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Cloning and Characterization of *Ancylostoma*-secreted Protein

A NOVEL PROTEIN ASSOCIATED WITH THE TRANSITION TO PARASITISM BY INFECTIVE HOOKWORM LARVAE*

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The developmentally arrested third stage infective larva of hookworms resumes development upon entry into the definitive host. This transition to parasitism can be modeled *in vitro* by stimulating infective larvae with a low molecular weight ultrafiltrate of host serum together with methylated glutathione analogues. When stimulated to resume development *in vitro*, activated larvae of the hookworm *Ancylostoma caninum* released a 42-kDa protein, termed *Ancylostoma*-secreted protein (ASP). ASP was the major protein released by activated hookworm larvae. Degenerate oligonucleotide primers, based on a partial internal amino acid sequence of the protein, were used together with flanking vector sequence primers to amplify a fragment from a third stage larval cDNA library by polymerase chain reaction. The fragment was used as a probe to isolate a longer clone from the larval cDNA library. The full-length ASP cDNA was found to encode a 424-amino acid protein with homology to the antigen 5/antigen 3 family of proteins from hymenopteran venoms and a family of cysteine-rich secretory proteins. ASP was expressed in bacterial cells, and a polyclonal antiserum against purified recombinant ASP was produced. The antiserum, which was demonstrated to be specific for ASP, was used as a probe to measure the kinetics of ASP release by hookworm larvae. ASP is released within 30 min of stimulation, with the majority released by 4 h. Low levels of ASP were released continuously following activation, but only if the stimuli were present in the incubation medium. The compound 4,7-phenanthroline, previously shown to inhibit larval activation, also inhibited release of ASP. The specific, rapid release of ASP by activated infective larvae suggests that this molecule occupies a critical and central role in the transition from the external environment to parasitism.

The early events of the hookworm infectious process are poorly understood, especially at the molecular and biochemical level. A better understanding of the molecules released by invading larvae, and the host's responses to them, would allow for the rational design of immuno- and chemotherapeutic intervention strategies. However, the inability to culture hookworms beyond the third-stage larvae (L_3)[1] *in vitro*, together with the difficulties associated with the isolation of sufficient parasitic stages for molecular and biochemical studies, has, until recently, hampered investigations of the critical first steps in the establishment of the parasitic relationship.

The initial events of the infectious process can now be modeled *in vitro*, using the resumption of feeding as a marker for the transition from the free-living L_3 to the developing parasitic L_3 (1–3). When free-living L_3 of the canine hookworm *Ancylostoma caninum* are stimulated to resume feeding *in vitro*, they release several molecules into the culture medium. Recently, a zinc metalloprotease activity has been reported from activated *A. caninum* L_3 ES products (4). Here we report the isolation, cloning, and expression of a 40-kDa protein from activated ES products, referred to as *Ancylostoma*-secreted protein (ASP), that is homologous to the major venom allergen of hymenopteran insects. ASP is the major protein released by activated hookworm L_3. Its release during invasion of the host, together with its partial homology to previously described allergens from insect venoms, suggest that it may orchestrate key events in the patho- and immunobiology of hookworm infection.

MATERIALS AND METHODS

Larval Activation—*A. caninum* was maintained as described previously (3). L_3 were collected from 1–4 wk-old coprocultures by the Baermann technique, and decontaminated with 1% HCl in BU buffer (50 mM Na_2HPO_4, 22 mM KH_2PO_4, 70 mM NaCl, pH 6.8) (5) for 30 min at 22 °C. Approximately 3500–7500 L_3 were incubated in individual wells of a 24-well tissue culture plate containing 0.5 ml of RPMI 1640 tissue culture medium, supplemented with 0.25 mM HEPES, pH 7.2, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, and 2.5 μg/ml amphotericin B. The L_3 were activated to resume development and feeding by including 15% (v/v) of a <10-kDa ultrafiltrate of canine serum and 25 mM *S*-methylglutathione (4). Nonactivated L_3 were incubated in RPMI alone (*i.e.* without the stimulus). The L_3 were incubated at 37 °C, 5% CO_2 for 24 h. The percentage of feeding L_3 was determined by incubating aliquots of L_3 with 2.5 mg/ml fluorescein isothiocyanate-labeled bovine serum albumin for 2–3 h, followed by counting the number of L_3 that had ingested the labeled bovine serum albumin by microscopic examination under epifluorescent illumination (6).

Collection of ES Products—Medium containing activated larvae was transferred to individual microcentrifuge tubes and centrifuged for 5

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U26187.

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[1] The abbreviations used are: L_3, third-stage larvae; ASP, *Ancylostoma*-secreted protein; rASP, recombinant ASP; bp, base pair(s); kbp, kilobase pair(s); SSC, salt/sodium citrate; pd, pixel density; SSPE, salt/sodium phosphate/EDTA; PAGE, polyacrylamide gel electrophoresis; SL, spliced leader; ES, excretory/secretory; ORF, open reading frame; Vesq Ag, *V. squamosa* antigen 5; IL, interleukin; GSP, gene-specific primer; CRISP, cysteine-rich secretory protein; RACE, rapid amplification of cDNA ends; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; NFDM-P, non-fat dried milk in phosphate-buffered saline.

TABLE I
Primers used for cloning and 5′-RACE

R = A or G; Y = C or T; N = A, C, T, or G. Restriction sites *Pst*I (ASP-5), *Eco*RI (GSP-3), and *Bam*HI (anchor) used for cloning are underlined.

