Supporting Information

Moreno et al. 10.1073/pnas.1011983107

SI Materials and Methods

Parasites. All animal procedures were approved by the Animal Care Committee of McGill University–Macdonald campus and were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Infected gerbils were obtained from the Filariasis Research Reagent Repository Center (Athens, GA). Mf and adult *B. malayi* were recovered >120 d postinfection from the peritoneal cavities of Mongolian jirds (*Meriones unguiculatus*) infected subcutaneously with 200 to 300 L3. Adult worms were washed several times in RPMI medium 1640 [supplemented with L-glutamine, 20 mM hepes, 100 μg/mL penicillin, 100 units/mL streptomycin (Gibco), pH 7.2] (henceforth, RPMI 1640) and separated by gender according to size. Mf were obtained by washing the peritoneal cavity with 37 °C RPMI 1640. The washes were centrifuged 5 min at 1,000 × *g* to pellet mf, which were subsequently purified from host cells by passage through PD-10 columns equilibrated with prewarmed RPMI 1640, as described (1).

Cloning and Sequencing of Bma-AVR-14 Subunits. Partial sequences for two glutamate-gated chloride channels (GluCls) (AVR-14) were obtained from the *B. malayi* genome project (Bm1_00335 and Bm1_15450). Sequence alignment of the predicted genomic and coding sequences allowed us to initially infer that the two gene models are splice variants of the same gene. Completion of the full coding sequences for these genes was carried out by following a strategy like that used for *avr-14* from *Dirofilaria immitis* (2).

B. malayi total RNA was extracted from adult worms or mf using the RNeasy Microkit (Qiagen) according to the manufacturer's instructions for tissue samples. Complementary DNA was obtained from reverse-transcription reactions at 50 °C on 100 to 500 ng RNA in a total volume of 20 μL with SuperScript III (Invitrogen), using either an oligo(dT)12-18 primer (Invitrogen) or the 3′RACE adaptor from a 3′RACE kit (Bioline) (Table S1). PCRs were performed (Fig. S2) with 1 μL of the reverse-transcriptase reaction and 0.02 U/μL Phusion High-Fidelity DNA polymerase (New England Biolabs), 1× Phusion HF Buffer, 200 μM dNTPs, 0.5 μM each primer (Table S1) in a total volume of 20 μL on a Mastercycler epgradient thermocycler (Eppendorf) following a program of initial denaturation at 98 °C for 1 min, followed by 35 cycles of denaturation at 98 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. PCR products were visualized under UV light after electrophoresis through a 1% agarose gel. Selected bands were purified using a QIAquick gel extraction kit (Qiagen) and cloned into pJET1.2/blunt using the CloneJET PCR cloning kit (Fermentas). Recombinant clones were sequenced in both directions using pJET1.2 forward and reverse primers and assembly was performed in NTIvector v.10 (Invitrogen).

Antibodies. Immune sera against on-site produced C-terminal His-tagged Bma-MIF-1, Bma-TCTP, and Bma-CPI-2 recombinant proteins were raised in rabbit, goat, and guinea pig, respectively, under contract with commercial sources (Pacific Immunology for Bma-MIF-1 and Bma-TCTP, Harlan Bioproducts for Bma-CPI-2). Immune sera were subsequently purified by affinity chromatography on antigen-coupled Affi-Gel-10 beads (Biorad) or Sulfolink (Pierce) according to the instructions of the manufacturers. Affinity purified rabbit anti-human-TPI (sc-30145; Santa Cruz) raised against the full-length human protein was cross-reactive toward Bma-TPI and was also used in this study. Purified rabbit antibody against Bm-VAL-1 was a kind gift from Murray Selkirk (Imperial College, London, United Kingdom).

A peptide with the sequence LRTKMILRREFS-cysteine, mapping to residues 239 to 250 of Bma-AVR-14A, was synthesized, coupled to KHL and used to immunize rabbits commercially (Antagene Inc.). Immune serum was subsequently purified by chromatography through a peptide-coupled Sulfolink resin (Pierce).

Goat anti-rabbit IgG and anti-guinea pig IgG-Alexa Fluor 488, donkey anti-goat IgG Alexa Fluor 488 (Invitrogen), and donkey anti-rabbit IgG HRP-linked (Amersham) were used as secondary antibodies for immunoassays.

