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Characterization of a Novel Filarial Serine Protease Inhibitor, Ov-SPI-1, from Onchocerca volvulus, with Potential Multifunctional Roles during Development of the Parasite*

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A novel filarial serine protease inhibitor (SPI) from the human parasitic nematode Onchocerca volvulus, Ov-SPI-1, was identified through the analysis of a molting third-stage larvae expressed sequence tag dataset. Subsequent analysis of the expressed sequence tag datasets of O. volvulus and other filariae identified four other members of this family. These proteins are related to the low molecular weight SPIs originally isolated from Ascaris suum where they are believed to protect the parasite from host intestinal proteases. The two Ov-spi transcripts are up-regulated in the molting larvae and adult stages of the development of the parasite. Recombinant Ov-SPI-1 is an active inhibitor of serine proteases, specifically elastase, chymotrypsin, and cathepsin G. Immunolocalization of the Ov-SPI proteins demonstrates that the endogenous proteins are localized to the basal layer of the cuticle of third-stage, molting third-stage, and fourth-stage larvae, the body channels and multivesicular bodies of third-stage larvae and the processed material found between the two cuticles during molting. In O. volvulus adult worms the Ov-SPI proteins are localized to the sperm and to eggshells surrounding the developing embryos. RNA interference targeting the Ov-spi genes resulted in the specific knockdown of the transcript levels of both Ov-spi-1 and Ov-spi-2, a loss of native proteins, and a significant reduction in both molting and viability of third-stage larvae. We suggest the Ov-SPI proteins play a vital role in nematode molting by controlling the activity of an endogenous serine protease(s). The localization data in adults also indicate that these inhibitors may be involved in other processes such as embryogenesis and spermatogenesis.

The cuticle is an extracellular hydroskeleton that overlays the hypodermis of all nematodes. Most nematodes molt their cuticles four times during pre-adult development. Although being fairly inert and structurally robust, the cuticle is also permeable to small compounds and expands during growth periods between molts (1). A number of enzymes have been implicated in the shedding of old cuticles and the remodeling process that occurs as the new cuticle develops (2–8). Proteolytic enzymes have been shown to play a vital role in these processes, and inhibitor studies and rational cloning strategies have identified several nematode proteases whose functions are required for molting (9–11).

To identify novel filarial proteins involved in the molting process, we adopted a transcriptomics approach. Thousands of expressed sequence tags (ESTs)4 have been sequenced from cDNA libraries constructed from the infective third-stage larvae (L3) and molting L3 (mL3) of the human filarial nematode Onchocerca volvulus (12, 13). Analysis of these datasets identified novel cysteine proteases involved in the molting process (14, 15). Also identified in these analyses was an O. volvulus small molecular weight serine protease inhibitor (SPI) with similarities to other nematode SPI; Ascaris suum chymotrypsin/elastase inhibitors and hookworm factors VII and Xa inhibitors (12, 16, 17).

Protease inhibitors play a variety of important biological roles by controlling endogenous and exogenous proteolytic activities. In parasitic nematodes they have been implicated in the parasite’s survival within the host by inhibition of exogenous host proteases normally found in their preferred microenvironments (18–22), the inhibition of enzymes found in plasma or secreted from immune effector cells (17, 23–25), and the modulation of immune responses (26–29). Their endogenous roles have been less studied. It has been postulated that proteases involved in the molting process would be controlled by maturation (through removal of inhibitory pro-regions) or via endogenous protease inhibitors. In O. volvulus a cystatin-like cysteine protease inhibitor, onchocystatin or Ov-CPI-2, has been identified, which localized to the cuticle of molting nematodes and developing embryos (30). It has been suggested that it might play a role in modulating the activity of cysteine proteases required for the L3 to fourth-stage larvae (L4) molt or eggshell morphogenesis in embryos. We now present data indicating that the SPI identified in the molting EST dataset belongs to a family of proteins that play vital roles in the O. volvulus L3 to L4 molt and are

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4 The abbreviations used are: EST, expressed sequence tag; L2, second-stage larva; L3, third-stage larva; mL3, molting third-stage larva; L4, fourth-stage larva; Mf, microfilariae; AM, adult male; AF, adult female; Ov, O. volvulus; Bm, B. malayi; Di, D. immitis; Ls, L. sigmodontis; SPI, serine protease inhibitor; TIL, trypsin inhibitor-like; IC/E, inhibitor of chymotrypsin/elastase; BLAST, basic local alignment search tool; RNAI, RNA interference; dsRNA, double-stranded RNA; AMC, 7-amino-4-methylcoumarin; Suc, succinyl; Z, benzoyloxycarbonyl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; aa, amino acid(s).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) DQ013154, DQ013161, DQ011671, and DQ011672.

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suggested to be involved in other processes such as embryogenesis and spermatogenesis.

