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Multiplex proteomics analysis of gender-associated proteins in *Brugia malayi*

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Abstract

Gender-associated (GA) genes are important for the development and reproduction of filarial nematodes. Identification and characterization of GA genes may provide insight into major pathways and processes involved in development and reproduction. The recent completion of the *Brugia malayi* genome has provided a good foundation for proteomics studies. Multiplex protein labeling and two-dimensional difference in-gel electrophoresis (2D-DIGE) combined with MALDI-TOF/TOF tandem MS were used to identify GA proteins. Thirty male and 32 female associated proteins were identified in this study. Many of these GA genes have homologues in *Caenorhabditis elegans* (83% male and 69% female), and most of the homologues have severe RNA interference (RNAi) phenotypes (72% male and 55% female) in *C. elegans*. Functional analysis showed that the male-associated genes are enriched for energy production, metabolic processes and cytoskeleton, while the female-associated genes are enriched for RNA modification and transcription. GA genes encode many excreted/secreted proteins. In situ localization studies showed that GA genes are mainly expressed in reproductive organs, and this is further evidence for their involvement in reproduction. Improved understanding of the basic biology of filarial nematodes may lead to improved tools for prevention and treatment of filarial infections. This study combined proteomics, in situ hybridization (ISH) and bioinformatics in a systems biology approach to improve understanding of gender differences and key proteins involved in reproduction in filarial worms. Advanced proteomics methods and bioinformatics led to the identification of 62 GA proteins for *B. malayi*. ISH revealed that most of those GA genes are expressed during embryogenesis or spermatogenesis. ISH results were consistent with RNAi data for *C. elegans* that linked the homologues of the *B. malayi* proteins to gamete production and embryogenesis.

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Graphical Abstract

Keywords

Brugia malayi; Multiplex proteomics analysis; Gender-associated proteins; Two-dimensional difference in-gel electrophoresis; In situ hybridization

1. Introduction

Filarial nematodes are important pathogens that cause lymphatic filariasis and onchocerciasis (river blindness), which affect approximately 150 million people in some 80 countries in tropical regions. These are serious diseases that are important causes of disability in the developing world. The Global Alliance to Eliminate Lymphatic Filariasis (GAELF, <http://www.filaria.org/>) was formed in 2000 to eliminate lymphatic filariasis by 2020 through mass drug administration (MDA), and there are similar programs for the control of onchocerciasis. Currently three drugs, diethylcarbamazine (DEC), ivermectin and albendazole, are used to kill or sterilize filarial parasites. There is concern that large-scale use of these drugs may result in drug resistance (Osei-Atweneboana et al., 2007). Furthermore, no new drugs for these neglected tropical diseases have been developed during the past three decades (Chirac and Torreele, 2006). Therefore, it is important to develop new drugs or alternative intervention approaches as a backup for the current MDA programs.

Brugia malayi is the only human filarial nematode species that can be maintained in the laboratory. Therefore, *B. malayi* has been extensively studied as a model organism for nematode parasites. The *B. malayi* genome project has provided a good foundation for proteomic studies on filarial parasites (Ghedini et al., 2007). Three proteomic studies have reported secretomes for adult worms and two larvae stages of *B. malayi*. Differences in secretomes by stage provide clues regarding mechanisms of parasite invasion, self-protection and manipulation of host immune responses (Hewitson et al., 2008; Moreno and Geary, 2008; Bennuru et al., 2009). Recently, a global proteomic analysis was done on the major developmental stages of *B. malayi* that identified 62% of the total proteins predicted from the draft genome (Bennuru et al., 2011).

Our laboratory is interested in the developmental and reproductive biology of filarial nematodes. Comparative studies of adult male and female *B. malayi* may provide insight into major pathways and processes involved in development and reproduction. Prior studies have focused on gene expression using microarrays (Li et al., 2005), quantitative real time PCR (qRT-PCR) (Li et al., 2004) and in situ hybridization (ISH) (Jiang et al., 2008) to characterize gender-associated (GA) genes. The current study used *B. malayi* genome data and advanced proteomics techniques to identify GA proteins. We also incorporated gene expression and localization data to gain insight regarding the function of these proteins.

2. Materials and methods

2.1. Parasites, protein extraction and labeling

Adult *B. malayi* worms were isolated from infected jirds (provided by the Filariasis Research Reagents Repository, University of Georgia, Athens, GA, USA) and separated by sex based on morphology. Fresh worms (60 females/sample, 120 males/sample) were washed with PBS, chilled in liquid nitrogen and ground into powder with a mortar and pestle. The worm powder was dissolved in lysis buffer (30 mM Tris-HCl pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS) plus a protease inhibitor cocktail (Roche, Indianapolis, USA). Dissolved samples were vortexed and placed on ice for 30 min. Samples were sonicated on ice until clear with a setting of 10% power for 20 s and pause for 20 s (Sonicator Ultrasonic Processor XL2010, Misonix Inc, Farmingdale, NY, USA). Sonicated samples were centrifuged for 10 min at 15,000 *g* at 4° C and supernatants were placed in clean 1.5 ml Eppendorf tubes. Protein concentration was determined with the Advanced Protein Assay Kit (Cytoskeleton Inc., Denver, USA). Protein samples were labeled with charge-matched cyanine dyes as previously described (Unlu et al., 1997; Tonge et al., 2001; Bredemeyer et al., 2004). Protein samples (50 µg/sample) were labeled with 400 pmol of Cy2, Cy3 or Cy5 cyanine dyes.