ASP-5	5′-GGTACTGCAGGARCCNGAYGCNYTNGG-3′
GSP-1	5′-CCGACTCCATTATAGATGG-3′
GSP-2	5′-CCACCAGCCGGAGAGC-3′
GSP-3	5′-CTTGAATTCCAGCGAACGCGC-3′
Anchor	5′-GAATCGATGGATCCTGCAGC^{17}

min at 16,000 rpm. The tubes were placed on ice for 10 min to slow the swimming motion of the larvae. The supernatant containing the ES products was carefully transferred to a new microcentrifuge tube, and inspected visually using a dissection microscope to assure that no L_3 had been transferred. Supernatants were pooled and stored at −20 °C. Prior to electrophoresis, supernatants were concentrated 4–10 times by lyophilization or ultrafiltration using Centricon 10 cartridges (Amicon, Beverley, MA).

Protein Sequencing—ES products from approximately 50,000 activated *A. caninum* L_3 were concentrated and electrophoresed in a 12.5% acrylamide gel (7) under nondenaturing conditions. The major band of M_r = 40,000 was visualized by Coomassie Blue staining and excised. Approximately 98 pmol of isolated protein were digested with trypsin *in situ* as described elsewhere (8, 9). Enzymatic digests were fractionated by reverse phase HPLC on a Hewlett Packard 1090 HPLC system, and several peaks were quantified by laser desorption mass spectrometry prior to sequencing, as described previously (8).

PCR—Degenerate oligonucleotide primers, corresponding to the EPDALG portion of protein sequence and synthesized in both orientations, were used to amplify an ASP gene-specific product from DNA isolated from an *A. caninum* L_3 cDNA library constructed in λZAP II (Stratagene, La Jolla, CA). The preparation of the cDNA library is described elsewhere (10). The degenerate primers were paired with flanking vector primers (T3 or T7 promoter) in a PCR. The PCR conditions were 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 unit of *Taq* DNA polymerase (Promega, Madison, WI), and 75 ng of phage DNA, containing the L_3 cDNA library, in a 20-μl reaction. Preliminary experiments indicated that amplification using the sense strand degenerate primer ASP-5 (Table I) and the T7 primer produced a 550-bp product. Ten identical reactions containing only the DNA and primers in 10 μl were subjected to "hot start" PCR (11). The reactions were heated at 94 °C for 5 min, then lowered to 85 °C for 5 min, during which time the remaining reaction components were added. This was followed by 30 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 45 °C, and 2 min of extension at 72 °C. The PCR reactions were pooled, digested with *Pst*I and *Kpn*I, and separated by electrophoresis in a 3% low melting point NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME) containing 0.5 μg/ml ethidium bromide. The 550-bp band was excised, transferred to a microcentrifuge tube, and melted at 65 °C. The melted agarose was frozen in a dry ice-ethanol bath and immediately centrifuged at 16,000 × *g* for 10 min. The aqueous phase was transferred to a new tube, ethanol-precipitated, resuspended in 7 μl of distilled H_2O, and cloned into pBluescript by standard methods. Double-stranded plasmid DNA was sequenced by the dideoxy method (12, 13), using the Sequenase 2.0 kit (U.S. Biochemical-Amersham Corp, Cleveland, OH) and sequential synthetic oligonucleotide primers.

Library Screening—The *A. caninum* L_3 cDNA library was propagated in XL1-BLUE cells, and plated according to standard methods (14). Approximately 2×10^5 plaques were screened with an RNA probe made by transcription of linearized pBluescript containing the 550 bp of PCR product with T3 RNA polymerase (Boehringer Mannheim) in the presence of α-[^{32}P]UTP. Hybridization conditions were as follows: 6 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 10 × Denhardt's solution (1 × = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400), 1.0% SDS, 200 μg/ml yeast tRNA, 50% formamide, and the radiolabeled probe (≈1.2×10^6 cpm/ml) at 42 °C for 18 h. Filters were washed with 2 × SSC, 0.1% SDS at 60 °C for 45 min, 0.2 × SSC, 0.1% SDS at 60 °C for 30 min, and 0.1 × SSC, 0.1% SDS at 60 °C for 60 min (1 × SSC = 150 mM NaCl, 15 mM sodium citrate), and exposed to XAR-5 film (Eastman Kodak Co.) with an intensifying screen for 7 h at −70 °C. Two rounds of plaque purification resulted in eight clones, which were rescued by *in vivo* excision of their plasmid DNAs (15). Plasmid DNAs (pBluescript) were isolated by standard procedures, and digested with *Eco*RI and *Xho*I to release the inserts. Two of the eight inserts were of different sizes, of approximately 1000 and 1200 bp. The 5′ and 3′ ends were sequenced using T3 and T7 primers, respectively. Both clones had 3′-poly(A) tails and identical 3′ sequences. The 5′ ends were different, but further sequencing indicated that the shorter clone was a truncated version of the longer clone, renamed pASP-1. Restriction mapping indicated internal *Eco*RI and *Nco*I restriction sites (Fig. 1), which were utilized for subcloning to facilitate sequencing. Both strands were sequenced completely.