Indirect ELISA. Indirect ELISA was performed to estimate affinity of the GluCl antibody for different subunits of the channel. High binding polystyrene 96-well plates (Costar) were coated with 50 μL of a 12-μM solution of either LRTKMILRREFS-cysteine or ARVMLLLRREYS-cysteine in 50 μM sodium carbonate buffer, pH 9.6. After extensive washing with PBS – 0.1% Tween 20, unbound sites in the plate were blocked by incubation with blocking buffer (1% BSA in PBS–0.1% Tween 20) for 2 h. One-hundred microliters of primary antibody diluted in blocking buffer was added to the plate in serial dilution and incubated for 2 h. After extensive washes with PBS – 0.1% Tween 20, 100 μL of a 1:5,000 dilution of anti-rabbit IgG-HRP in blocking buffer was added to each well and incubated for 1 h at room temperature. After incubation with secondary antibody, the wells were washed with PBS– 0.1% Tween 20 and 100 μL of a solution of 3,3′,5,5′-Tetramethylbenzidine (T0440; Sigma) were added to the plate. The coloring reaction was stopped by adding of 100 μL of 2 N HCl 10 min after the addition of substrate. Absorbance values were subsequently determined at 445 nm in a plate reader (EL808; Biotek).

Immunohistochemistry. The mf were fixed and permeabilized by an adaptation of the tube protocol for *Caenorhabditis elegans* (3). The mf were collected by centrifugation at 1,000 × *g* for 5 min and washed once with warm PBS. Freeze-cracking was performed in fixing solution [4% (wt/vol) paraformaldehyde in PBS] by immersing the tube in liquid nitrogen for 2 to 3 min followed by thawing in a 37 °C water bath (three times). The mf were incubated in the fixing solution for 4 h at 4 °C, collected by centrifugation, and washed several times with PBST (0.1% Triton X-100 in PBS). Permeabilization was done by incubation overnight at 37 °C in fresh 2-mercaptoethanol solution (5% 2-mercaptoethanol, 1% Triton X-100, 120 mM Tris, pH 7.0). After extensive washing in PBST, worms were incubated for 12 h in collagenase solution [1,000 U/mL collagenase type VII (Sigma), 1 mM $CaCl_2$, 0.1% Triton X-100, 100 mM Tris, pH 7.4] at 37 °C. The mf were washed with PBST and then incubated overnight with AbD solution (0.1% BSA, 0.1% sodium azide in PBST).

Incubations with primary affinity-purified antibodies diluted in AbD solution (5–16 μg/mL) were performed for 2 d at 4 °C. Removal of unbound antibodies was done by three to four successive centrifugation–resuspension steps in AbD and final incubation overnight at 4 °C. Incubation and removal of secondary antibodies (dilution 1:1,000 or 1:3,000) was performed the same way. Muscle tissue and nuclei were counterstained by incubation overnight with 100 nM rhodamine-phalloidin (Cytoskeleton) and 50 ng/mL DAPI (Sigma), respectively.

Specimens were washed in AbD as above and mounted in glycerol–PBS-DABCO 1%. Observations were performed on a Biorad Radiance 2100 confocal laser scanning microscope equipped with a Nikon E800 fluorescence microscope for confocal image acquisition and the LASERSHARP 2000 analyzing

software package. Controls include observation of mf with omission of primary antibody and, in the case of on-site produced proteins, with peptide- or protein-adsorbed primary antibodies. Preadsorption of the antibodies was performed by overnight incubation of 50 μg/mL of peptide (for anti-GluCl) or 5 to 40 μg/mL of protein (for anti-MIF-1, anti-TCTP and anti-CPI-2). Image processing was performed on ImageJ v1.42q (National Institutes of Health).

In Vitro Treatment of *B. malayi* with IVM. After initial incubation overnight, parasites were incubated for 72 h in volumes of 1 mL in 24-well plates at 37°, 5% CO_2. RPMI containing 0, 0.1, 0.32, or 1.0 μM IVM was used for drug treatments after dilution of a 10 mM stock solution dissolved in DMSO. Control wells contained 0.1% DMSO. Parasite densities were set at 250×10^3 mf/mL media from each plate were collected and replaced with fresh media with or without drug every 24 h. The medium in each well was treated as an independent sample; mf were recovered by centrifugation at $1,000 \times g$ for 5 min, the supernatant was filtered on 0.22-μm membranes. Both the media and collected mf were stored at −80 °C until analysis.