2.2. Two-dimensional difference in-gel electrophoresis (2D-DIGE) and image analysis

One male and two female protein samples (50 µg each) were separated on the same 2D-DIGE gel. Samples were loaded on to a pH 3–11 non-linear immobilized pH gradient strip (GE Healthcare, Pittsburgh, USA) followed by isoelectric focusing for a total of 65,000 Volthour (Vh) using a Protean IEF cell (BioRad, Hercules, CA, USA). After focusing, proteins were reduced, alkylated with iodoacetamide and layered on a 10–20% polyacrylamide gel for SDS-PAGE separation. Samples were imaged with a Typhoon Imager (GE Healthcare) using specified excitation/emission wavelengths: 488/520 nm for Cy2, 520/580 nm for Cy3, 620/670 nm for Cy5. DeCyder (version 6.5, GE Healthcare) software tools were used for image analysis. The differential in-gel analysis (DIA) module was used to align and normalize distinct images within each gel. DIA was set to identify 10,000 spots and then filtered to include only those with spot volumes > 10,000. The DIA module can also calculate abundance ratios of spots using a previously-described normalization algorithm (Alban et al., 2003).

2.3. Selection and excision of spots for protein identification

Protein features were selected for MS based on the following criteria: baseline resolution from other features, reproducible spot volumes for the two female samples, and at least a two-fold difference between spot volumes in male and female samples (Fig. 1). Selected spots were excised robotically (ProPic, Genomic Solutions, Ann Arbor, USA) using a triangulation algorithm implemented with in-house software.

2.4. Protein identification

Excised gel pieces were digested in situ with trypsin (Havlis et al., 2003). Samples were processed and analyzed using nano-LC-FTMS/MS. The “raw” files were processed using MASCOT Distiller, version 2.2.04 (Matrix Science, Oxford, U.K.) with the following settings: 1) MS processing: 200 data points per Da; no aggregation method; maximum charge state = +8; minimum number of peaks = 1. 2) MS/MS processing: 200 data points per Da; time domain aggregation method enabled; minimum number of peaks = 10; precursor charge and m/z, try to re-determine from the survey scan (tolerance = 2.5 Da); charge defaults = +2/+3; maximum charge state = +2. 3) Time domain parameters: minimum precursor mass = 700; maximum precursor mass = 16,000; precursor m/z tolerance for grouping = 0.1; maximum number of intermediate scans = 5; minimum number of scans in a group = 1. 4) Peak picking: maximum iterations = 500; correlation threshold = 0.90; minimum signal-to-noise = 3; minimum peak m/z = 50; maximum peak m/z = 100,000; minimum peak width = 0.001; maximum peak width = 2; and expected peak width = 0.01. The resulting Mascot generic files (.mgf) were exported to MASCOT version 2.2.04. The tandem MS data from the LTQ-FT mass spectrometer were searched against a *B. malayi* protein database (updated on 11 November 2007 and provided by Elodie Ghedin (University of Pittsburgh, Pittsburgh, Pennsylvania, USA)) using MASCOT version 2.2.04 (Matrix Sciences) with the following constraints: MS tolerance = 10 ppm, MS/MS tolerance = 0.8 Da with fixed modifications of cysteine residues (carbamidomethylation) and variable oxidation of methionine residues. The searches performed allowed up to five missed cleavages. An aliquot of the peptide pool was also analyzed using capillary reversed-phase HPLC-MS/MS interfaced to an electrospray-quadrupole-time-of-flight mass spectrometer (Q-STAR XL, Applied Biosystems, Carlsbad, USA). Criteria used for protein identification included: two or more peptide matches, MASCOT score > 60, and $P < 0.05$.

2.5. MS data analysis methods: peptide intensity (PI) and mass spectral counting (MSC)

For spots with multiple protein matches, PI and MSC analysis methods were used. Prior studies have demonstrated that PIs and MSC correlate well with protein abundances (Bondarenko et al., 2002; Chelius and Bondarenko, 2002; Ghaemmaghami et al., 2003; Wang et al., 2003; Liu et al., 2004). Criteria for choosing dominant proteins by PI were: peptides with intensity ≥ 2 ; proteins/peptides were considered to be dominant if their intensity was at least three times as high as those of non-dominant proteins. Proteins identified by MSC were considered to be dominant if they had two or more peptide matches and if MSC ≥ 2 higher than other proteins identified in the spot.

2.6. ISH

ISH was performed as previously described (Jiang et al., 2008). Briefly, adult *B. malayi* worms (12 weeks p.i.) were cut into frozen sections of 10 µm thickness and digoxigenin labeled RNA probes were used for ISH. Sense probes were used as negative controls. No hybridization signals were detected with negative control probes.