**EXPERIMENTAL PROCEDURES**

**Identification and Cloning of the Filarial Serine Protease Inhibitor Genes, Ov-spi-1, Ov-spi-2, Bm-spi-1, Di-spi-1, and Ls-spi-1—**Analysis of ESTs from an *O. volvulus* mL3 cDNA expression library (SL96MLW-OvmL3) identified a potential serine protease inhibitor (Ov-spi-1, *O. volvulus* clusters VOC00017 and VOC03875), which is highly expressed in the mL3 (12). Subsequent BLAST (31) searches of the *O. volvulus* EST dataset revealed a second closely related sequence, which has been designated Ov-spi-2. BLAST searches of other filarial EST datasets identified further members of this gene family in *Brugia malayi*, *Dirofilaria immitis*, and *Litomosoides sigmodontis*. Representative EST clones for Ov-spi-1 (cDNA clone SWOv3MAC0255K, GenBank™ accession AA294761), Ov-spi-2 (SWOv3MAC7385K, AA294327), Bm-spi-1 (kb65g12y1, CB338347; and SYWACAL06G12SK, AW874776), Di-spi-1 (ke26b11y1, BQ456080,) and Ls-spi-1 (Ls_AM1_09G07_T7, CD203523) were obtained from the Blaxter laboratory (University of Edinburgh, Edinburgh, UK), the Filarial Genome Project Network repository (Smith College, Northampton, MA), or the Genome Sequencing Center Washington University (St. Louis, MO). The plasmid inserts were fully sequenced on both strands using both vector-specific and gene-specific primers. The confirmed EST sequences have been deposited in GenBank™ (Ov-spi-1, DQ011671; Ov-spi-2, DQ011672; Bm-spi-1, DQ013154; Di-spi-1, DQ013156; and Ls-spi-1, DQ013157).

The genomic sequences of the filarial spi genes were obtained by PCR amplification from total genomic DNA from each species using Long Range Taq (Stratagene, La Jolla, CA) and gene-specific primers (Bm-spi-1.F1: 5′-CATCATATTGTTAAGTCTTGAAG-3′; Bm-spi-1.R2: 5′-CTCAGAAAACTTTATCCAGTATA-3′; Ls-spi-1.F1: 5′-ATCAGAAATCTACAGAATG-3′; Ls-spi-1.R1: 5′-ATTGAACTCTCAGATTTGAGTCA-3′; Di-spi-1.F1: 5′-TTAAAATCTAAGGAAAA-3′; Di-spi-1.R1: 5′-GCATATTTTATCTACGGTTTG-3′). PCR fragments were gel-purified (Qiagen kit, Qiagen Inc., Valencia, CA) and cloned into pCR4-TOPO vector (Invitrogen). For each fragment clones were selected and fully sequenced using vector- and gene-specific primers.

**Sequence and Phylogenetic Analysis of the Filarial SPI Dataset**—The five filarial SPI peptide sequences were compared with each other and the public datasets (NCBI GenBank™, pfam, SMART) using BLAST and ClustalX (www.sanger.ac.uk/Software/Pfam/ and smart.embl-heidelberg.de/) (31–34). The inhibitory domain identified within the filarial SPIs has been designated TIL (trypsin inhibitor-like) within pfam (PF01826). The previously identified members isolated from the filarial SPIs has been designated TIL (trypsin inhibitor-like) within Genes, Ov-spi-1, Ov-spi-2, Bm-spi-1, Di-spi-1, and Ls-spi-1. TIL sequences were designated as an outgroup because of their low sequence similarity to the other sequences and their lack of some of the conserved structural cysteine residues. T. suis is a member of the Dorylaimida family and only distantly related to the other (rhadophilid) nematodes considered (39).

**Homology Modeling of the Filarial Serine Protease Inhibitors—**X-ray crystal structure of the ASCSU_IC/E-1 inhibitor (AsC/E-1; accession P07851) bound to porcine elastase was retrieved from the Protein Data Bank (PDB; code 1EAI) (40). Homology models of the filarial SPIs, Ov-spi-1, Bm-spi-1, Di-spi-1, and Ls-spi-1 were built based on the x-ray crystal structure of ASCSU_IC/E-1 using the automated software Modeler (41) within Quanta 2000 (Accelrys, San Diego, CA) running on a Silicon Graphics Octane with a dual R12000 processor (sgi, Mountain View, CA). The “Refine 3” option in Modeler, which uses a conjugated gradient together with molecular dynamics by a simulated annealing technique, was used to optimize the models. Five models for each run were developed, and the model with the lowest Objective Function was selected. The resulting models were evaluated for their stereochemical properties by Procheck software (42) at 2.0 Å and by Quanta Protein Health programs within Quanta2000.

After initial modeling, each modeled inhibitor was superimposed on the x-ray crystal structure of the AsC/E-1 inhibitor, the latter was then removed, and the structure of the modeled inhibitor and elastase were merged. These steps were performed to investigate the interactions of the critical residues in the inhibitors with the elastase. The Quanta Protein Health Check revealed few close contacts of the modeled inhibitors with elastase, and high energy. Polar hydrogens were added to the model and optimized by 200 steps of the Steepest Descent method to remove the close contacts. The protein backbone was kept fixed during optimization. The structures of modeled inhibitors bound to elastase showed no further close contacts and low Charmm energies. These optimized structures were used for further analysis.

**Expression and Purification of the Recombinant Ov-spi-1 (rOv-SPI-1)—**The mature serine protease inhibitor domain of Ov-spi-1 (aa 15–118) was cloned in-frame into both the pProEX HTb (BamH1/HindIII sites) and the pRSET A (BamH1/EcoR1 sites) expression vectors (Invitrogen) and expressed as a fusion with an N-terminal polyhistidine tag. Overexpression of the rOv-SPI-1 fusion protein was as previously described with modifications (12). Briefly, expression was induced from an overnight culture of BL21 (DE3) pLyS.Escherichia coli (Sigma) with isopropyl 1-thio-galactopyranoside at a final concentration of 1 mM for 3 h at 37 °C. Recombinant protein was purified by affinity chromatography on a nickel column (ProBond resin, Invitrogen) according to the manufacturer’s instructions and dialyzed overnight at 4 °C against phosphate-buffered saline.