For 2D-DiGE experiments, ES products were recovered from three independent incubations with or without 0.1 μM IVM. Media were collected each 24 h for three consecutive days, as mentioned above. The spent media were immediately passed through a 0.22-μm filter; all collected samples from each independent replicate were pooled before concentration for subsequent analysis. Proteins were concentrated to 1 to 1.5 mL in an Amicon Ultra 3000 MWCO (Millipore) and then precipitated with trichloroacetic acid (TCA, 20% final concentration) as described previously (1). Proteins were resuspended in 30 mM Tris-HCl pH 8.5 containing 7 M urea, 2 M thiourea, and 4% CHAPS.

Protein Determination. Protein determinations were performed with an EZQ protein quantitation kit (Invitrogen) with some modifications. For protein quantitation in ESP, 250 μL of either sample or RPMI were spiked with 50 ng of ovalbumin as an internal standard and then concentrated to dryness in a speed-vac. Samples were resuspended in 5-μL doubled distilled water. The total sample was serially spotted in volumes of 2 μL on the assay paper in the sampling plate. The assay paper was washed twice with MeOH for 5 min and then incubated with the EZQ reagent for 30 min. Final 2-min washes were performed three times with 10% MeOH, 7% acetic acid. The assay paper was placed into the sampling plate and fluorescence was determined in a plate fluorimeter (FlexStation II; Molecular Devices) set at 450 nm for excitation and emission at 610 nm. Protein amounts were estimated from extrapolation from the standard curve, corrected according to the internal standard readings of RPMI alone.

Two-Dimensional DiGE. For 2D-DiGE, 0.5 μg of each protein sample was labeled with 40 pmol either Cy3 or Cy5, alternating the dyes to avoid labeling bias, on ice for 30 min in the dark, as described (4). A pooled sample that was included as an internal standard in the gel runs was generated by combining an equal amount of each sample and labeling it with Cy2. Labeled samples were combined, randomized, and diluted with rehydration buffer [1% IPG buffer (GE Healthcare), 7 M urea, 2 M thiourea, 2% DTT (wt/vol), 4% CHAPS (wt/vol), 0.002% bromophenol blue (wt/vol)] to 250 μL. For isoelectrofocusing, samples were actively rehydrated into IPG strips (pH 3–10 NL, 24 cm length; GE Healthcare) and focused with an Ettan IPGphorII Isoelectric Focusing System (GE Healthcare) using a step-gradient protocol ranging from 30 to 8,000 V for ≈26 h (30 V 10 h; 500 V 1 h; 1,000 V 1 h; 8,000 V 3 h; 800 V 3 h).

IPG strips were rehydrated in 10 mL equilibration buffer-1 [6 M urea, 5 mg/mL DTT, 30% glycerol (vol/vol), 2% SDS (wt/vol), 0.002% bromophenol blue (wt/vol), 100 mM Tris-HCl, pH 6.8] for 10 min followed by a second incubation with 10 mL equilibration buffer-2 [6 M urea, 45 mg/mL iodoacetamide, 30% glycerol (vol/vol), 2% SDS (wt/vol), 0.002% bromophenol blue (wt/vol), 100 mM Tris-HCl, pH 6.8] and proteins were separated on 4–6% SDS/PAGE gels (24 cm × 20 cm × 1 mm). The SDS/PAGE gels were run at 1 W per gel for 60 min and then at 2 W per gel at 25 °C until the bromophenol blue dye front reach the bottom of the gel (~16 h). Proteins were visualized with a Typhoon 9400 (GE Healthcare) fluorescence scanner. The Cy2-, Cy3-, and Cy5-labeled images for each gel were scanned at the excitation/emission wavelengths of 488/520 nm, 532/580 nm, and 633/670 nm, respectively. The images were imported and analyzed with DeCyder software v.7.0 (GE Healthcare).