2.7. Bioinformatic analysis

To search for *C. elegans* homologues, *B. malayi* nucleotide sequences were compared by BLAST analysis against the non-redundant (NR) *C. elegans* database at the National Center for Biotechnology Information (NCBI), USA and optimized for “Somewhat similar sequences (BLASTn)” (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome). Criteria for the potential homologues were: score > 50 and E-value < 5.00E-5. For functional annotation of the *B. malayi* proteins, protein sequences were searched against conserved protein domains in the eukaryotic orthologous group (KOG) database (cdsearch/oasis_kog v2.25) at NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The search was done with low-complexity regions filtered with an E-value cut-off of 0.01. One functional annotation was assigned for each protein based on the E-value and Bitscore.

3. Results

3.1. 2D-DIGE analysis of *B. malayi* male and female proteins

Each image had approximately 7,000 protein spots; 4,829 spots remained in the analysis after removal of artefactual and low abundance spots. Gel pictures for male and female samples are shown in Fig. 2. When spots produced by two female samples were compared, the volume ratio was less than 1.6 fold for 95% of the spots (high reproducibility between samples). When the same spots were compared between female and male samples, this value increased to 2.9 fold, showing substantial differences by gender for shared proteins. Ninety-six protein spots (49 male and 47 female) that were differentially present in the gel were selected for MS analysis. For the 49 male-associated spots, the ratios between male and female ranged from 2.89 to 25.05. Ratios for 47 female-associated spots ranged from 2.73 to 10.86 (Supplementary Table S1).

3.2. MS analysis of GA proteins

The results of the MS analysis are summarized in Table 1. Ten spots selected for MS analysis did not generate any MS data. Fifty-nine (or 60) spots contained one dominant protein. Several spots had no protein matches and 22 contained two or more proteins with no clear dominant protein.

3.3. Comparison of results obtained by PI and MSC analyses

The MS data were analyzed using PI and MSC. The two methods generated the same results for most of the MS data. Twenty-four male- and 25 female-associated proteins were identified by PI, and 27 male and 25 female were identified by MSC. Overall, the two

methods identified 30 male-associated and 32 female-associated proteins (Table 2). We further classified the GA proteins into different functional groups (Tables 3 and 4, Supplementary Table S2). For male-associated proteins, the major groups were cytoskeleton (11/30 or 37%), metabolism (7/30 or 23%), and protein modification (6/30 or 20%). In contrast, the major groups represented in female-associated proteins were RNA modification (11/32 or 34%), uncharacterized (6/32 or 19%), protein modification (5/32 or 16%), cytoskeleton (3/32 or 9%), and metabolism (4/32 or 13%).

3.4. Expression patterns of *B. malayi* genes encoding GA proteins

To further analyze the GA proteins, genes that encode GA proteins were cloned and the expression of these genes was localized by ISH. These genes were classified into five groups based on their expression patterns: exclusively expressed in male, exclusively expressed in female, more highly expressed in male than female, more highly expressed in female than male, and similar expression levels in male and female worms. For the 29 male-associated protein genes, three genes were only expressed in male worms, seven genes showed higher expression in male than female worms, nine genes had higher expression signals in females, and 10 genes displayed similar expression levels in male and female worms (Table 5). For the 32 female-associated protein genes, two genes were exclusively expressed in females, 24 exhibited higher expression levels in females than in males, and six genes showed similar expression levels in male and female worms (Table 6). The in situ expression patterns of one female and one male exclusively expressed protein gene are shown in Fig. 3. Gene 13325.m00230 (**Bm1_07685**, DJ-1 family protein) produced an expression signal in ovaries (Fig. 3Aa) and morula stage embryos (Fig. 3Ab) but not in stretched microfilariae and not in any male sections (Fig. 3Ad, spermatocytes and Fig. 3Ae, spermatids). Gene 14015.m00091 (**Bm1_13605**, major sperm protein cytoskeletal) had a similar expression pattern to other major sperm proteins reported previously (Jiang et al., 2008) with strong expression signals in spermatocytes (Fig. 3Bd, arrow) and spermatids (Fig. 3Be, arrow) and no expression signal in any female sections (Fig. 3Ba-c). The expression patterns of three male-associated protein genes are shown in Fig. 4. For gene 14972.m07803 (**Bm1_40465**, cuticular glutathione peroxidase precursor), the expression pattern was similar in male (Fig. 4Ad-f) and female worms (Fig. 4Aa-c) with signal in muscles and intestine, and the signal was stronger in males than in females. For gene 14231.m00144 (**Bm1_16060**, carbohydrate phosphorylase), expression was only detected in muscles in males (Fig. 4Bc,d), while gene expression was detected in muscles, morulae and early pretzel stage embryos in females (Fig. 4Ba,b). The expression pattern for gene 14176.m00093 (**Bm1_15350**, fructose-bisphosphate aldolase 1) differed in male and female worms. In females (Fig. 4Ca,b), gene expression was detected in oocytes and morula stage embryos. In contrast, the gene was expressed in spermatogonia and spermatocytes in males (Fig. 4Cc,d).

Expression patterns of four female biased protein genes are shown in Fig. 5. Gene 14977.m04994 (**Bm1_43730**, lactate dehydrogenase) was expressed in oocytes, morula stage embryos and uterus epithelium in females (Fig. 5Aa-c) and in spermatogonia and spermatocytes in males (Fig. 5Ad,e). The genes 14979.m04609 (**Bm1_45460**, prolyl 4-hydroxylase 2 precursor, Fig. 5Ba-c), 13066.m00247 (**Bm1_04850**, hypothetical FAD-dependent oxidoreductase YEL047C, Figure 5Ca-c) and 13409.m00048 (**Bm1_08695**,

trehalose-6-phosphate synthase, Fig. 5Da-c) had similar expression patterns in females with signals in oocytes, morulae and pretzel stage embryos. In males, these genes had expression signals in spermatocytes (14979.m04609 (Fig. 5Bd), 13066.m00247 (Fig. 5Cd) and 13409.m00048 (Fig. 5De)) and in spermatogonia (13409.m00048, Fig. 5Dd).