5′-RACE—A modified 5′-RACE technique was used to isolate the 5′ end and start codon of the ASP cDNA (16–18). Approximately 400 mg of frozen *A. caninum* L_3 were ground to a powder in a mortar chilled in liquid nitrogen, and total RNA isolated using the TRIzol reagent (Life Technologies, Inc.). First strand cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (Superscript II, Life Technologies, Inc.) and 1 pmol of ASP gene-specific primer 1 (GSP-1) (Fig. 1 and Table I) according to the manufacturer's protocol. The cDNA was ethanol precipitated in the presence of 10 μg of yeast tRNA and resuspended in 200 μl of distilled H_2O. A poly(dG) tail was added to the 3′ end of the cDNA using terminal deoxytransferase. A 50-μl reaction containing 33.5 μl of cDNA, 1 mM dGTP, and 15 units of terminal deoxytransferase (Life Technologies, Inc.) was incubated for 1 h at 37 °C. Following tailing, the reaction was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), ethanol-precipitated, and resuspended in 150 μl of distilled H_2O. Two μl were used as template for the first PCR reaction, containing 25 pmol of GSP 2 antisense primer, located internally to the primer used for RT (Fig. 1 and Table I), together with 25 pmol of a 5′-poly(G) anchor primer (Fig. 1 and Table I). The 50-μl reaction was subjected to 40 cycles of 1 min at 94 °C, 1 min at 48 °C, and 1 min at 72 °C. Following amplification, 1 μl of the reaction was used in a second PCR reaction, which was identical to the first, except that a third nested antisense primer, GSP-3 (Fig. 1 and Table I) with a 5′-*Eco*RI restriction site, was substituted for GSP-2, and the annealing temperature increased to 55 °C. Four reactions were pooled, extracted with phenol and chloroform, ethanol-precipitated, and digested with *Bam*HI and *Eco*RI. The inserts were gel purified, cloned into pBluescript, and sequenced as above.

Computer Analysis—Sequence editing, alignments, and comparisons were performed using the Wisconsin Genetics Software Package (Genetics Computer Group, Madison, WI) on a VAX 7610 computer. The potential signal sequence cleavage sites was determined using the PSIGNAL program (PC/GENE Release 5.18, Intelligenetics, Mountain View, CA) on a personal computer.

Expression and Purification of Recombinant ASP—A 1-kb *Eco*RI/*Xho*I fragment containing the 3′ end and poly(A) tail of ASP-1 (Fig. 1) was cloned in-frame into the pET28(c) expression vector (Novagen, Madison, WI) and used to transform competent BL21 *Escherichia coli* cells (F^- *ompT hsdS*$_B$ ($r^-_B m^-_B$) *gal dcm*). Log-phase cells were induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside and incubated for 3 h at 30 °C. One-ml aliquots were removed at 0, 1, 2, and 3 h of induction and examined by SDS-PAGE (7) and Western blotting (see below).

For purification of rASP, a 2-liter culture of BL21 cells containing the recombinant plasmid pET28:ASP was induced as described above. The cells were centrifuged, the pellet resuspended in 60 ml of 50 mM Tris (pH 8.0), 2 mM EDTA, 0.1% Triton X-100, 6 mg of lysozyme, and incubated at 30 °C for 30 min. The solution was sonicated using a Branson 450 Sonifier (30% duty cycle, output setting 3) until it was no longer viscous and centrifuged at 12,000 × *g* for 15 min. The pellet was resuspended in 60 ml of 1.0% SDS, 0.5% 2-mercaptoethanol, boiled for 5 min, and cooled to 22 °C. The extract was dialyzed (M_r = 12,000–14,000) against 2 liters of 0.1% SDS in phosphate-buffered saline for 2 days at 22 °C, with two changes of buffer. The cell extract was applied to a 10-ml HisBind resin column (Novagen), and chromatography was conducted according to the manufacturer's instruction, except that 0.1% SDS was added to all buffers. The expressed protein, containing a 6-residue histidine tag (His-Tag, Novagen) that bound it to the Ni^+ resin, was eluted from the column with 0.1–1.0 M stepwise gradient of imidazole, dialyzed as above, and isolated by preparative SDS-PAGE on 11% acrylamide gels.

Antisera Production—rASP antiserum was produced by immunization of a New Zealand White rabbit with gel-purified rASP. The gel slices were macerated and injected subcutaneously with complete Freund's adjuvant. Additional boosts with purified rASP in incomplete Freimd's adjuvant were administered at 3, 6, and 9 weeks. Vesq Ag5 antiserum was produced by immunization of a New Zealand White rabbit with purified *Vespula squamosa* Ag5 (Vesq Ag5) in complete Freund's adjuvant in multiple intradermal sites (19). The antigen was estimated to be over 98% pure by SDS-PAGE and protein sequence analysis (20).

Specificity of the antisera was confirmed by its adsorption with purified rASP. rASP was coupled to 3 M Emphaze beads (Pierce, Rockford, IL) by incubating 0.2 g of beads with 0.27 mg of rASP in phosphate-buffered saline, 0.1% SDS, 0.8 M sodium citrate, pH 8.0, overnight at 22 °C. The beads were sedimented by gentle centrifugation, the supernatant was discarded, and the coupling reaction was stopped by adding 10 ml of 1.0 M Tris, pH 8.0, followed by incubation at 22 °C for 2.5 h. The beads were washed twice with phosphate-buffered saline and stored at 4 °C. rASP antiserum (1:500) and Vesq Ag5 antiserum (1:2000) were diluted in 5% NFDM-P, and 10 ml were incubated with 0.2 ml of ASP-conjugated beads overnight at 4 °C. The beads were removed by centrifugation, and the supernatant was used in a Western blot (see below).

