared using a Kruskal–Wallis one-way analysis of variance on ranks using SigmaStat program (Jandel Scientific, San Rafael, CA). $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Identification of a novel class of small HSP from *B. malayi* as a huIL10R binding protein

In this study we attempted to identify clones from *B. malayi* phage display library that bind to huIL10R by a biopanning approach. This approach resulted in sequential enrichment of 200 fold increase in huIL10R binding phage clones after four rounds of biopanning (Table 1). Ten clones from fourth round of panning were randomly selected and DNA sequenced. Sequencing results showed that all the ten clones encoded a partial length cDNA showing significant homology to novel heat shock protein family of *C. elegans*. Therefore, the newly identified protein was designated *Brugia malayi* HSP12.6 (BmHSP12.6). Sequence analysis of this cDNA showed that there were 43 ESTs deposited from the L3, L4 and adult life cycle stages of *B. malayi*. Four of these ESTs were reported from the L3 stage, 34 from the L4 and five from the adult stages. The predicted ORFs of these ESTs were used to demonstrate that these had significant sequence similarity with *C. elegans* HSP12.6. The cloned sequence was deposited in Genbank under the accession number AY692227.

Subsequent sequence analysis showed that BmHSP12.6 sequences were 100% identical to the *W. bancrofti* HSP12.6 and 53% identical to *C. elegans* HSP12.6 (Fig. 1). In addition, BmHSP12.6 showed similarity to the ESTs in many gastrointestinal nematodes including *Ancylostoma caninum* (57% identity), *Ascaris lumbricoides* (44% identity) and *A. suum* (44% identity) (Fig. 1). PROSITE analysis showed that BmHSP12.6 has several putative post-translation modifications such as N-glycosylation, protein kinase c phosphorylation, casein kinase II phosphorylation and N-myristoylation sites.

Small heat shock proteins (sHSP) constitute a diverse family of proteins ranging from 12–43 kDa proteins found in several organisms [16,17]. These sHSP which are related to eye lens alpha-crystallins often act as molecular chaperones to prevent the aggregation and activate the renaturation of unfolded proteins [18]. Unlike the prominent molecular chaperones such as HSP90, HSP70, HSP60 and HSP40, the sHSP family of proteins are structurally divergent and encoded by multigene families [19]. The existence of an unusually sHSP was first reported from *C. elegans* by Caspers *et al* [20]. Since then several sHSP variants have been identified from different organisms [21–23]. Interestingly, a previous study show that *B. malayi* organism also express low molecular size HSP (SHSP18) [24], and this sHSP is shown to be developmentally regulated and heat inducible. Structurally, sHSP contains three domains of variable length and sequence. However, the central alpha-crystallin domain which is usually 80–100 amino acids is highly conserved among the sHSP. As would be expected, the BmHSP12.6 identified in this study has the conserved central alpha-crystallin domain with a short N-terminal domain and an unusually small C-terminal extension, a structure that appears to be novel for the filarial sHSPs.

One of the objectives of the present study was to determine whether *B. malayi* derived parasite-encoded proteins resemble huIL10 in their ability to bind to huIL10R and express huIL10-like function or block huIL10-like function. Initial sequence analysis showed that BmHSP12.6 shares 44% sequence similarity with huIL10 (Fig 2) suggesting that BmHSP12.6 may carry huIL10R binding epitopes similar to huIL10.

3.2 rBmHSP12.6 binds to huIL10R in vitro

The binding of BmHSP12.6 to huIL10R was initially identified by biopanning of the *B. malayi* L3 phage display expression library with huIL10R (above results). Subsequently, we cloned and expressed recombinant BmHSP12.6. Ability of the purified recombinant BmHSP12.6 to bind to huIL10R was then re-confirmed by ELISA. Our results show that rBmHSP12.6 binds to huIL10R in a dose dependent fashion (Fig 3). This binding was specific as rBmNIP3, a similarly expressed non-specific recombinant protein failed to bind to huIL10R (Fig 3). We then asked the question whether rBmHSP12.6 can interfere with the binding of huIL10 to huIL10R receptor. We used a competitive ELISA to determine the binding kinetics. These results show that rBmHSP12.6 significantly ($P < 0.05$) interfered with the binding of huIL10 to huIL10R compared to a control rBmNIP3 (Fig 4).

3.3 Stage-specific expression of BmHSP12.6 in different life-cycle stages of *B. malayi*

The exact roles played by sHSP *in vivo* are not clearly understood, but they are likely to mediate protective function under stress conditions [25]. Many sHSPs are known to be expressed in a developmentally regulated fashion [17,19,24]. Analysis of the number of EST homologues from different life cycle stages of *B. malayi* suggested that there might be differential expression in BmHSP12.6 in different life cycle stages of the parasite. To examine this more closely, PCR was performed from the cDNA libraries of different life cycle stages of *B. malayi*. These data showed that based on the intensity of normalized bands, BmHSP12.6 transcripts were predominantly expressed in post infective stages of the parasite (L4, adults and mf) compared to infective L3 stages (Fig 5). Previous studies with another heat shock protein, BmHSP18 also showed that expression of BmHSP18 is up regulated in the vertebrate stages of the parasite at 37°C compared to the stages recovered from the mosquito vector [24]. The fact that BmHSP12.6 expression appears to be highly up regulated in vertebrate host might suggest that BmHSP12.6 may have an important role in stress adaptation of the parasite in the mammalian host. HSP12.6 homologue of *C. elegans* is also developmentally regulated [19] suggesting that the sHSP family of proteins may be differentially expressed.