3.5. RNA interference (RNAi) phenotypes of *C. elegans* homologues of *B. malayi* GA proteins

Little direct functional information is available for *B. malayi* genes. However, phenotypes of *C. elegans* homologues may provide clues to functions of GA proteins in *B. malayi*. Twenty-five of 30 male-associated proteins had *C. elegans* homologues. Twenty of the 25 homologues had RNAi phenotypes, and 18 had severe phenotypes. Twenty-two of the 32 female-associated proteins had homologs in *C. elegans*. Seventeen had RNAi phenotypes (12 severe) (Table 7 and Supplementary Table S3).

4. Discussion

We compared protein profiles of male and female adult *B. malayi* through 2D-DIGE combined with MALDI-TOF/TOF tandem MS. With multiplex labeling, several samples can be separated and compared in the same gel. This makes it easier to identify differences in protein profiles between two samples. The Decyder software is superior to the human eye for viewing 2D gels; Decyder identified more than 7,000 spots in each of our protein samples. Decyder is also useful for comparing the relative abundance of proteins that are present in different samples. The 3D view of the proteins and quantitative abundance ratios were useful for selecting proteins in this study.

MALDI-TOF/TOF tandem MS is a very sensitive technique that can detect trace amounts of peptides. PI and MSC were used to identify the dominant protein within each spot. The two methods identified the same dominant protein for most spots, and they provided consistent results for other spots. It is better to use both of these methods to confirm the identity of dominant proteins.

Cytoskeleton proteins were prominent among male-associated proteins (37% of male GA proteins versus 9% of female), and most of these were muscle related proteins such as actin (**Bm1_21725**, spot 6; **Bm1_16810**, spot 77; **Bm1_21705**, spot 77), calponin (**Bm1_49075**, spot 5), myosin (**Bm1_40715**, spot 73), and troponin (**Bm1_09610**, spot 80; **Bm1_35060**, spot 9). This result is consistent with the relatively higher muscle content and more rapid movement in males. Another major group associated with males was metabolism, which includes metabolic processes and energy production (23% in males versus 13% in females). This suggests that energy needs are higher in male worms. The male-associated list also contained two major sperm proteins (MSP) that are well known to be important components of sperm, and this finding is consistent with previous results (Li et al., 2005, 2011). Among the 32 female-associated proteins, 34% belong to the RNA modification group. This may indicate vigorous RNA transcription and processing associated with embryogenesis and embryo development in female worms. Among the 62 GA proteins, one protein (**Bm1_35060**, troponin family protein) was present in both the male- and female-associated protein lists. We checked the MS data and found that this protein was present in three

different spots (9, 10 and 11). Two spots showed male versus female abundance ratios of 25.05 (spot 9) and 8.6 (spot 10); the third spot had a female versus male ratio of 6.57 (spot 11). All three spots were on the same vertical level in the gels (Fig. 6), which means that they have the same molecular mass. The different horizontal positions for these spots means that they have different isoelectric points, and this suggests that male and female worms may differ in their post-translational modification of this protein. Gender differences in phosphorylation have been reported in *Homo sapiens* (Lagranha et al., 2010) and mice (Zhong et al., 2003; Ceylan-Isik et al., 2006).

ISH is a useful technique for understanding gene function in filarial nematodes. ISH was used to analyze expression patterns for genes encoding 62 GA proteins. Expression pattern comparisons between male and female worms must be performed cautiously due to the different sizes and internal structures of these worms. Comparisons are most reliable when genes are expressed in only one sex or when genes are expressed in the same tissues in both sexes. Gene expression results sometimes differ from protein results. Therefore, the main purpose of the ISH was to provide additional information about these GA protein genes (location of expression) and not to confirm the differential expression of the proteins. For the 30 male-associated protein genes, four were exclusively expressed in somatic tissues, but most (26 out of 30) were expressed in reproductive tissues. These included three genes that were only expressed in males. All 32 female-associated protein genes were expressed in reproductive tissues, but only two were exclusively expressed in females.

Caenorhabditis elegans is an intensively investigated model organism with a well annotated genome (C. elegans Sequencing Consortium, 1998). Gene expression patterns and RNAi phenotypes are available for most *C. elegans* genes (<http://nematode.lab.nig.ac.jp/>, <http://www.wormbase.org/>). This information may provide clues to the function of *B. malayi* genes. Most of the GA proteins (25/30 or 83% for male and 22/32 or 69% for female) have homologues in *C. elegans*. Many of the homologues have severe RNAi phenotypes (Table 7). It is likely that many of these GA proteins have conserved functions in *B. malayi*. The homologues of the *B. malayi* GA protein genes were checked against the germline gene list for *C. elegans* (Reinke et al., 2000) and it was found that nine of those were involved in early embryogenesis, two in spermatogenesis and five in oogenesis (Supplementary Table S3). Our ISH data also showed that most of the GA genes were expressed during embryogenesis or spermatogenesis. These results suggest that many of the GA proteins are involved in reproduction and that homologues have conserved functions in distantly related nematodes.