Analysis consisted of initial spot detection and pair-wise comparisons of treated and untreated mf to the pooled internal standard on each gel. Calculation of individual protein abundance represented by normalized spot volumes (Cy3:Cy2 and Cy5:Cy2 ratios) was initially performed with the DeCyder differential in-gel analysis module. Subsequently, the three gels were matched using the internal standard pool spot map with the DeCyder biological variation analysis module. Protein-spot matches were confirmed and in some cases adjusted manually using the spot editing features of DeCyder v 7.0. Comparative cross-gel statistical analyses of the spot maps were used to determine specific changes in abundance. Paired Student's *t* test *P* values for each normalized spot volume across the gels were calculated.

SI Text

Determination of Changes in Protein Abundance in Excretory-Secretory Products from *Brugia malayi* Microfilariae After Incubation with 0.1 μM Ivermectin. To determine the effect of ivermectin (IVM) on the relative abundance of specific proteins in microfilariae (mf) excretory-secretory (ES) products, mf were incubated with or without 0.1 μM IVM. Media were recovered and exchanged each 24 h for three consecutive days, pooled, concentrated, and analyzed using 2D-difference gel electrophoresis (DiGE) methods. Incubation with 0.1 μM IVM decreased by ~30% the amount of protein released by mf compared with unexposed controls in three independent replicates ($P < 0.05$, paired *t* test).

Analysis of protein maps using the DeCyder V. 7.0 software allowed the comparison of the normalized volume in 61 major protein spots from treated and untreated samples (Fig. S4). As indicated in Table S2, there were no significant differences in any of the analyzed spots. This finding indicates that, although there is a drop in the amount of protein released following exposure of mf to IVM, the relative composition of proteins in ES products remains unchanged.

Taking into account the association of the IVM receptor with the muscle structure encompassing the ES-vesicle, it is expected that the effect of IVM on protein release would result in a drop in the relative abundance of proteins that are released through the ES-pore. The absence of changes in the protein composition of the ES products indicates that protein release through a different anatomical path is negligible compared with the release through the ES-apparatus. This result supports our proposal that the mf ES apparatus is the predominant anatomical path for protein release to the host environment.

1. Moreno Y, Geary TG (2008) Stage- and gender-specific proteomic analysis of *Brugia malayi* excretory-secretory products. *PLoS Negl Trop Dis* 2:e326.
2. Yates DM, Wolstenholme AJ (2004) An ivermectin-sensitive glutamate-gated chloride channel subunit from *Dirofilaria immitis*. *Int J Parasitol* 34:1075–1081.
3. Duerr JS (2006) Immunohistochemistry. in *WormBook*, ed The *C. elegans* Research Community (WormBook), 10.1895/wormbook.1.105.1. Available at http://www.wormbook.org.
4. Tannu NS, Hemby SE (2006) Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling. *Nat Protoc* 1:1732–1742.