Western Blotting—Concentrated ES products or rASP were separated in 11% SDS-polyacrylamide gels and transferred to Immobilon-P poly(vinylidene fluoride) membranes (Millipore, Bedford, MA) by electroblotting at 20 V for 16–18 h at 4 °C (21). The membranes were blocked with 5% NFDM-P for 6–8 h at 4 °C. Following blocking, the membrane was incubated with a 1:2000 dilution of rabbit anti-Vesq Ag5 antiserum or a 1:500 dilution of rabbit anti-rASP antiserum in NFDM-P for 16–18 h at 4 °C. After three washes in NFDM-P, the membrane was incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma) for 1 h at 22 °C. Bands were visualized using enhanced chemiluminescence (Amersham Corp.).

In Vitro Activation Experiments—*In vitro* activation experiments were conducted to determine the kinetics of ASP release. In these experiments, the presence of ASP in ES products was determined by Western blotting using rASP antiserum as a specific probe. To determine when ASP is first released by activated L_3, individual wells containing approximately 6000 activated L_3 were harvested at 30 min and 1, 4, and 24 h of incubation, and the ES products were removed and concentrated by ultrafiltration. Nonactivated L_3 incubated for 24 h in RPMI alone were used as a negative control. The percentage of feeding L_3 in the population was determined at 24 h.

To determine if ASP was released continuously during the activation process, the ES products of a single population of L_3 were sampled over time. A well containing approximately 6500 L_3 was incubated under standard activation conditions. At 1, 4, and 24 h of incubation, the L_3 were removed and pelleted by centrifugation, and the supernatant was transferred to a new tube and concentrated by ultrafiltration. The L_3 were washed three times with 15 ml of RPMI and returned to a new well. Fresh medium containing the stimuli was added, and the incubation was resumed, except at 24 h, which was the terminal time point. A negative control (no stimuli) and a positive control (entire 24-h activated L_3 ES product output) were harvested at 24 h. The percentage of feeding L_3 was determined at 24 h.

To determine whether the presence of the stimulus was required for continuous ASP release, two wells containing 6000 L_3 were incubated with the stimulus for 1 or 4 h. At these times, the ES products were harvested, and the L_3 were washed three times with RPMI and reincubated in RPMI alone (*i.e.* without the activation stimuli). At 24 h, the ES products were harvested again, and the percentage of feeding L_3 was determined. Activated and nonactivated larvae incubated for 24 h were used as controls, with their ES products collected at 24 h only.

4,7-Phenanthroline, a known feeding inhibitor (4), was tested for its effect on ASP release. Approximately 6000 L_3 were incubated with 0.5 mM 4,7-phenanthroline and activation stimuli for 24 h. Following incubation, the ES products were harvested and concentrated by ultrafiltration. The ES products from all experiments were examined by Western blotting using rASP antiserum as described above.

Image Analysis—Autoradiographs of Western blots were scanned using a HP Scanjet IIcx digital scanner (Hewlett-Packard, Corvallis, OR) using HP DeskScan version 2.0 software on a Power Mac 6100 (Apple Computers, Cupertino, CA). The image was saved as a TIF file, and analyzed using NIH Image software version 1.54 (Wayne Rasband, NIH). Areas of the bands were selected by drawing a rectangle around the band. An area of identical size, to be used as a measure of the background level, was selected from the negative control lane at the same position as the positive bands. Therefore, values are expressed relative to the negative control, which although not visible, might register in the scan. Total intensity of the area was calculated by NIH Image, the background value was subtracted, and the corrected pd was used for comparisons.

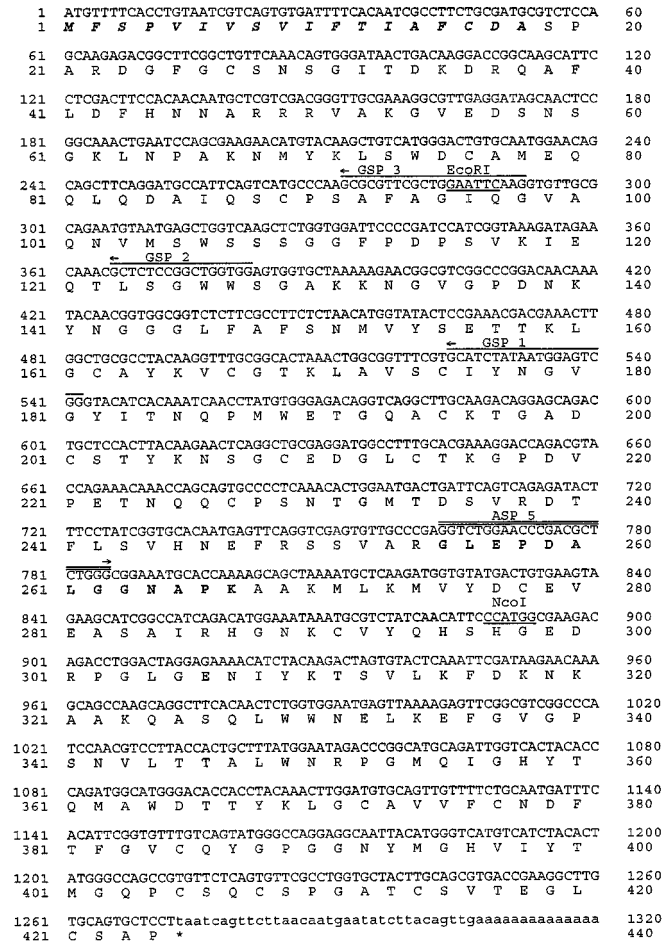

FIG. 1. **The complete DNA and deduced amino acid sequence of the ASP cDNA.** The sequenced peptide (amino acids 255–267) from which PCR primers were derived is in *bold type*, and the sequence of the primer used for PCR is *double overlined*. The location of primers used for 5′ RACE are *overlined*, and the restriction enzyme sites *Eco*RI and *Nco*I used for subcloning are *underlined*. The 18-amino acid secretory signal sequence (amino acids 1–18) is in *bold italic type*. The complete cDNA sequence was submitted to GenBank™ with the accession no. U26187.