The completion of the *B. malayi* genome and improvements in MS techniques provided a strong foundation for this study. Several recent papers have reported proteomes of *B. malayi* excretory and secretory (ES) products and worm proteins (Hewitson et al., 2008; Moreno and Geary, 2008; Bennuru et al., 2009). We crosschecked our protein list with these published protein databases (Supplementary Table S4). Only 28 of the 62 (45%) GA proteins in this study appear on lists of secreted proteins in these prior publications. However, the other 34 proteins appeared in a recent global proteomics study (Bennuru et al., 2011). We further cross-referenced our identified proteins with other publications and found that 11 GA proteins were listed as potential drug candidates (Crowther et al., 2010; Kumar et al., 2007). Two other proteins (**Bm1_41425**, nematode fatty acid retinoid binding protein

and **Bm1_40465**, cuticular glutathione peroxidase precursor) were involved in worm and host immune interactions (Maizels et al., 2001) (Supplementary Table S4). Thus, many of the GA proteins identified in this study may have potential as targets for new drugs or vaccines. A recent paper has provided a global comparison of gene expression in male and female *B. malayi* (Li et al., 2011).

In this study, we identified 62 GA proteins that shared features with GA genes identified by transcription profiling: i) GA genes and genes that encode GA proteins have a higher frequency of severe RNAi phenotypes than other genes (Li et al., 2011); ii) Energy production, metabolic processes and cytoskeletal proteins are enriched in males; RNA modification and transcription are enriched in females; and a recent global proteomics paper reported similar findings (Bennuru et al., 2011). iii) Many GA proteins have been reported to be present in adult worms as ES proteins. iv) Many GA genes and proteins are involved in reproduction. These parallels reflect the fact that GA proteins tend to be encoded by genes with GA gene expression. While this was true for most of the female-associated proteins identified in our study (26 of 32), only 10 of 29 male-associated proteins identified in this study had male-associated gene expression confirmed by in situ studies.

In summary, advanced proteomics methods and genome information led to identification of 62 GA proteins in the filarial nematode *B. malayi*. ISH revealed that most of the genes that encode these proteins are expressed during embryogenesis or spermatogenesis. ISH results were consistent with RNAi data for *C. elegans* that link genes that encode homologues of GA in *B. malayi* proteins to gamete production and embryogenesis. This study used several different methods (proteomics, ISH and bioinformatics) in a systems biology approach to improve the understanding of gender differences and key proteins involved in reproduction in filarial worms. Improved understanding of the basic biology of filarial nematodes may lead to new tools for disease prevention and treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Proteomics methods and bioinformatics led to identification of 62 gender-associated (GA) proteins for *Brugia malayi*.
- In situ hybridisation (ISH) revealed that most of these GA genes are expressed during embryogenesis or spermatogenesis.
- RNAi data for *C. elegans* linked the homologues of the *B. malayi* proteins to gamete production and embryogenesis.
- This study combined proteomics, ISH and bioinformatics in a systems biology approach.

Fig. 1.

Representative examples of two-dimensional difference in-gel electrophoresis (2D-DIGE) images and corresponding 3D images of spots from protein samples of *Brugia malayi* worms selected for MS. A total of 96 spots were selected. F1, F2: female samples 1 and 2; M: male sample. The lines surrounding the protein spots and the lines below the peaks indicate the outer borders.

Fig. 2.

Two-dimensional difference in-gel electrophoresis (2D-DIGE) images of proteins from male and female *Brugia malayi* worms. (A) One protein sample from female worms. (B) Protein sample from male worms. Numbers point to the spots selected for MS. The pI range from 3–11 is indicated with arrows and MW (kDa) to the right.

Fig. 3.

In situ hybridization (ISH) patterns for female exclusively expressed protein genes **Bm1_07685** (13325.m00230, Aa-e), and male exclusively expressed protein genes **Bm1_13605** (14015.m00091, Ba-e) in adult *Brugia malayi*. Transcripts of **Bm1_07685** were exclusively in female worms at ovaries (Aa) and morula stage embryos (Ab, arrows), but not in stretched microfilariae (Ac, arrows); no signals were detected in male worm sections (Ad,e, arrows point to spermatocytes and spermatids, respectively). In contrast, transcripts of **Bm1_13605** were only detected in male sections in spermatocytes (Bd, arrow) and spermatids (Be, arrow); no signals were detected in female sections (Ba, ovaries; Bb, arrows point to morula stage embryos; Bc, arrows point to stretched microfilariae). O, ovary; i, intestine. Scale bar = 20 μ m.

Fig. 4.