**Enzyme Inhibitory Activity of rOv-SPI-1—**The inhibitory activity of rOv-SPI-1 was tested against a panel of serine proteases. The enzymes...
Multiple Roles of Ov-SPI-1 in O. volvulus Development

Expression Profile of Ov-spi-1 and Ov-spi-2 throughout the O. volvulus Life Cycle—Primer sets specific for Ov-spi-1 (OvSPI1F; 5′-ACGTCG-TGGATGCGGATC-3′ and OvSPI1R2; 5′-GTTCGATTACGATCC-G-3′) and Ov-spi-2 (OvSPI1F; and OvSPI2R; 5′-GAGATCTCC-TGACATC-3′) were used to amplify fragments from O. volvulus cDNA libraries. The specificity of the primers for Ov-spi-1 and Ov-spi-2 was confirmed by PCR using plasmid DNA encoding the full-length cDNA sequences of Ov-spi-1 or Ov-spi-2. Each set of primers did not amplify the non-target spi transcript (data not shown). These include second-stage larvae (L2, SA98MLW-OvL2), infective third-stage larvae (L3, SA94WL-OvL3), molting third-stage larvae (L3, SL96-MLW-OvmL3), adult male (AM, SA98MLW-OvAM), adult female (AF, SA98MLW-OvAF), and microfilariae (MF, SA98MLW-OvMf). The cDNA libraries were constructed by the Filarial Genome Project. PCR with primers specific to Ov-spi-1 (OvSPI1F2; 5′-AATATGTCGACCGAGGATC-3′ and TubR1; 5′-CGAGTACCTCTCACGATTT-3′) was used as a constitutively expressed transcript qualitative control. The PCR conditions used were 95 °C for 3 min 1× cycle; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min 40× cycles; 72 °C for 10 min.

Polyclonal Sera Production and Immunolocalization of the Ov-SPI Proteins—Polyclonal sera raised against rOv-SPI-1 was produced in mice using the immunization protocol previously described (43). Briefly, 6- to 8-week-old male BALB/cByJ mice were injected subcutaneously with 25 μg of rOv-SPI-1 in 0.1 ml of phosphate-buffered saline with 0.1 ml of (Sigma). Mice were boosted 14 days later with 25 μg of rOv-SPI-1 in Freund’s complete adjuvant. Antibody specificity was screened by Western blot analysis against the rOv-SPI-1 and other recombinant proteins. It was specific to rOv-SPI-1 only (data not shown).

Stage-specific O. volvulus worms (L3, M3, adult female, and adult male) were isolated as described previously (30, 44). Worms were fixed for 30 min in 0.25% glutaraldehyde, 1% sucrose in 0.1 m phosphate buffer, pH 7.4, and then processed for immunoelectron microscopy as previously described (30, 45). For the immunolocalization of the native parasite proteins corresponding to Ov-SPI, thin sections (70 nm) of embedded worms were incubated with antibodies raised against rOv-SPI-1 (mouse anti-Ov-SPI-1), followed by incubation with rabbit anti-mouse IgG, and then incubation in a suspension of 15-nm gold particles coated with protein A (Amersham Biosciences). Pre-immune serum was used as the control.

Double-stranded RNA Preparation and RNA Interference—RNAi treatment of O. volvulus L3 was carried out using dsRNA as described by Lustigman et al. (46). The mature Ov-SPI-1 in pProEX HTb expression vector was used to amplify the cDNA corresponding to the region encoding mature Ov-SPI-1 (position 67–362), which also corresponds to bp 67–349 of the cDNA encoding Ov-SPI-2, using primers specific for Ov-spi-1 (OvSPI1F; as above and OvSPI1R; 5′-GAAATTTCAAGAGTCCATC-3′), and the PCR fragment was then subcloned into pCR4-TOPO vector (Invitrogen) to use as the template for RNA synthesis. The cDNA was amplified with M13 forward and M13 reverse primers (Invitrogen) and then used with either T3 and T7 RNA polymerase for the single-stranded sense or antisense RNA synthesis using the MEGAscript high yield transcription kit (Ambion Inc., Austin, TX). Large quantities of dsRNA were prepared as previously described (47). Soaking with dsRNA was performed on O. volvulus L3 as described (46), with modifications. Briefly, cryopreserved L3 were thawed and washed 5× with culture medium (1:1 NCTC 135 and Iscove’s modified Dulbeccco’s media, plus 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine) before their transfer to 96-well plates (5–15 larvae per well in 50 μl). Sterile dsRNA in culture medium was added in 50 μl at a final concentration of 0.5 mg/ml, and the larvae were cultured at 37 °C in a humidified 5% CO2 incubator. After 20 h, 50 μl of culture medium was removed and 1.5 × 10^6 normal human peripheral blood mononuclear cells in 100 μl of complete medium (culture medium plus heat-inactivated fetal calf serum, final 10%) were added to the culture as previously described (10). Uptake of dsRNA into the parasite was examined using fluorescent-labeled Cy3-dsRNA as previously described (46). The melting phenotype and morphology was observed under dissecting microscope every 24 h until the seventh day when the experiment was terminated. On day 7 larval viability was assessed visually after the uptake of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) and its reduction into the blue formazan derivative (48), as described previously (49). Briefly, after incubation for 18 h at 37 °C, 5% CO2, in 100 μl of 0.1% MTT, larval viability was scored as live when larvae stained blue uniformly along their entire length and dead when they remained unstained. Ov-spi dsRNA (targeting both Ov-spi-1 and Ov-spi-2 transcripts) was used as the test RNAi treatment, with negative controls being either culture medium containing RNA damage buffer or dsRNA of an unrelated Plasmidium falciparum gene Pf-eba-140 (position 2914 to 3396; accession number MAL13P1.60), and a positive control corresponding to the cathepsin L of O. volvulus, Ov-cpl (position 219–984, accession number U71150), which has been shown to inhibit molting of L3 (46). Each RNAi experiment was repeated at least twice. The average molting of the control group is considered 100% of molting in each experiment, and the relative reduced molting rate of each experimental group is calculated accordingly. The results shown are the mean percent inhibition of molting in each experimental group, and the standard deviation reflects the variation between experiments.