RESULTS

When *A. caninum* L_3 are stimulated to resume feeding *in vitro*, they release a major product of approximately M_r = 40,000, referred to as ASP. In contrast, no proteins are detected in the ES products of nonactivated L_3 (not shown). Microsequencing of a trypsin digestion product of this protein revealed a peptide with the sequence GLEPDALGGNAPK. A degenerate primer derived from the EPDALG portion of this sequence, together with a primer complementary to flanking vector sequence, amplified a fragment of approximately 550 bp from phage λ DNA containing an *A. caninum* L_3 cDNA library. This product was used as a probe to isolate a 1.2-kb cDNA clone encoding ASP from the *A. caninum* L_3 cDNA library. The cDNA contained a 3′ poly(A) tail, but lacked a 5′ initiation codon. The 5′ end containing the ATG (Met) start codon was isolated from L_3 cDNA by 5′-RACE (16–18). Four 5′ end clones were sequenced, and found to encode identical sequences that were in frame with and overlapped the cDNA clone. Unlike another hookworm cDNA recently cloned in our laboratory (10), none of the 5′ sequences contained the conserved nematode SL 1 or SL 2 (22–24), nor could the 5′ end be amplified from cDNA using SL 1 primer and an internal gene-specific primer.

The full-length cDNA encodes an ORF of 424 amino acids, with a predicted molecular weight of 45,735, a pI of 7.13, and a

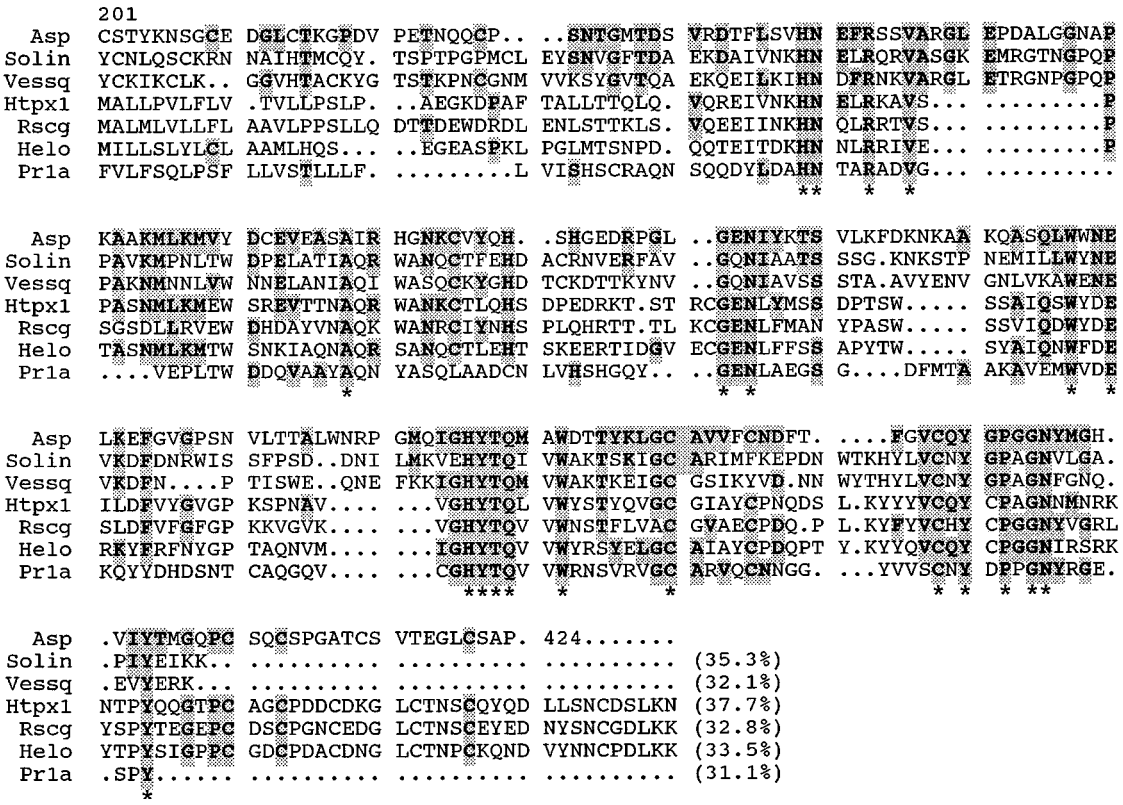

FIG. 2. **Comparison of the ASP deduced amino acid sequence with the amino acid sequences of selected homologous molecules.** *Shaded residues* are identical to those in ASP, and positions marked with an asterisk (*) are conserved in all molecules. Numbers in parentheses represent the percent identity with ASP, calculated using pairwise comparisons between ASP and the homolog using the GAP program of the Wisconsin Genetics program. *Solin*, antigen 3 from the red imported fire ant, *Solenopsis invicta* (accession no. P35778); *Vessq*, Vesq Ag5 from the yellow jacket *V. squamosa* (P35786); *Htpx*, human testes-specific protein (B33329); *Rscg*, rat sperm-coating glycoprotein (acidic epididymal glycoprotein, A24609); *Helo*, helothermine from Mexican beaded lizard (*Heloderma horridum horridum*) salivary gland (U13619); *Pr1a*, tobacco pathogenesis-related protein 1a precursor (S00513).