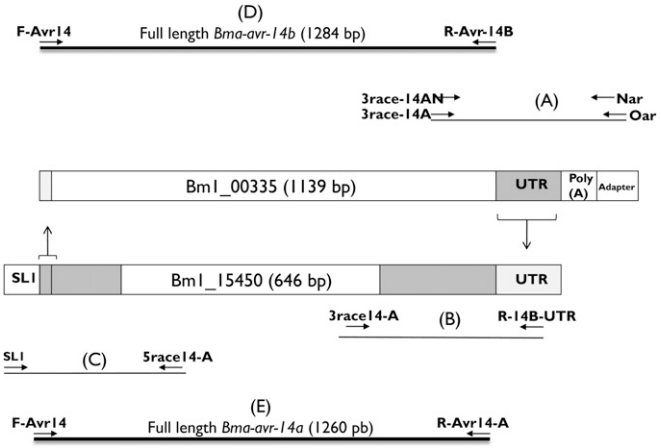

Fig. S1. Strategy for cloning two splice variants of the *B. malayi avr-14* gene. Mining the *B. malayi* genome database revealed two partial sequences (Bm1_00335 and Bm1_15450) sharing homology with AVR-14 subunits from *C. elegans*. Sequence alignment of the predicted genomic and coding sequences showed that these gene models are splice variants of the same gene. The strategy for cloning the two full ORFs consisted of: (*A*) Assessing the sequence by cloning the amplified UTR of the *avr-14* transcripts by 3′RACE using nested PCR with cDNA obtained from retrotranscription with a commercial adaptor (Bioline) and primers annealing to both the *avr-14b* (3race-14 A and AN) and the adaptor sequence (Nar and Oar). (*B*) The 5′ region of *avr-14a* containing the stop codon was assessed by cloning and sequencing the PCR product obtained from cDNA using a forward primer designed on the reported sequence (3race14-A) and a reverse primer designed on the UTR of the *avr-14b* (R-14B-UTR). (*C*). The 3′ end of the *avr-14* variants was found by cloning and sequencing the PCR product resulting from the amplification of cDNA using a forward primer annealing to the Splice Leader sequence (SL1) and a reverse primer designed on the *avr-14a* reported sequence. (*D* and *E*) The full coding sequences for *avr-14 a* and *b* were obtained by cloning and sequencing the PCR products resulting from the amplification of cDNA using a common forward primer (F-Avr14) and reverse primers specific for each subunit (R-Avr14 A and B).