Loss of Ov-spi-1 and 2 Transcripts and Native Ov-SPI Proteins following RNAi—Loss of Ov-spi-1 and Ov-spi-2 transcripts following RNAi treatment were examined by quantitative real-time PCR, where Ov-spi dsRNA treatment was expected to affect transcript levels of both Ov-spi-1 and Ov-spi-2 due to their high degree of identity. Primers used were specific for either Ov-spi-1 (OvSPI1F2; 5′-AATGTGACCGAGGATC-3′ and TubR1; 5′-CGAGTACCTCTCACGATTT-3′) and OvSPI1R2; as above), which were designed outside...
FIGURE 1. A, a comparison of the filarial SPI sequences. The schematic shows a representation of the filarial SPI-1s. The boxes show a schematic representation of the three different regions: signal peptide (SP, yellow); mature inhibitory (TIL, blue) domain; and C-terminal region (white), found in the protein sequences. The length of the boxes is proportional to the length of the domains in the corresponding protein group. The conserved positions of introns within the filarial SPI-1 are shown with empty inverted triangles. The position of the first intron in Ls-spi-1 is shifted 3 bp forward relative to the other filarial SPIs. The positions of the ten conserved cysteines (C, red), and the disulfide bonds they are predicted to form are shown with connecting bars. The closed inverted triangle shows the position of the predicted inhibitory loop found between fifth and sixth cysteines. The open circle with a bar indicates the position of the 10-bp deletion in Ov-spi-2, which causes a shift in-frame and thus truncates the predicted SPI-2 protein by removing 11 aa of the C-terminal region. The hexagons show the two potential N-glycosylation sites found in Ov-spi-1 with the second site also being conserved in Bm-spi-1.

B, SPI Reactive Site

C, Bayesian phylogenetic analysis of nematode SPI domains. The tree shown in this figure is a consensus built from 9,750 trees saved.
Multiple Roles of Ov-SPI-1 in O. volvulus Development

The fully sequenced genomic fragments containing the predicted exons of Ov-spi-1, Ov-spi-2, Bm-spi-1, Di-spi-1, and Ls-spi-1 have been deposited in GenBank™ (accession numbers: DQ013160, DQ013161, DQ013155, DQ013159, and DQ013158, respectively). Analysis of the fragments establishes that Ov-spi-1 and Ov-spi-2 are not splice variants, because the deletion does not occur at either of the verified intron splicing sites. Although the sequence of the second intron of Ov-spi-1 and Ov-spi-2 show a high level of sequence conservation, four single base changes and a 2-bp insertion were found in Ov-spi-2 relative to Ov-spi-1 genomic sequence. In addition, the 3′-untranslated region of Ov-spi-2 has four base changes, two single base insertions, and a single 16-bp insertion relative to Ov-spi-1. The two intron positions in the two Ov-spi sequences are also conserved in Bm-spi-1 and Di-spi-1, but not in Ls-spi-1 (Fig. 1A).

Searches of the GenBank™ non-redundant and EST datasets revealed that TIL-like domains could be found in predicted proteins from the majority of nematode EST datasets as well as some arthropod and vertebrate. Within the nematode sequences the filarial SPIs showed the highest sequence similarity to the A. suum chymotrypsin and elastase inhibitors (IC/E) and the Anisakis simplex serine protease inhibitors (41–68% sequence similarity). Comparisons of the filarial SPI sequences to the predicted proteins of the C. elegans genome showed that Ov-spi-1 and -2, Bm-spi-1, and Di-spi-1 showed the highest similarity to R10H1.4 (47–59%), whereas Ls-spi-1 was most similar to C25E10.10 (52%).