In situ hybridization (ISH) patterns for male biased protein genes, **Bm1_40465** (14972.m07803, Aa-f), **Bm1_16060** (14231.m00144, Ba-d) and **Bm1_15350** (14176.m00093, Ca-d), in adult *Brugia malayi*. Transcripts of **Bm1_40465** were detected in lateral cords and intestine of various segments of female worms (Aa-c) and male worms (Ad-f). Cross-sections with ovaries (Aa), morula stage embryos (Ab) and stretched microfilariae (Ac) of female; cross-section with spermatocytes (Ad), spermatozoa (Ae) and longitudinal section (Af) of male worms. Hybridization signals for gene **Bm1_16060** were seen in muscles (Ba), morulae (Ba, arrows) and early pretzel stage embryos (Bb, arrows) in females and only seen in muscles in males (Bc, cross section and Bd longitudinal section). Hybridization signals for gene **Bm1_15350** were detected in oocytes (Ca) and morula stage embryos (Cb, arrows) in females, and in spermatogonia (Cc, arrow) and spermatocytes (Cd, arrow) in males. O, ovary; i, intestine; m, muscle; lc, lateral cord. Scale bar = 20 μ m.

Fig. 5.

In situ hybridization (ISH) patterns for female-biased protein genes, **Bm1_43730** (14977.m04994, Aa-e), **Bm1_45460** (14979.m04609, Ba-d), **Bm1_04850** (13066.m00093, Ca-d) and **Bm1_08695** (13409.m00048, Da-e), in adult *Brugia malayi*. Transcripts of **Bm1_43730** were detected in oocytes (Aa), morula stage embryos (Ab, arrows) and uterus epithelium (Ac, arrow heads) in female worms and detected in spermatogonia (Ad, arrow) and spermatocytes (Ae, arrow) in males. Hybridization signals for gene **Bm1_45460** were seen in oocytes (Ba), morulae (Bb, arrows) and pretzel stage embryos (Bc, arrows) in females and were also seen in spermatocytes in males (Bd, arrow). Hybridization signals for gene **Bm1_04850** were detected in oocytes (Ca), morula stage embryos (Cb, arrows), and early pretzel stage embryos (Cc) in females, and signals were also detected in spermatocytes (Cd, arrow) in males. Transcripts of **Bm1_08695** were localized in female worms in oocytes (Da), morula stage embryos (Db, arrows) and early pretzel stage embryos (Dc, arrows, arrowheads point to late pretzel embryos which have no hybridization signals); transcripts were also localized in spermatogonia (Dd, arrow) and spermatocytes (De, arrow) in males. O, ovary; i, intestine. Scale bar = 20 μ m.

Fig. 6. One protein was present in three spots in a two-dimensional gel electrophoresis gel. Spots 9 and 10 came from a male protein sample and spot 11 (within the dotted circle) came from a female protein sample. This figure only shows spots from a male worm sample.

Table 1

Comparison between results obtained by peptide intensity and spectral count in identifying dominant proteins^a for 96 spots from *Brugia malayi* protein samples selected for mass spectrometry.

Protein features	Peptide intensity	Spectral count
No MS data obtained	10	10
No protein matches	5	4
One protein	59	60
Two proteins	14	12
> Two proteins	8	10
Total	96	96

^aRefer to Section 2.5 for criteria used for selecting dominant proteins.

Table 2

Dominant proteins identified in each spot of *Brugia malayi* protein samples by peptide intensity and spectral count methods.

Proteins	Peptide intensity	Spectral count	Sub total
Male	24	27	30
Female ^a	25	25	32
Sub total	49	52	
Total ^{b,c}	62		

^aOne protein (Bm1_35060, troponin family protein) is present in both the male and female lists.

^bTotal protein numbers were obtained by subtracting redundancies between the two methods and between sexes.

^cOne protein (Bm1_21705, actin 1) was present in the male list by the spectral count method and in the female list by the peptide intensity method. Based on in situ hybridization (ISH) data, this protein was later removed from the female list.

Table 3Classification of male-associated proteins of *Brugia malayi* based on functional annotation.

Classification	Pub locus	Annotation
Cytoskeleton		
	Bm1_19065	conserved hypothetical proteins
	Bm1_49075	Calponin homolog OV9M, putative
	Bm1_21725	actin 2, putative PF00022
	Bm1_13600	major sperm protein 2, putative cytoskeletal MSP PF00635
	Bm1_17055	hypothetical protein
	Bm1_57260	ATP synthase delta chain, mitochondrial precursor, putative TIGR01216
	Bm1_40715	myosin heavy chain, putative PF01576
	Bm1_09610	Troponin T, putative
	Bm1_35060	Troponin family protein
	Bm1_16810	actin, putative PF00022
	Bm1_21705	actin 1, putative PF00022
Protein modification		
	Bm1_43675	heat shock 70 kDa protein, putative PF00012
	Bm1_40465	Cuticular glutathione peroxidase precursor, putative PF00255
	Bm1_17805	proteasome subunit alpha type 1, putative PF00227
	Bm1_11450	chaperonin homolog HSP60, mitochondrial precursor, putative PF00118
	Bm1_26325	Thioredoxin family protein PF00085
	Bm1_27325	p27, putative PF00011 Hsp20/alpha crystallin family
Metabolism		
	Bm1_15350	fructose-bisphosphate aldolase 1, putative PF00274
	Bm1_41940	Glyceraldehyde 3-phosphate dehydrogenase, putative TIGR01534
	Bm1_29130	triosephosphate isomerase, putative PF00121
	Bm1_16060	carbohydrate phosphorylase, putative TIGR02093
	Bm1_13790	adenylate kinase isoenzyme 1, putative TIGR01360
	Bm1_50960	vacuolar ATP synthase catalytic subunit A, osteoclast isoform, putative
	Bm1_34625	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex,
RNA processing and modification		
	Bm1_31425	KH domain containing protein PF00013
Transcription		
	Bm1_23945	hypothetical protein
Cell cycle control, cell division, chromosome partitioning, Nuclear structure		
	Bm1_56985	cytoplasmic intermediate filament protein, putative PF00038
Intracellular trafficking, secretion, and vesicular transport		
	Bm1_13605	Major Sperm Protein (MSP), putative cytoskeletal MSP PF00635
Uncharacterized		
	Bm1_43025	PDZ domain PF00595
	Bm1_46705	thioredoxin, identical