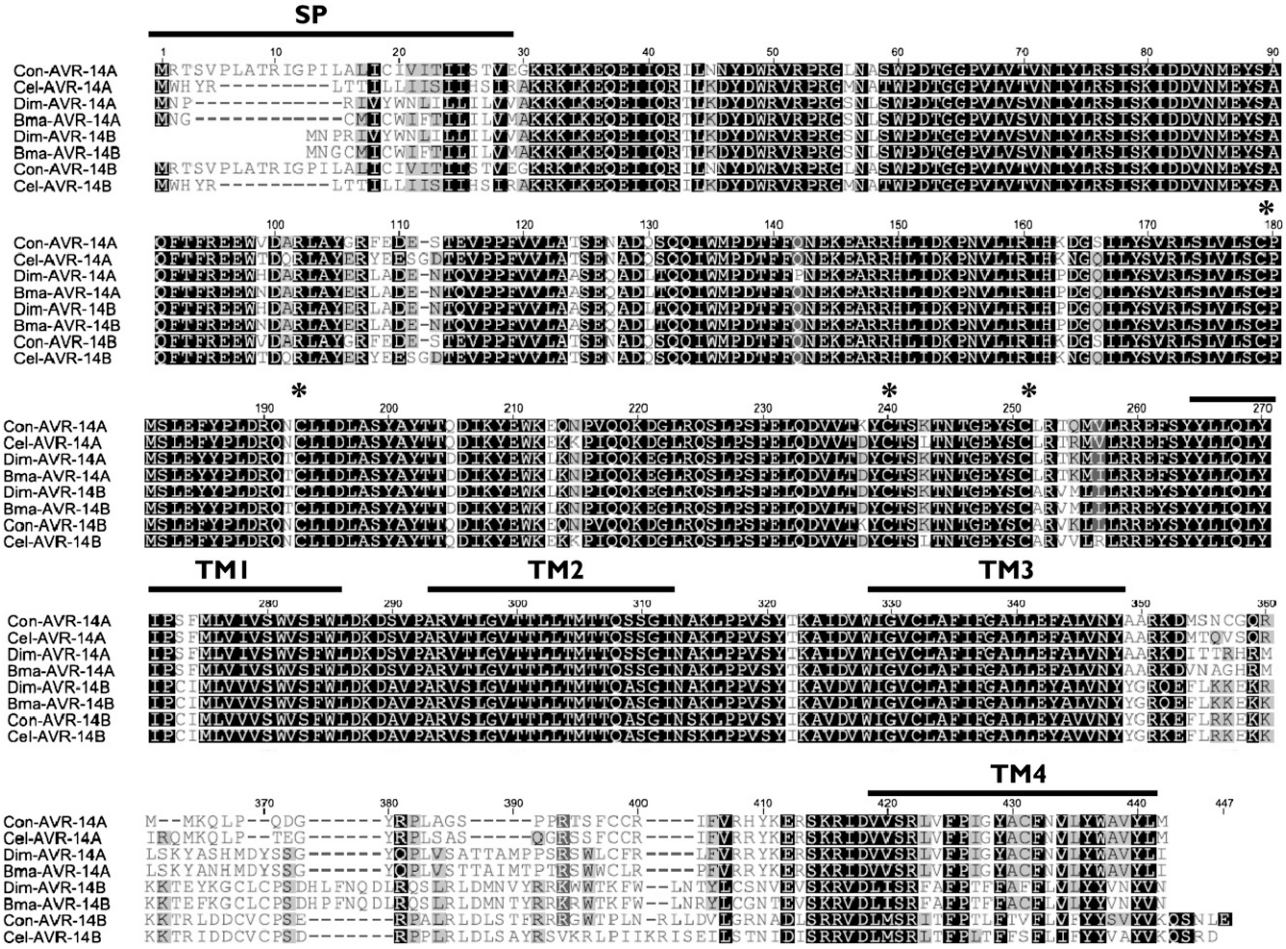

Fig. S2. ClustalX alignment of AVR-14 subunit amino acid sequences from *B. malayi* (Bma), *D. immitis* (Dim), *C. elegans* (Cel), and *Cooperia oncophora* (Con). Characteristic features of the ligand-gated ion channel superfamily were predicted for Bma-AVR-14 subunits, including the presence of a signal peptide (SP), two pairs of cysteine residues in the N-terminal region, one pair characteristic of ligand-gated ion channel (*), and a second pair diagnostic for glutamate-gated chloride channels and glycine receptors (+). Four transmembrane helices (TM1–4) are predicted in the C-terminal regions.