The reactive site of SPIs is designated as P1-P2-P3/P1′-P1′-P′-P′-P′ (50). Fig. 1B shows an alignment of the reactive site loops of the filarial SPIs (Ov-spi-1 and -2 amino acid sequence, CALRC; Bm-spi-1, CFLIC; Ls-spi-1, CYKK; and Di-spi-1, CTLIC) and other nematode SPIs with characterized protease specificities (ascard, hookworm, and trichurid SPIs: ASCSU_IC/E-1, CPLMC; ASCSU_IC/E2–5, CALMC; ASCSU_ATI, CTREC; ANSSI_SPI-1, CPAMC; ANSSI_SPI-2/3, CATIC; ANCCA_AP2c and ANCCA_AP3, CLVRIC; ANCCA_AP4, CLRLC; ANCCA_AP5, CRSRCG; ANCCA_AP6, CFSFSC; TRISU_TCI1, CTRQC; and TRISU_CE11, CTMOCQ) (51, 52). Two hookworm and ten C. elegans SPIs with unknown protease specificities have also been included in the alignment. The sequences have been grouped according to the characteristics of the amino acid residues found in the P1′/P′ positions, and their potential target enzymes were deduced from the sequences found in the characterized inhibitors. In three of the filarial inhibitors and the Ascaris IC/E inhibitors the amino acid leucine occupies the critical P1 position. The P1′ site displays more variation with allphatic amino acids in Ascard SPIs and two of the filarial SPIs (methionine in the Ascaris C/E inhibitors and Anisakis SPI-1, isoleucine in Anisakis SPI-2/3, Bm-spi-1, and Di-spi-1), and charged residues in the filarial Ls-spi-1, and Ov-spi-1 and -2 (lysine or arginine). The reactive site sequence of the A. suum trypsin inhibitor (ATI) differs with the charged amino acids arginine and glutamic acid occupying the P1 and P1′ reactive sites, respectively (Fig. 1B) (53, 54). Based on the composition of their reactive sites we speculated that the Onchocerca SPI-1 and -2 target a chymotrypsin/elastase-like protease. Using these criteria we also predict the majority of the C. elegans inhibitors included in our study (eight out of ten) will have similar target protease preferences. The remaining C. elegans inhibitors CAEE_L10H1_4 and CAEE_F23D8_3 have arginine or lysine in the P1 position indicating that they may inhibit trypsin-like proteases.

during Bayesian analysis of the SPI protein alignment, rooted using the two T. suis sequences. Posterior probability values for each node >0.65 are shown in black. The bootstrap values (>65) of a distance analysis (neighbor joining; 10,000 replicates) of the same alignment are shown in red. The name includes a five-letter species identifier and the SPI gene name. Based on the results of the phylogenetic analysis a subset of the TIL domains have been divided into three groups: Ia, C. elegans T0666.10 and hookworm TILs; Ib, seven C. elegans TILs; and II, C. elegans R10H1.4, ascard, and filarial TILs.
Multiple Roles of Ov-SPI-1 in O. volvulus Development

Phylogenetic Analysis of the Nematode Serine Protease Inhibitor Protein Sequences—Phylogenetic analysis of the nematode TIL domains from C. elegans, ascarid, and filarial SPIs, using the Bayesian and neighbor joining analysis, indicates that the filarial SPI sequences are part of a large clade, which includes the previously isolated ascarid TILs from A. suum and A. simplex, as well as the C. elegans open reading frame R10H1.4 (Fig. 1C). All nodes yielding posterior probability value of >0.65 or bootstrap values of >65 are indicated. Three supported groups have been designated Ia, Ib, and II. Group I contains most of the SPI sequences derived from Clade V nematodes (39). Group Ia consists of the hookworm factors Xa and VIIa inhibitors and C. elegans T06E06.10, whereas the poorly supported group Ib (probability value of 0.65) consists of seven C. elegans sequences. Group II consists of the C. elegans R10H1.4, ascarid, anisakid, and filarial SPIs. The A. simplex and most of the filarial SPIs form their own subgroups. The overall low sequence similarity (33.8–40.9%) of Ls-SPI-1 to the other filarial SPIs, its unique intronic splice sites, and its exclusion from the filarial clade indicate that it may not be part of the same TIL subgroup as the other filarial sequences.

Homology-based Modeling of Ov-SPI-1 and Bm-SPI-1—The homology-based modeling of Ov-SPI-1, Bm-SPI-1, and Di-SPI-1 when merged with the x-ray crystal structure of porcine elastase (PDB code 1EAI), showed mutual penetration characteristics as observed in the A. suum chymotrypsin/elastase inhibitor bound crystal structure. The P1 residue, Leu-31, of the inhibitor penetrates into the S1 substrate specificity pocket, and R217A of elastase penetrates through a pore formed by the A. suum IC/E-1 inhibitor (40). The later penetration is a unique feature and has not been reported for any other protein inhibitor-protease complex. Although the residues in the P1 site vary in all three inhibitors studied here, one of the key residues, Leu-31 (P1), reported as indicative of the inhibitory activity of rOv-SPI-1 was confirmed using a reverse zymography technique, where the inhibitory activity of rOv-SPI-1 was detected as a single band with an apparent molecular mass of 16 kDa (including 4 kDa for the histidine tag extension) on stained substrate gel slices, that was resistant to both elastase and chymotrypsin, but not trypsin activity (data not shown).

Profile of Ov-spi-1 and Ov-spi-2 Expression throughout the Parasites Life Cycle—Primers were designed to specifically amplify either Ov-spi-1 or Ov-spi-2 from different developmental stages of O. volvulus. PCR on O. volvulus stage-specific cDNA libraries indicated that the transcripts of Ov-spi-1 are present in all life-cycle stages from microfilariae to adults, with expression increasing during molting of L3 (ml3) and in adult stages (Fig. 3). Ov-spi-2, however, was only found to be transcribed in L2 and the post-human-infection parasite stages (ml3 to adult) (Fig. 3). Previous analysis of the O. volvulus EST databases (13, 55) indicates that Ov-spi-1 and Ov-spi-2 may be up-regulated in ml3 (clusters OVC00017, OVC03875, and OVC08442; 41 ESTs, 33 of which are derived from the ml3 dataset), and they represent the third most abundant transcript in the ml3 dataset (12). Because of the subtle differences between the Ov-spi-1 and Ov-spi-2 transcripts the EST clustering algorithms have not successfully differentiated between them. However, by specifically searching the O. volvulus EST databases we identified 21 ESTs encoding Ov-spi-1, 11 ESTs encoding Ov-spi-2, and 9 that could not be assigned to either gene because of lack of sequence covering the 10-bp deletion site. In both cases the majority of ESTs were isolated from the ml3 dataset indicating that both are highly expressed at that stage.