Protein sequences were searched for conserved domains in the eukaryotic orthologous group (KOG) database. For each protein only a single annotation was assigned based on the E-value and Bitscore (details in Supplementary Table S2). Unknown, hypothetical and function unknown proteins are classified as uncharacterized; metabolism includes amino acid, carbohydrate, nuclear and energy metabolism.

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Table 4

Classification of female-associated proteins of *Brugia malayi* based on functional annotation.

Classification	Pub locus	Annotation
Cytoskeleton		
	Bm1_21620	Profilin family protein PF00235
	Bm1_03430	dihydrolipoamide S-acetyltransferase precursor, putative PF00364
	Bm1_57220	Src substrate cortactin, putative PF02218
Protein modification		
	Bm1_28935	transglutaminase, putative TIGR01130
	Bm1_14535	Small heat shock protein OV25-1, putative PF00011
	Bm1_32895	FKBP-type peptidyl-prolyl cis-trans isomerase-30, BmFKBP-30 PF00254
	Bm1_25705	peptidyl-prolyl cis-trans isomerase 11, putative PF00160
	Bm1_51495	heat shock protein 90, putative
Metabolism		
	Bm1_45460	prolyl 4-hydroxylase 2 precursor, putative
	Bm1_11460	cytidine deaminase, identical TIGR01354
	Bm1_29970	oxidoreductase, short chain dehydrogenase/reductase family protein PF00106
	Bm1_43730	lactate dehydrogenase., putative TIGR01771
RNA processing and modification		
	Bm1_04840	KH domain containing protein PF00013
	Bm1_20785	hypothetical protein
	Bm1_04850	Hypothetical FAD-dependent oxidoreductase YEL047C, putative TIGR01813
	Bm1_08695	trehalose-6-phosphate synthase, putative
	Bm1_08205	Probable ATP-dependent RNA helicase p47 homolog, putative PF00270
	Bm1_31255	RNA recognition motif containing protein, putative PF00076
	Bm1_45785	SF2, putative PF00076 RNA recognition motif
	Bm1_22805	hypothetical protein
	Bm1_47275	Splicing factor, proline-and glutamine-rich, putative PF00076
	Bm1_07305	KH domain containing protein PF00013
	Bm1_33950	heterogeneous ribonuclear particle protein, putative PF00076
Replication, recombination and repair		
	Bm1_39065	SET protein, putative PF00956 Nucleosome assembly protein
Chromatin structure and dynamics		
	Bm1_57630	retinoblastoma-binding protein, putative PF00400
Uncharacterized		
	Bm1_38615	hypothetical protein
	Bm1_51000	conserved hypothetical protein
	Bm1_42615	MGC64450 protein, putative PF07933
	Bm1_41425	BM20, putative PF05823 Nematode fatty acid retinoid binding protein
	Bm1_21695	hypothetical protein
	Bm1_26585	Hypothetical UPF0205 protein C40H1.5 in chromosome III precursor, putative PF01060
Defense mechanisms		

Classification	Pub locus	Annotation
	Bm1_07685	DJ-1 family protein TIGR01383

Protein sequences were searched for conserved domains in the eukaryotic orthologous group (KOG) database. For each protein only a single annotation was assigned based on the E-value and Bitscore (details in Supplementary Table S2). Unknown, hypothetical and function unknown proteins are classified as uncharacterized; metabolism includes amino acid, carbohydrate, nuclear and energy metabolism.

Table 5

Expression patterns of *Brugia malayi* genes encoding male-associated proteins.

Group	Pub_locus	Female reproductive system						Muscle	Male reproductive system				
		Uterus-epithelium	Oocytes early	Oocytes late	Morulae early	Morulae late	Pretzel early		Pretzel late	Spermatogonia	Spermatocytes	Spermatids	Testis
M	Bm1_13790									1		2	
	Bm1_13605											3	3
	Bm1_13600											3	3
M>F	Bm1_49075							3m,2f					
	Bm1_21705							3m,2f					
	Bm1_21725							3m,2f					
	Bm1_40465							lc3m,lc2f					
	Bm1_35060	1						3m,2f					
	Bm1_09610	1				1		lc2f,lc3m					
	Bm1_17805				1	1		2m	2		2		
	Bm1_16060				1	2	1	3m,fl					
	Bm1_29130	3		2					1				
	Bm1_50960		2	2	2	2	1				2		
M<F	Bm1_43675	1	1	2	3	2	1				3		
	Bm1_57260		1	1	1	1					1		
	Bm1_16810			3	2	1	1				3		
	Bm1_31425		1	3	2	2	1				2		
	Bm1_46705		2	1	2	2	2					2	
	Bm1_34625		2	3	2	2	1	1	1				
	Bm1_43025		1	1	2	2	1	1	1	1	1		
	Bm1_15350		1	1	2	2			1		2		
	Bm1_40715					2	2	1			2		
	Bm1_56985	2	2	2	2	2			2		2	1	1 vd
M=F	Bm1_27325	1	1	1	1	2	1			1	1		
	Bm1_41940	1	1	2	2	2	1	1		1	2	2	
	Bm1_26325		1			1					1		
	Bm1_11450		1			1							2