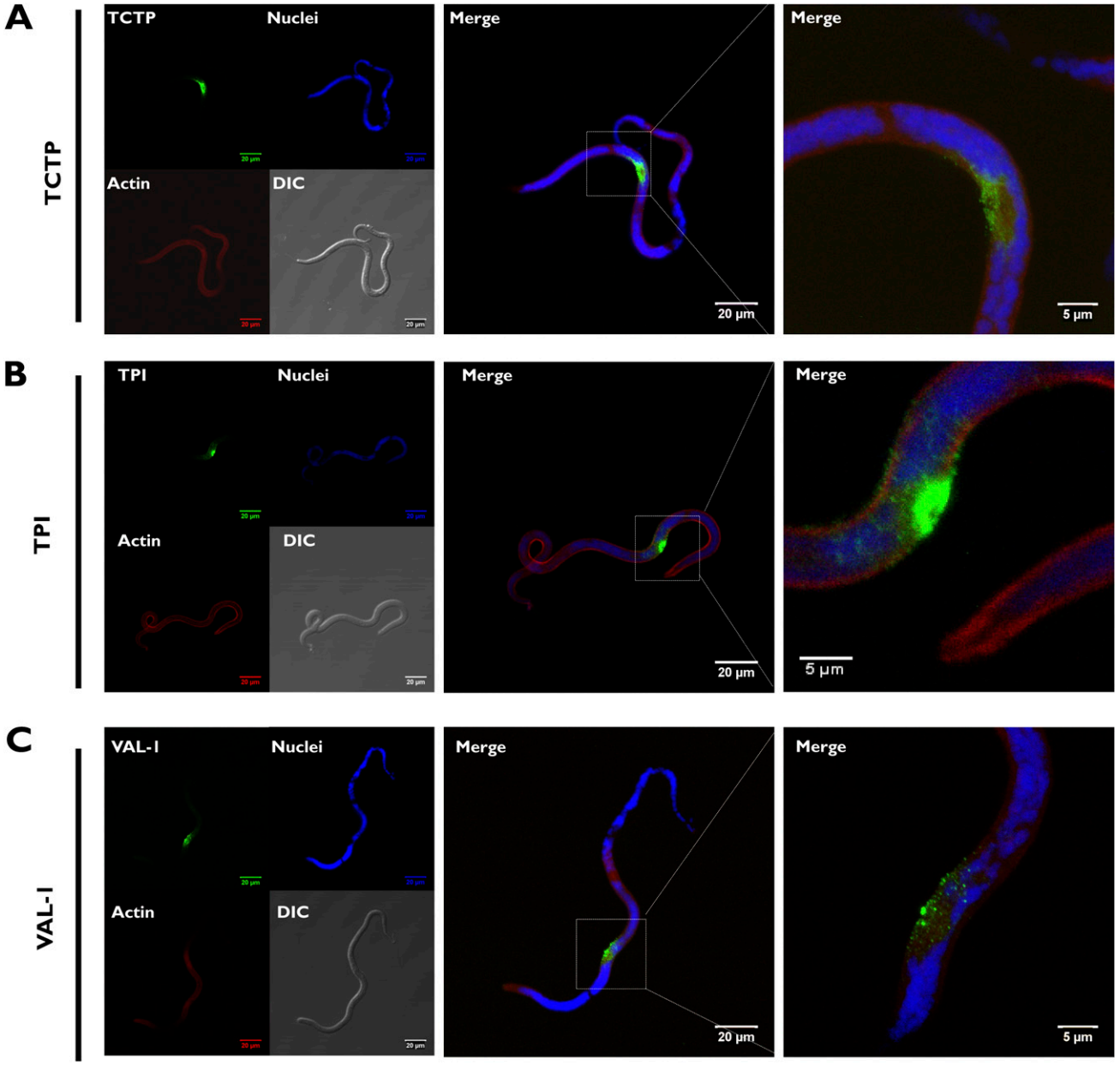

Fig. S3. Immunolocalization in the ES apparatus of *B. malayi* mf of three ES proteins. In addition to CPI-2, this localization pattern was also found with (*A*) TCTP, (*B*) TPI, and (*C*) VAL-1.

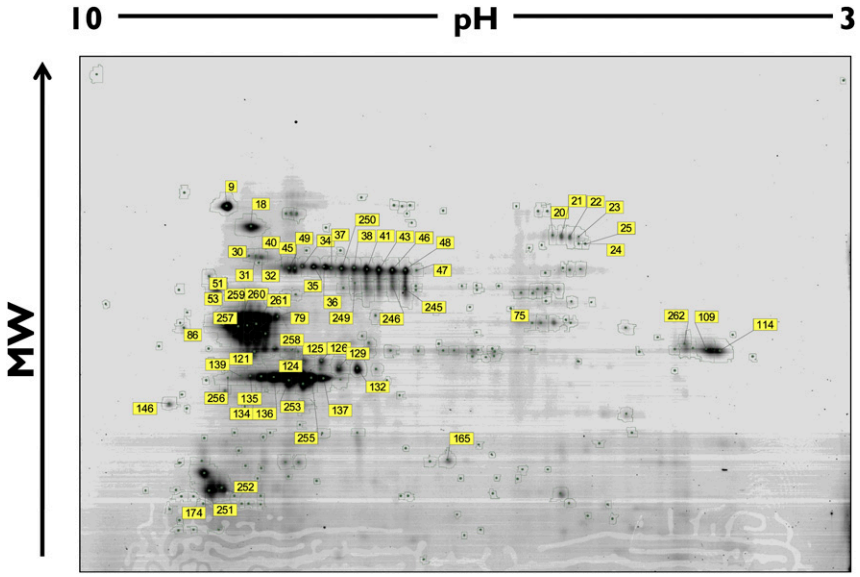

Fig. S4. Master gel (Cy2 label) showing the protein map obtained by difference gel electrophoresis analysis of excretory-secretory proteins from *B. malayi* microfilariae treated or untreated with 0.1 μM ivermectin. Labels represent the master numbers of the 61 spots analyzed.

Table S1. Primer sequences used for cloning the two splice variants of the *B. malayi avr-14* gene

Experiment	Primer name (source)	Primer sequence
3′ RACE for Bma-AVR-14B	3RACE adaptor (Bioline)	5′CCCTGTTCAAGCGCATCTGAGGTGAACCATGAACCGTG CTTTTTTTTTTTTTTTTT-3′
	Oar (Bioline)	5′CAGTCGGTCCTGCAGGGCATCTGAGGTGAACCATGA-3′
	Nar (Bioline)	5′CAGTCGGTCCTGCAGGGCATCTGAGGTGAACCATGA-3′
	3race-14B	5′-TGGTTAAATAGATACCTGTGCGG-3′
	3race-14BN	5′-GTTGACCTCATTTCACGATTTGC-3′
3′RACE for Bma-AVR-14A	R-14B-UTR	5′-GCGGCCGCATTCATTTACCGCAACAAAACAG-3′
	3race-14A	5′-ATACCCGAACTTTGTGTGGTCATCG-3′
5′ end of Bm-AVR-14A	SL1	5′-GGTTTAATTACCCAAGTTTGAG-3′
	5race-14A	5′-TTCCTCTCGAAACGTAAACTGTGCG-3′
Bma AVR-14 full-length amplification	R-AVR-14B	5′-TCAATTCACATAATTCACATAGTA-3′
	R-AVR-14A	5′-TTAAATGAGATAAACAGCCCA-3′
	F-AVR-14	5′-ATGAATGGTTGTATGATTTGTTGG-3′