34-bp 3′-untranslated region (Fig. 1). The entire sequence of the original peptide purified from ES products was present in the ORF (amino acids 255–267), confirming that this cDNA encodes the ASP molecule. The amino-terminal 18 amino acids are highly hydrophobic (11/18), and a potential eukaryotic signal sequence cleavage site is located between amino acids 18 (A) and 19 (S) (25), consistent with the secretory nature of the protein. There are no *N*-linked glycosylation sites present in the ORF.

Data base comparison (SwissProt) revealed significant homology between the COOH-terminal 215 amino acids of the ASP-deduced amino acid sequence and the antigen 5/antigen 3 family of molecules from *Hymenoptera* venoms (26–28), a family of cysteine-rich secretory proteins, called CRISPs (29–34), and pathogenesis-related proteins in plants (35) (Fig. 2). The amino-terminal 191 amino acids failed to show significant homology to any proteins in the data base. Because of the apparent homology to hymenopteran venom Ag 5, a polyclonal antiserum against Vesq Ag5 was tested for its ability to recognize secreted and recombinant ASP. As shown in Fig. 3*b*, Vesq Ag5 antiserum cross-reacted with both the recombinant and native ASP molecules, although recognition was not entirely specific. In addition to ASP, the heterologous serum also recognized a protein of $M_r \approx 28{,}000$ in ES products from activated L_3. The antiserum also cross-reacted with rASP, as well as a higher molecular weight protein. However, adsorption of the Vesq Ag5 antiserum with rASP removed all reactivity with the ES products, and decreased binding to rASP by 78%, in addition to abrogating the high M_r reactivity in the rASP lane. The Vesq Ag5 antiserum failed to recognize any proteins in the ES products from nonactivated L_3.

In contrast, the rabbit antiserum prepared against purified rASP exclusively recognized both the recombinant and the native molecules. As seen in the Western blot in Fig. 3*a*, the antiserum recognized a band of $M_r \approx 40{,}000-42{,}000$ in ES products from activated L_3, and a slightly lower M_r band in purified rASP, but nothing in the ES products from nonactivated L_3. Adsorption of the antiserum with rASP completely abrogated the reaction (Fig. 3*a*), indicating that the antiserum was specific for ASP.

The rASP antiserum was used investigate the kinetics and biology of ASP release. The first experiment was designed to determine when ASP is first released by activated L_3. ES products were collected at several time points, and analyzed by Western blotting with rASP antiserum as the probe. Infective L_3 released ASP as early as 30 min following exposure to activating stimuli (Fig. 4). Image analysis indicated that by 4 h, ASP release had reached levels equal to that released by 24 h in the positive control (pd at 4 h = 118, 24 h = 116).

A second experiment was conducted to determine whether ASP was released continuously during the incubation, or whether it was primarily released early in the activation process. In this experiment, ES products were harvested from a single population of activated L_3 at several time points. The ES products were first harvested at 1 h, and the L_3 were thoroughly washed and returned to the tissue culture plate with fresh medium containing the activation stimuli. This was repeated on the same population of L_3 at 4 h. At 24 h, the ES products were collected, and the L_3 were assayed for feeding. Therefore, the ES products contained the ASP secreted between 0 and 1 h of incubation, 1–4 h of incubation, and 4–24 h of incubation. As shown in Fig. 5, ASP was present in the ES

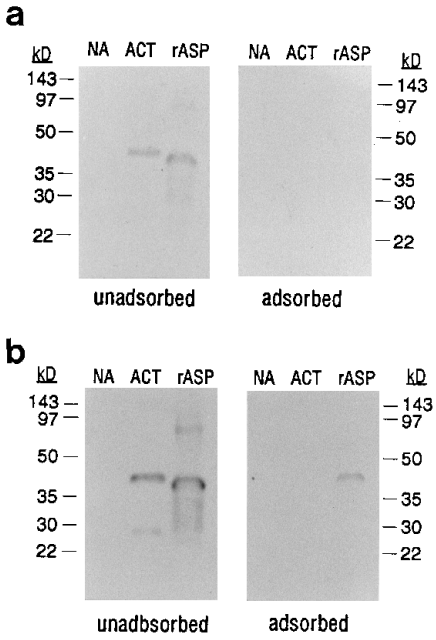

FIG. 3. **Adsorption of antisera with rASP.** Concentrated ES products from 6000 nonactivated (*NA*) or activated (*ACT*) *A. caninum* L_3, together with 8 μg of purified rASP, were separated in an 11% polyacrylamide gel. Following transfer to poly(vinylidene fluoride) membranes, the blots were probed with neat antiserum or antiserum adsorbed against rASP. *a*, anti-rASP antiserum, 1:500; *b*, anti-Vesq Ag5 antiserum, 1:2000.