Immunolocalization of Native Ov-SPI Proteins in O. volvulus—Specific antibodies raised against rOv-SPI-1 (anti-Ov-SPI-1), which has 99% identity to Ov-SPI-2 over their first 104 amino acids, were used to detect their corresponding native proteins in the parasite. The endogenous protein corresponding to Ov-SPI-1 and -2 was localized by immunoelectron microscopy on sections taken from different life-cycle stages of O. volvulus. In L3, Ov-SPI-1 and -2 were present within the body channels (Fig. 4, A and B), within the multivesicular bodies (Fig. 4B), and in the basal layer of the cuticle (Fig. 5B). Intense labeling was also seen in the basal layer of the cuticle of ml3 (Fig. 4, C and D). After separation of the L3 and L4 cuticles, the Ov-SPI-1 and -2 proteins were present in the basal layer of both the old L3 cuticle and the new L4 cuticle (Fig. 4D). Interestingly, intense labeling was also observed in the processed mate-
which was statistically significant (Mann-Whitney test, U = 19–33% molting in the normal culture media control groups, dsRNA-treated larvae exhibited less molting compared to the control group.

Transcripts and Native Proteins

Molting and Viability Due to a Specific Depletion of Ov-spi-1 and -2

Analysis of specific gene transcript levels following RNAi treatment showed that both Ov-spi-1 and Ov-spi-2 transcripts in Ov-spi dsRNA-treated L3 were severely diminished, ~200-fold, when compared with RNAi treatment with the negative control dsRNA (Pf-eba-140), or dsRNA corresponding to the O. volvulus cathepsin L (Ov-cpl) (Fig. 5C). Transcript levels in the medium control were comparable to those in the negative control dsRNA (data not shown). Immunoelectron microscopy demonstrated that the native Ov-SPI-1/SP-2 proteins were highly reduced following Ov-spi dsRNA treatment (Fig. 5D). In normal control molting larvae Ov-SPI-1/SP-2 was localized to the cuticles of both L3 and L4 (Fig. 5D, panels a and b), whereas in Ov-spi RNAi-treated L3 the staining of Ov-SPI-1/SP-2 was almost absent (Fig. 5D, panel c). Notably, larvae that did not successfully molt after dsRNA treatment exhibited incomplete separation between the L3 and L4 cuticles (Fig. 5D, panel c). Interestingly, in control larvae intense labeling was also observed in the processed material, which appears between the L3 and L4 cuticles during molting (Fig. 5D, panel b). The labeling of Ov-SPI-1/SP-2 in L3 and mL3 confirmed our previous localization of the endogenous protein (Fig. 4), however, in addition the preservation of material being degraded between the cuticles during molting shows that the endogenous inhibitors are present during the degradation of cuticular material during molting. The localization of O. volvulus cathepsin L- and Z-like proteases, known to have an essential function during molting, were not affected by the Ov-spi dsRNA treatment (Fig. 5D, panels d and e).

DISCUSSION

Parasite-derived protease inhibitors have been shown to play a variety of roles in the survival of the parasite by inhibition of exogenous host proteases (18, 20, 29). They are also believed to control endogenous proteases involved in the development and reproduction of the parasite (30). Here we describe the identification and characterization of a novel family of low molecular weight serine protease inhibitors (SPIs), Ov-SPI-1 and Ov-SPI-2, from the human parasitic nematode O. volvulus.
Subsequent searching of other filarial EST datasets allowed us to identify three other members of this family from *B. malayi*, *D. immitis*, and *L. sigmodontis* (*Bm*-SPI-1, *Di*-SPI-1, and *Ls*-SPI-1, respectively). This group of low molecular weight serine protease inhibitors was first identified in *A. suum* and *A. lumbricoides* (16, 18, 51, 54) where they are believed to protect the nematode from intestinal serine proteases. Members of the family have been identified in other parasitic nematodes, including the ascaridid *A. simplex* (20, 52, 56), the strongyloid hookworms *A. caninum* and *A. ceylanicum* (17, 23, 25), and the whipworm *T. suis* (21, 57). These SPIs are also believed to be involved in controlling exogenous host proteases in these nematodes. The inhibitory portions of these proteins have been designated TIL domains (trypsin inhibitor-like) within Pfam. The completed *C. elegans* genome contains nineteen different genes with TIL domains (36), none of which have been purposely characterized functionally in regard to their specific roles during development. Regardless, they presumably play a variety of roles in the biology of this free-living nematode. There were no reported RNAi phenotypes associated with any of these genes in the recently published genome-wide screens (www.wormbase.org). Members of this family have been identified in arthropods such as the honeybee *Apis mellifera* (58, 59), *Drosophila melanogaster* (60), gall wasps (61), and scorpions (62). Their functions in arthropods have not yet been fully elucidated, although they are reported to be involved in determining lifespan or are components of insect venoms. TIL domain proteins have also been isolated from frogs, where they are believed to exert anti-microbial properties (63, 64). In mammals TILs have been identified in a variety of proteins such as von Willebrand factor or zonadhesin, which have roles in blood clotting and sperm gamete recognition, or adhesion (65, 66). The mammalian TILs have always been found in the context of large multidomain proteins (see entry for PF01826). This