3.4 rBmHSP12.6 has huIL10-like stimulatory effects on MC/9 mast cells

IL10 has pleiotropic effects on various cells and growth stimulatory activity on mast cells [15]. Our above results show that BmHSP12.6 shares significant similarity with huIL10 (Fig 2), also it binds and competes with huIL10 in binding to huIL10R (Fig 5). MC/9 mast cells express IL10R which is highly identical to huIL10R [26]. Addition of rhuIL10 to the MC/9 mast cell cultures stimulates these cells to proliferate. Therefore, we next tested whether rBmHSP12.6 has any stimulatory effect on the MC/9 cells. These studies showed that rBmHSP12.6 enhanced the growth of MC/9 mast cells similar to that of rhuIL10 (Fig 6). When MC/9 cells were cultured in the presence of both rBmHSP12.6 and rhuIL10, no significant differences in mast cell growth was observed. However, the MC/9 growth stimulating activity of rBmHSP12.6 was abolished when soluble rhuIL10R was added to the cultures. These results thus suggest that rBmHSP12.6 has huIL10-like activity on MC/9 cells potentially mediated via IL10R.

In addition to their roles in classical chaperone activity and stress regulation, HSPs play an important role in innate immunity [27] by forming receptor complexes with TLR2, TLR4 and CD91 on target cells. As TLR expression and signaling have been shown to be important in mediating the pathology associated with lymphatic filariasis [28], this molecule may provide a link between the parasite and the host's innate immune system. Further studies will identify the intracellular signaling events following binding of rBmHSP12.6 to huIL10R and its significance in lymphatic filarial infection.

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Figure 1.

Multiple alignments (CLUSTAL) of the amino acid sequences of HSP12.6 family of proteins from *B. malayi* (BmHSp12.6, accession no. AY692227), *W. bancrofti* (WbHSP12.6, accession no. CD374831), *C. elegans* (CeHSP12.6, accession no. U92044), *A. caninum* (AcHSP12.6, accession no. BG232458), *A. lumbricoides* (AlHSP12.6, accession no. BU585661), *A. suum* (AsHSP12.6, accession no. BQ095038). (*) Indicates identical amino acids, (:) indicates strongly similar amino acids and (.) indicates weakly similar amino acids.

Figure 2.

Amino acid sequence alignment (CLUSTAL) of BmHSP12.6 (accession no. AY692227) and *Homo sapiens* IL10 (HsIL10, accession no. NM_000572). (*) Indicates identical amino acids, (:) indicates strongly similar amino acids and (.) indicates weakly similar amino acids.

Figure 3.

Dose dependent binding of rBmHSP12.6 to huIL10R. The binding of rBmHSP12.6 to huIL10R was determined by ELISA. huIL10R coated wells were incubated with various concentrations of either rBmNIP3 or rBmHSP12.6 (0.5 to 10 ug/ml) for 2h at room temperature. Penta Histidine-HRP conjugate was used to detect the binding of rBmHSP12.6 to huIL10R. This study shows that rBmHSP12.6 binds to huIL10R in a dose dependent manner. Results presented are representative of one of three similar experiments.

Figure 4.

BmHSP12.6 interferes with rhuIL10 binding to huIL10R. The ability of rBmHSP12.6 to inhibit the binding of rhuIL10 to huIL10R was determined by ELISA. huIL10R coated wells were incubated with varying concentration of rBmHSP12.6 (0.1ug/ml to 10ug/ml) for 2h at room temperature. rBmNIP3 served as control protein. After incubation, 100ng/ml of rhuIL10 was added to the wells and the binding of rhuIL10 was detected using biotinylated goat anti-huIL10 antibody followed by the addition of streptavidin HRP conjugate. Results show that rBmHSP12.6 prevents the binding of huIL10 to huIL10R in a dose dependent fashion. Data presented is representative of one of three similar results.

Figure 5.

Expression of BmHSP12.6 mRNA in various life cycle stages of *B. malayi*. **A.** BmHSP12.6 and BmGAPDH transcripts were amplified by PCR from the cDNA libraries of various life cycle stages (L3, L4, adult and Mf) of *B. malayi* using primers specific for BmHSP12.6 and BmGAPDH. 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 represent results from one of three similar experiments.

Figure 6.

BmHSP12.6 enhances the growth of MC/9 mast cells *in vitro*. The effects of rBmHSP12.6 on MC/9 cells were determined by a cell viability assay. 5×10^3 MC/9 cells/ml were stimulated with rBmHSP12.6 (10 μ g/ml) or rhuIL10 (100ng/ml) for 72h at 37° C. Cells were also cultured with a combination of rBmHSP12.6 and rhuIL10 or a combination of rBmHSP12.6 and soluble huIL10R. rBmNIP3 stimulated cells served as negative control. Results show that rBmHSP12.6 enhanced the growth of MC/9 cells and this growth was abrogated in presence of soluble huIL10R. Data presented is representative of one of three similar experiments.

Table 1Selection of huIL10R specific clones by biopanning of *B. malayi* L3 phage display library

Round of Panning	Phage applied	Phage Eluted	Enrichment
1	10^{11}	1×10^6	1×10^{-5}
2	10^{11}	3×10^6	3×10^{-5}
3	10^{11}	4×10^7	4×10^{-4}
4	10^{11}	2×10^8	2×10^{-3}