Table S2. Paired *t*-test values for 61 spots analyzed by DiGE

Spot	Master no.	Status	Spot appearance (total number of maps)	Paired *t* test	Paired av ratio
1	75	Confirmed	9 (9)	0.18	1.25
2	32	Confirmed	9 (9)	0.43	1.09
3	23	Confirmed	9 (9)	0.5	1.25
4	86	Confirmed	9 (9)	0.51	1.12
5	256	Confirmed	9 (9)	0.51	1.31
6	51	Confirmed	9 (9)	0.53	1.33
7	139	Confirmed	9 (9)	0.55	1.34
8	252	Confirmed	9 (9)	0.59	1.29
9	121	Confirmed	9 (9)	0.6	−1.1
10	53	Confirmed	9 (9)	0.6	1.27
11	251	Confirmed	9 (9)	0.6	1.32
12	49	Confirmed	9 (9)	0.61	1.51
13	174	Confirmed	9 (9)	0.63	1.27
14	79	Confirmed	9 (9)	0.64	1.41
15	45	Confirmed	9 (9)	0.65	1.37
16	134	Confirmed	9 (9)	0.65	1.39
17	218	Confirmed	9 (9)	0.66	1.18
18	24	Confirmed	9 (9)	0.66	1.45
19	245	Confirmed	9 (9)	0.73	1.24
20	40	Confirmed	9 (9)	0.73	1.33
21	137	Confirmed	9 (9)	0.73	1.41
22	38	Confirmed	9 (9)	0.74	−1.03
23	255	Confirmed	9 (9)	0.74	1.31
24	129	Confirmed	9 (9)	0.74	1.36
25	261	Confirmed	9 (9)	0.74	1.39
26	35	Confirmed	9 (9)	0.75	1.21
27	253	Confirmed	9 (9)	0.77	1.3
28	258	Confirmed	9 (9)	0.77	1.4
29	22	Confirmed	9 (9)	0.79	1.18
30	34	Confirmed	9 (9)	0.79	1.21
31	246	Confirmed	9 (9)	0.8	1.07
32	21	Confirmed	9 (9)	0.81	1.13
33	132	Confirmed	9 (9)	0.81	1.47
34	114	Confirmed	9 (9)	0.82	1.37
35	249	Confirmed	9 (9)	0.83	1.07
36	260	Confirmed	9 (9)	0.83	1.25
37	257	Confirmed	9 (9)	0.83	1.32
38	31	Confirmed	9 (9)	0.83	1.33
39	259	Confirmed	9 (9)	0.83	1.35
40	250	Confirmed	9 (9)	0.84	1.03
41	41	Confirmed	9 (9)	0.84	1.04
42	25	Confirmed	9 (9)	0.84	1.12
43	47	Confirmed	9 (9)	0.85	1.24
44	109	Confirmed	9 (9)	0.86	1.3
45	43	Confirmed	9 (9)	0.87	1.06
46	46	Confirmed	9 (9)	0.87	1.07
47	37	Confirmed	9 (9)	0.88	1.02
48	36	Confirmed	9 (9)	0.88	1.09
49	125	Confirmed	9 (9)	0.88	1.27
50	135	Confirmed	9 (9)	0.89	1.12
51	30	Confirmed	9 (9)	0.89	1.14
52	124	Confirmed	9 (9)	0.91	1.14
53	126	Confirmed	9 (9)	0.92	1.25
54	48	Confirmed	9 (9)	0.94	1.15
55	18	Confirmed	9 (9)	0.94	1.16
56	136	Confirmed	9 (9)	0.95	1.15
57	165	Confirmed	9 (9)	0.97	1.08
58	9	Confirmed	9 (9)	0.97	1.18
59	262	Confirmed	9 (9)	0.98	1.36
60	146	Confirmed	9 (9)	0.99	1.02
61	20	Confirmed	9 (9)	0.99	1.06