![FIGURE 5. RNAi treatment of *O. volvulus* L3 with Ov-spi dsRNA results in an inhibition of molting and viability due to Ov-spi-1 and -2 gene specific knock-down. *O. volvulus* L3 were soaked in dsRNA at a final concentration of 0.5 mg/ml as described under “Experimental Procedures.” Larvae were cultured in the presence of dsRNA for 20 h at 37 °C in humidified 5% CO2, after which 1.5 × 10^6 normal human peripheral blood mononuclear cells were added and L3 were cultured for an additional 6 days. A, Ov-spi RNAi led to a significant inhibition of molting when compared with the Pf-eba-140 dsRNA control group. The data are presented as percent inhibition relative to the normal molting control group, where the average of molting is considered 100% of molting, and the relative reduced molting rate of the two experimental groups is calculated accordingly. B, Ov-spi dsRNA-treated L3, which had not molted by day 7 showed significantly reduced viability. C, quantitative real-time PCR was carried out on total RNA isolated from ~200 control or RNAi-treated *O. volvulus* L3 after 20-h incubation with medium alone or dsRNA. PCR primers were specific to Ov-spi-1, Ov-spi-2, or to the control gene Ov-calponin. Ov-spi expression was normalized to Ov-calponin expression and normalized expression in the control group was set to 1.0. Both Ov-spi-1 and Ov-spi-2 RNA were depleted in Ov-spi dsRNA-treated L3s compared with Pf-eba-140 or Ov-cpl dsRNA-treated L3s. D, immunoelectron microscopy was performed on control (a and b) and Ov-spi RNAi-treated (c) L3 collected on day 5 as described under “Experimental Procedures.” The native Ov-SPI-1/SPI-2 proteins were localized to the new L4 cuticle, in-between the old and new cuticles in the control larvae (a and b), but their expression was almost completely reduced in the Ov-spi RNAi-treated larvae (c). The presence and localization of Ov-CPZ-1 (d) and Ov-CPL-1 (e) in the Ov-spi RNAi-treated L3s was similar as in normal mL3. The regions where the cuticles of L4 and L3 separate are marked by an arrowhead and an arrow, respectively. Each bar represents 500 nm.](http://www.jbc.org/content/280/49/40852/fig/5)
Multiple Roles of Ov-SPI-1 in *O. volvulus* Development

The endogenous enzyme could be a potential target of Ov-SPI-1 and/or Ov-SPI-2. In *C. elegans*, blisterase is encoded by the bli-4 gene, which gives rise to at least nine protein isoforms by alternative splicing (72), and these enzymes are involved in the processing of hormones and structural components required for molting and the genesis of the new cuticle (72, 73). More recently it has been shown that bli-4 is essential for molting in *C. elegans* using RNAi (77). Moreover, elastase-like serine protease activity has been detected in the secretions of *Onchocerca lienalis* L3s (78). Interestingly, serine proteases have been shown to be involved in the control of molting and larval development in insects (79–84). In addition, Kunitz-type serine protease inhibitors are constituents of the molting fluid in *D. melanogaster* where they are believed to control enzymes involved in tissue remodeling (85). Recently, using genome wide RNAi screening, it has been shown that serine protease inhibitors containing the Kunitz/bovine pancreatic trypsin inhibitor domains are also essential for molting in *C. elegans* (77). We suggest that the Ov-SPIs might act in a similar manner regulating the activity of a serine protease(s) involved in nematode molting. In addition to their potential role in molting, it is possible that the Ov-SPIs are secreted onto the surface of the nematodes cuticle or into the immediate vicinity to prevent autodigestion of its cuticle by its own secretions.

We directly addressed the function of the Ov-SPIs in molting by RNAi targeting of the Ov-spi-1 and Ov-spi-2 transcripts. When compared with the untreated control or the Pf-eba-140 control (dsRNA treatment control) the Ov-spi RNAi treatment of L3s significantly inhibited molting. The treated L3s failed to shed their cuticles, displayed aberrant morphology, and reduced viability. These phenotypic differences were correlated with a loss of both Ov-spi-1 and Ov-spi-2 transcripts, and a specific reduction in native Ov-spi-1/SPI-2 proteins. As described previously by Lustigman et al. (46) we noted some nonspecific toxic effect of unrelated dsRNA on molting. It is unclear what the mechanism for this nonspecific effect is, however, reverse transcription-PCR of the targeted transcripts does not indicate an indirect knockdown in their transcription. Also the protein expression of two previously characterized cysteine proteases involved in molting (Ov-CPL-1 and Ov-CPZ-1) (46) was unaffected. Off-target effects have been reported to occur in RNAi of mammalian cells (86, 87), and it possible that the AT-rich *P. falciparum* gene fragment is acting on other AT-rich transcripts found in *O. volvulus* (88). Whereas we have not specifically explored the mechanism of Ov-spi RNAi lethality, it is probable that it results from the unsuccessful emergence of the L4 from the old L3 cuticle.