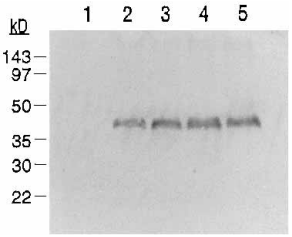

FIG. 4. **Time course of ASP release.** Western blots containing ES products from 6000 activated *A. caninum* L_3 were probed with rASP antiserum (1:500). *Lane 1*, nonactivated L_3, 24 h of incubation; *lane 2*, 30 min of incubation; *lane 3*, 1 h of incubation; *lane 4*, 4 h of incubation; *lane 5*, 24 h of incubation.

products from all time intervals, although the 0–1 h fraction contained more than the other time intervals. Comparison of the 0–1 h band (*lane 2*, pd = 65.4) with the total ASP released (*lanes 2–4*, total pd = 111) by image analysis indicated that nearly 60% of the total ASP released over 24 h was released in the 1st h following exposure to stimuli. Furthermore, almost 90% of the total ASP is released by 4 h (sum of *lanes 2–3*, pd = 98.9, *versus lanes 2–4*). The number of the manipulated L_3 that were feeding at 24 h was similar to the percentage of undisturbed, activated L_3 that fed (88 *versus* 95%), indicating that the larvae suffered no untoward effects during harvesting of the ES products.

A third experiment was designed to determine if ASP release was dependent on the presence of the activation stimuli. In this experiment, ES products were harvested from individual populations of L_3 at either 1 or 4 h of incubation, and the L_3 were washed and reincubated in the absence of stimuli. The ES products from the reincubated L_3 were harvested at 24 h and examined by Western blot with rASP antiserum. As seen in Fig. 6, rASP antiserum recognized strong bands in the 1-, 4-, and 24-h (positive control) ES products. However, no further ASP was released when the ES products from L_3 reincubated without the stimuli were examined at 24 h, despite the fact that

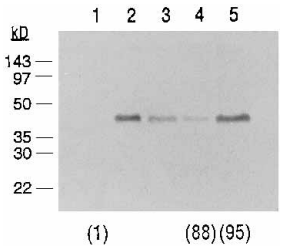

FIG. 5. **Kinetics of ASP release by a single population of *A. caninum* L_3.** ES products from 6500 L_3 were collected at various time points, the L_3 were washed, and the incubation was continued in the presence of activation stimuli. *Lane 1*, nonactivated L_3, 24 h of incubation (negative control); *lane 2*, ES products harvested at 1 h of incubation (0–1-h output); *lane 3*, ES products harvested at 4 h of incubation (1–4-h output); *lane 4*, ES products harvested at 24 h of incubation (4–24-h output); *lane 5*, ES products incubated for 24 h (0–24-h output, positive control).

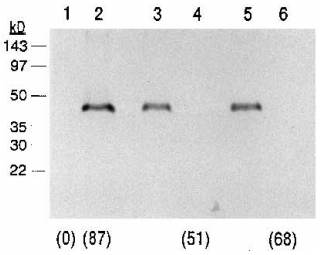

FIG. 6. **Continuous ASP release requires the stimulus.** ES products were harvested at specified times, the L_3 were washed, and incubation was continued in the absence of the activation stimuli. ES products were examined by Western blotting. *Numbers in parentheses* indicate percentage feeding when assayed at 24 h. *Lane 1*, ES products from nonactivated L_3 incubated for 24 h; *lane 2*, activated L_3 ES products incubated 24 h; *lane 3*, activated L_3 ES products, 0–1-h output; *lane 4*, activated L_3 ES products, 1–24 h without stimulus; *lane 5*, activated L_3 ES products, 0–4-h output; *lane 6*, activated L_3 ES products, 4–24 h without stimulus.

significant proportions of the L_3 had been triggered to resume feeding when assayed at 24 h (1 h, 51% feeding; 4 h, 68%; 24-h control, 87%).

Inclusion of 2.5 mM 4,7-phenanthroline in the incubation decreased ASP release by 58% (Fig. 7, uninhibited pd = 106.8, inhibited pd = 45.3). Feeding was decreased from 96% in uninhibited L_3 to 13% in L_3 exposed to the inhibitor.

DISCUSSION

During invasion of a definitive host, hookworm infective L_3 encounter signals that initiate developmental pathways that were previously suspended in the free-living L_3 stage (2, 36, 37). The resumption of feeding by parasitic L_3 associated with exposure to hostlike conditions functions as a marker for activation and the transition to parasitism (2, 3). Coincident with *in vitro* activation, L_3 release a 40-kDa molecule, known as ASP, as the major component of ES products. In order to investigate its role in the transition to parasitism, the cDNA encoding ASP was cloned and analyzed. The cloned molecule was expressed in *E. coli* cells, purified, and used to produce a polyclonal antiserum.

Sequencing of the ASP cDNA revealed an open reading frame of 424 amino acids. The 5′ end of the cDNA encoded a highly hydrophobic (11/18) amino acid NH_2 terminus. The presence of a probable cleavage site (25) between Ala-18 and Ser-19 suggests that the 18 NH_2-terminal amino acids represent the signal peptide that targets ASP as a secretory protein. The calculated molecular mass of the complete peptide encoded by the ORF is 45,735 daltons, whereas the calculated molecular mass of the protein without the signal sequence is 43,793 daltons, which agrees more closely with the apparent M_r of

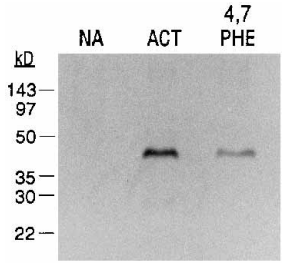

FIG. 7. **Effect of 0.5 mM 4,7-phenanthroline on release of ASP by *A. caninum* activated L_3.** *NA*, ES products from 6000 nonactivated L_3; *ACT*, ES products from 6000 activated L_3; *4,7-PHE*, ES products from 6000 activated L_3 incubated with 2.5 mM 4,7-phenanthroline. The percentage of L_3 feeding at 24 h was: NA, 3.5%; ACT, 96%; 4,7-PHE, 13%.