Group	Pub_Locus	Female reproductive system						Muscle			Male reproductive system		
		Uterus-epithelium	Oocytes early	Oocytes late	Morulae early	Morulae late	Pretzel early	Pretzel late	Spermatogonia	Spermatocytes	Spermatids	Testis	
	Bm1_19065		1	2	1	1			1	1			
	Bm1_23945		2	2	2	2	2		2	1			
	Bm1_17055	2	1	1	2	2	1	1	1	1		2	

Signal intensity was scored as follows: 1, weak; 2, moderate; 3, strong; blank, no signal. M, male only; M>F, transcripts are more abundant in males than in females and M<F is vice versa; M=F, transcripts are similar in abundance. Gene expression patterns of Bm1_43675 (**BM003899**, heat shock protein 70) and Bm1_13600 (**BM014209**, major sperm protein 2) were published in Jiang et al. (2008) and the expression pattern for gene Bm1_35060 was reported in Li et al. (2011). Gene Bm1_40465 was also weakly expressed in the intestine of male and female worms. Genes expressed in muscles and lateral cords were grouped under the muscle category. Vd, vas deferens; lc, lateral cord; m, male; f, female; 3m, strongly expressed in males; lc2f, moderately expressed in the lateral cord of females.

Table 6

Expression patterns of *Brugia malayi* genes encoding female-associated proteins.

Group	Pub_locus	Female reproductive system					Muscle			Male reproductive system			
		Uterus-epithelium	Oocytes early	Oocytes late	Morulae early	Morulae late	Pretzel early	Pretzel late	Muscle	Spermatogonia	Spermatocytes	Spermatids	Testis
F	Bm1_45785				1	1							
	Bm1_07685		2	2	1	1							
F<M	Bm1_41425								3m,3f				3
F=M	Bm1_26585	2							1m				2
	Bm1_21620		2	1	1	1					1		2
	Bm1_21695				2	1	1			2	2		
	Bm1_32895		1	1	2	2	2	1				2	
	Bm1_43730	2	2	3	3	2				2	3		
	Bm1_08695		1	1	1	1	1			1	1		
	Bm1_51000	3	3	3	3	2			3m,2f				
	Bm1_22805		1	1	1	2				1			
	Bm1_20785			1	2	1	1			1			
	Bm1_29970		1	2	2	2	1			1			
	Bm1_57220				1	2	1				1		
	Bm1_25705			1	2	1	1				1		
	Bm1_04840		2	2	2	2					1		
	Bm1_42615		2	2	2	2	1				1		
	Bm1_08205		1	2	2	2	1				2		
	Bm1_03430		2	2	2	2	1	1			2		
	Bm1_51495	2	3	1	3	3	2				2		2
	Bm1_45460		1	1	2	1	1	1			1		
	Bm1_33950		1		1	2	1	1			1		
	Bm1_31255		2	3	1	2	2	1			1		
	Bm1_28935		2		2	1	1	1			1		
	Bm1_47275	1	2		2	1	1	1			1		
	Bm1_14535	2	1	2	2	2	2	1			1		1
	Bm1_57630		1	2	1	2	1			1	1		

Group	Pub. locus	Female reproductive system					Muscle		Male reproductive system			Testis
		Uterus-epithelium	Oocytes early	Oocytes late	Morulae early	Morulae late	Pretzel early	Pretzel late	Spermatogonia	Spermatocytes	Spermatids	
	Bm1_39065		2	2	2	2	2	1	2	1		
	Bm1_11460		1	1	1	2				1	1	
	Bm1_04850		2	2	2	2	1			1		
	Bm1_38615		1	1	2	2	2	1		1	1	
	Bm1_07305	1	2	3	3	2	1		1	2	2	

Signal intensity was scored as follows: 1, weak; 2, moderate; 3, strong; blank, no signal. F, female only; M>F, transcripts are more abundant in males than in females and M<F is vice versa; M=F, transcripts are similar in abundance. M, male; f, female; 3m, strongly expressed in males; 2f, moderately expressed in females. Gene expression patterns for Bm1_25705 (peptidyl-prolyl cis-trans isomerase 11) and Bm1_5785 (SF2, putative PF00076 RNA recognition motif) were reported previously (Li et al., 2011).

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RNA interference (RNAi) phenotypes of *Caenorhabditis elegans* homologues for the *Brugia malayi* gender-associated (GA) proteins identified by MS.

Table 7

<i>B. malayi</i> proteins		<i>C. elegans</i> homologues	RNAi phenotype		
Proteins			Severe (failed development, reproduction or paralysis)	Other	No phenotype
Male	30	25	18	2	5
Female	32	22	12	5	5