An interesting finding in this study was the overall similarity between the RNAi phenotypes of Ov-spi, Ov-cpl-1, and Ov-cpz-1 (46) in molting L3s. All display a similar failure in the separation of L3 and L4 cuticles. The knockdown of Ov-spi does not affect the transcription of the other two genes or the localization of their native proteins. It seems likely the Ov-SPIs regulate molting via inhibition of their unidentified endogenous target enzyme, and this activity appears to be independent of the presence of the cysteine proteases Ov-CPL-1 and Ov-CPZ-1 in the same locations. It is possible that these proteins act in independent pathways that subsequently converge when the cuticles begin separation. Alternatively, the Ov-SPIs could be involved in controlling the maturation of these cysteine proteases by a serine protease and while they are successfully targeted to the cuticle interfaces in its absence the cysteine proteases may not be fully active and thus molting is inhibited.

The expression of the Ov-spi transcripts in the other lifecycle stages of *O. volvulus* suggests they have functions outside of molting. The native proteins were also immunolocalized to the eggshells surrounding developing embryos within the uterus of adult female worms, and to

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5 Y. Oksov, unpublished observation.
6 S. Lustigman and C. Poole, unpublished data.
sperm within the testis of adult male worms suggesting a role in embryogenesis, microfilarial development, and spermatogenesis. \textit{Bm-spi-1} is also expressed in a variety of lifecycle stages suggesting that these inhibitors may have similar multifunctional roles in the other filariae. The \textit{A. suum} chymotrypsin/elastase inhibitors have been localized to the eggs, egg-shell-derived sheath of the L2 stages, and sperm, either through specific antibodies or treatment with fluorescently labeled proteases (89, 90). However, these studies did not address whether or not the \textit{A. suum} inhibitors were also inhibiting endogenous nematode proteases participating in the tissue remodeling processes taking place in the developing embryos. Although we do not know what functions the \textit{Ov}-SPIs have in nematode sperm, another TIL domain-containing protein has been identified as a component of the seminal fluid of \textit{D. melanogaster} (60) suggesting TILs might have conserved functions in sperm biology. Serine proteases are known to be involved in embryonic development (91) in a variety of organisms. In \textit{C. elegans}, blisters have been shown to be involved in early development of the nematodes as well as in cuticle formation (reviewed in Refs. 72–74). It is possible that there are targets of the \textit{Ov}-SPIs in the adults as well as the molting larvae.

Moreover, it is possible that, like other previously characterized nematode protease inhibitors, such as the cystatin family (29, 30) and the serpin family of serine protease inhibitors (27, 28, 92), the \textit{Ov}-SPIs have exogenous as well as endogenous functions during the lifecycle of the parasite. This could involve the control of both host and parasite ecto-proteases. Filarial cystatins have been shown to have immunomodulatory properties either through direct effects on immune cell activation or by blocking antigen processing and presentation on class II major histocompatibility complex (29, 93–98). Nippocystatin, a cysteine protease inhibitor from \textit{Nippostrongylus brasiliensis}, was also shown to inhibit antigen processing and modulate antigen-specific immune responses to the parasite (99). Serine protease inhibitors belonging to the serpin family, such as viral serpins (100 –102), have also been implicated in immunomodulation. A filarial serpin identified in \textit{B. malayi} (26) acts as a major T cell antigen (103), whereas a serpin from the intestinal nematode \textit{Trichosstrongylus vitrinus} inhibits host serine proteases (104). We are currently exploring the immunomodulatory properties of the \textit{Ov}-SPIs and their ability to specifically inhibit proteases released from activated neutrophils and monocytes, such as elastase and cathepsin G (reviewed in Refs. 105 and 106), which may be involved in the control of inflammation (107, 108). Neutrophils have been found to be important effector cells in the killing of \textit{O. volvulus} larvae (109, 110). They are also involved in the pathology associated with nodule formation around adult \textit{Onchocerca} worms (111), microfilaria-mediated ocular pathology (112, 113), and are involved in the immune responses generated after anti-filarial treatment (114, 115). Control of proteases released from these cells would be vital for establishment and long term survival of the parasites within the host.

Indirect evidence of the involvement of the \textit{Ov}-SPIs in immune regulation is that \textit{Ov}-SPIs are antigenic and highly recognized by individuals exposed to \textit{O. volvulus} indicating exposure to the inhibitor,7 and thus suggesting they are released from the parasite during the early stages of parasite establishment in the host. Interestingly, antibody responses to \textit{Ov}-SPIs are higher in putatively immune individuals when compared with age-matched infected individuals, and the responses are up-regulated with age in chronically infected individuals7 suggesting a role in protective immunity. \textit{Ov}-SPI-1 is one of the vaccine candidates against \textit{O. volvulus}.

\footnotesize{7 L. Ford and S. Lustigman, unpublished data.}

In conclusion, we have identified a novel filarial serine protease inhibitor family with potential multifunctional roles in the development of the parasite, including molting, establishment in the final host, embryogenesis, and reproduction. Future work will focus on identifying the one or more potential target enzymes for the filarial serine protease inhibitors, including both endogenous parasite and exogenous host serine proteases. Identification of their target proteases may be important in the identification of potential targets for drug and vaccine development as well as providing further information on the biology of molting and adult reproductive processes in nematodes.

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Multiple Roles of Ov-SPI-1 in O. volvulus Development

Characterization of a Novel Filarial Serine Protease Inhibitor, *Ov*-SPI-1, from *Onchocerca volvulus*, with Potential Multifunctional Roles during Development of the Parasite

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