ASP in ES products (≈40,000–42,000) determined by gel electrophoresis (Fig. 3). Comparison of the ASP predicted amino acid sequence to protein data bases revealed significant homology (≈50–56% similarity, ≈30–35% identity) between the carboxyl-terminal 230 amino acids of ASP and the Ag 3/Ag 5 proteins of hymenopteran venoms (Fig. 2). Venom Ag5 is the major protein of several hymenopteran venoms and as such represents the major antigen (38). Its function is unknown, although it has been hypothesized to be an invertebrate neurotoxin (39).

The homology to venom proteins was confirmed by the ability of antiserum directed against Ag 5 of the yellow jacket *V. squamosa* to recognize ASP on Western blots of ES products and expressed rASP (Fig. 3*b*). The Vesq Ag5 antiserum also recognized a protein of lower M_r in ES products from activated L_3 that is not recognized by the rASP antiserum. Amino acid sequencing indicated that this lower M_r component is closely related to ASP (not shown) and probably represents another member of a family of ASP-like secretory proteins. This is supported by the elimination of cross-reactivity by adsorption of the antiserum with rASP, indicating that the low M_r molecule contains an epitope that is recognized by the polyclonal Vesq Ag5 antiserum, but not by the rASP antiserum.

ASP is also homologous to a family of CRISPs isolated from testis (mouse and human TPX-1, 29.7 and 37.7% identity; mouse CRISP-1, 31.7%) and salivary glands of mice (CRISP-3, 29.1%) and beaded lizards (helothermine, 33.5%) (29–34). The latter peptide is a salivary toxin that inhibits Ca^{2+}-induced Ca^{2+} release by blocking the ryanodine receptor on the sarcoplasmic reticulum. ASP contains 7 of the 16 invariant cysteine residues, but less than half of the cysteine-rich COOH-terminal domain, that are hallmarks of the CRISP family of proteins. However, all of the molecules, including ASP and the insect venoms, share a common sequence (HYTQ) corresponding to amino acids 358–361 of ASP (31).

ASP release occurs rapidly in the activation process, with large amounts secreted within 30 min of exposure to the activation stimuli. Nearly all of the total ASP secreted is released by 4 h of incubation. These data suggest that ASP might be produced and stored in secretory granules that are released in response to activation, as occurs during activation and molting of other parasitic nematodes (37). Indeed, isolation of the ASP message from a cDNA library of nonactivated L_3 indicates that mRNA-encoding ASP is present in the free-living stage prior to activation. However, it is possible that ASP mRNA is masked and translated only in response to the activation signal. Also, there is a low level of stimulus-dependent ASP released between 4 and 24 h, suggesting new, activation-associated synthesis of ASP. One possible explanation is that the activation stimulus causes the release of stored ASP from secretory granules, but also initiates ASP gene expression, resulting in continued low levels of ASP production and release. Confirmation of this hypothesis awaits the results of more detailed analysis of ASP stage-specific expression and ASP immunolocalization experiments.

ASP release occurs much earlier than the resumption of feeding, which typically begins at 6–8 h (6). Although feeding and ASP release both occur following exposure to the activation stimulus, the two events differ in that, once initiated, the presence of the stimulus is not required for feeding to continue (6), whereas the stimulus is required for continuous release of ASP. Studies are underway to determine whether ASP secretion can be uncoupled from feeding, or whether both phenomena occur in response to the same stimuli.

The rapid and constitutive release of ASP suggests an important function early in the infective process, perhaps as a modulator of the host immune response. There are numerous reports of ES products from nematode parasitic stages that alter host physiology or suppress host immunity, either by inhibiting immune effector mechanisms (40, 41) or by direct immunosuppression (42–44). Recently, adult *A. caninum* were shown to secrete a neutrophil inhibitory factor that binds to CD11/CD18 receptor of neutrophils and inhibits their ability to mediate phagocytosis (45, 46). Sequence comparison indicated that there is a 30.7% identity between ASP and neutrophil inhibitory factor (not shown). Thus, ASP might serve a similar function in pre-adult parasitic stages, thereby allowing invading L_3 to interfere with the host inflammatory response and evade destruction. Because ASP also exhibits homology with the peptide toxin helothermine (33.5% identity) (31), it is also possible that ASP exerts a direct toxic effect on host immune effector cells.

Alternatively, because of its close homology to Vesq Ag5, ASP might function as an allergen. Allergens elicit immediate-type hypersensitivity inflammatory host responses comprised of IgE production and a so called TH2 cytokine profile, including IL-4, Il-5, and Il-10. It is of interest that intestinal nematode infections also bias the host inflammatory response to produce Il-4 and Il-5 for the stimulation of IgE production and eosinophilia, respectively (47). Studies are underway to determine whether the TH2-like responses found in hookworm infection might arise in response to ASP.

Determination of the actual *in vivo* function of ASP will require further investigation. However, because its release coincides with infection and the transition to parasitism, ASP is a promising candidate for a recombinant vaccine against hookworm disease. ES products of several nematodes elicit some degree of host protection when used as vaccine antigens (40, 48–52). The only successful hookworm vaccine to date, against canine hookworm, employed radiation-attenuated infective L_3, and although a commercial failure, did reduce worm burden and the resultant pathology (53). One possibility is that the immune response against the irradiated L_3 was directed against an ES product, perhaps ASP, released during invasion. Evaluation of ASP as a vaccine candidate is currently under way.

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