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MOLECULAR CLONING AND PURIFICATION OF Ac-TMP, A DEVELOPMENTALLY REGULATED PUTATIVE TISSUE INHIBITOR OF METALLOPROTEASE RELEASED IN RELATIVE ABUNDANCE BY ADULT *ANCYLOSTOMA* HOOKWORMS

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Abstract. A cDNA encoding a putative tissue inhibitor of metalloprotease was cloned from an *Ancylostoma caninum* adult hookworm cDNA library by immunoscreening with anti-hookworm secretory products antiserum. Ac-TMP (*A. caninum* tissue inhibitor of metalloproteinase) is encoded by a 480-bp mRNA with a predicted open reading frame of 140 amino acids (molecular weight, 16,100 Da) that contains one potential N-linked glycosylation site and an N-terminal Cys-X-Cys consensus sequence. The open reading frame corresponds to a putative hookworm tissue inhibitor of metalloproteases (TIMP) with 33% identity and 50% similarity to the N-terminal domain of human TIMP-2. Analysis by reverse transcriptase-polymerase chain reaction indicates that transcription of *Ac-tmp* is restricted to the adult stage. The protein was isolated from *A. caninum* adult secretory products by reverse-phase high-performance liquid chromatography and identified as one of the most abundant proteins released by the parasite. To our knowledge, this is the first description of a TIMP from a parasitic invertebrate.

INTRODUCTION

Human hookworm infection is a major cause of anemia, developmental delays, and malnutrition in the developing world.[1] It is estimated that there are > 1 billion cases of hookworm infection worldwide, with 194 million cases in China alone.[2] In some regions of China, such as Hainan Province in the South China Sea, > 60% of the population harbor hookworms.[3] Most of the pathology caused by hookworm results from the adult stages of the parasite in the human intestine. The attachment of adult *Ancylostoma* hookworms to the mucosa and submucosa of the vertebrate small intestine is one of the best-defined examples of host-parasite relationships in all of parasitology.[4,5]

Work by several groups has begun to unravel the biochemical events linked to the resultant blood loss that develops as a consequence of parasite attachment.[6] Studies over the last decade have revealed that adult *Ancylostoma* hookworms release a battery of pharmacologically active agents into host tissues. These include anti-inflammatory polypeptides that bind Mac-1 ligands,[7] anticoagulants and anti-platelet aggregating agents,[8–11] and connective tissue hydrolases such as a hyaluronidase,[12] cathepsins,[13–15] and a brush border membrane zinc metalloprotease. Here, we describe a 16-kDa polypeptide secreted by adult *Ancylostoma caninum* hookworms that has significant homology to the N-terminal domain of vertebrate tissue inhibitor of matrix metalloproteases (TIMPs). Hookworm TIMP (Ac-TMP) is among the most abundant proteins released by the parasite, and to our knowledge, this is the first TIMP to be described from a parasitic invertebrate.

MATERIALS AND METHODS

Immunoscreening of adult *A. caninum* library. *Preparation of adult hookworm secretory products.* One hundred living, adult-stage *A. caninum* hookworms were recovered from the intestines of an infected dog at necropsy (6 weeks after infection), as described previously.[16] The adult worms were washed 3 times in sterile phosphate-buffered saline (PBS), then maintained in 15 mL RPMI 1640 containing 25 mM HEPES, 100 U/mL of ampicillin, and 100 μg/mL streptomycin at 37°C (5% CO_2) for 24 hr. The supernatant was collected, concentrated with PEG6000, and dialyzed against 1 L PBS (pH 7.2) overnight at 4°C. After dialysis, the secreted products were centrifuged at 10,000 × g for 10 min, and the supernatant was recovered.

Preparation of anti-secretory products antiserum. A rabbit was immunized by subcutaneous injection with the hookworm-secreted proteins (400 μg) emulsified with complete Freund adjuvant. Subsequently, the rabbit was immunized at 2-week intervals with the same quantity of hookworm-secreted proteins emulsified with incomplete Freund adjuvant for a total of 3 immunizations. The final bleed was obtained 10 days after the final immunizations, and the serum was separated from whole blood and stored at −20°C.

Immunoscreening of expression cDNA library. Construction of the cDNA expression λZapII (Stratagene, La Jolla, CA) library was reported previously.[9] An estimated 5×10^5 plaques were screened with the rabbit anti–*A. caninum* adult secretory product antibody according to manufacturer's instructions. Briefly, 5×10^4 plaques were plated on an Lennox L Broth (LB) agar plate. *Ancylostoma caninum* antigen expression was induced by covering the plaques with nitrocellulose membranes soaked with 10 mM isopropylthio-β-galactoside IPTG. Four hours after incubation at 37°C, the membranes were lifted, blocked with 5% nonfat milk in PBS, and then incubated with the rabbit antibody (1:500 dilution) for 1 hr at 24°C. The membranes were washed 3 times with PBS buffer containing 0.1% Tween-20 (PBS-Tween) and then incubated with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical, St. Louis, MO) at a 1:1,000 dilution at 24°C for another hour. The membranes were washed again 3 times with PBS-Tween and then developed with 3,3′-diaminobenzidine substrate and hydrogen peroxide. The putative positive clones were cored and isolated for secondary screening.

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AGCATATCAGCATGAGAGTGGCTATTGTTTTCATTGCATGCTTCGGCAGTA  50

          M  R  V  A  I  V  F  I  A  C  F  A  V   13
GCACACGCATGCAAGTgCGAAAAGAAACCTCGTCCTCCATTGGAGAAACT 100
 A  H  A  C  K  C  E  K  K  P  R  P  P  L  E  K  L  30
GCTTTGCCAATCACAATTTGTTACTCACGCGAAAGTGACGAAGAAGAGAA 150
  L  C  Q  S  Q  F  V  T  H  A  K  V  T  K  K  R   46
TTGATGGTTACTTCATCTATTACGACTTGGAGCATAAGGaAGTTTATAAG 200
I  D  G  Y  F  I  Y  Y  D  L  E  H  K  E  V  Y  K  63
CCCAAAGATAGGAGTATCCCAATCGAACTCTTCTCATGGAGGGAAAAGGA 250
 P  K  D  R  S  I  P  I  E  L  F  S  W  R  E  K  E  80
AAATTGTGGTGTTCCGGATCTCGAAGAAGGCAAAGAATACCTGATAGGAG 300
  N  C  G  V  P  D  L  E  E  G  K  E  Y  L  I  G   96
GTAAAGTGACGGATTATGGCGACGGTGATTTGGTAATTTCTGTTTCACGG 350
G  K  V  T  D  Y  G  D  G  D  L  V  I  S  V  S  R  113
TGCGACCTTCTCCGAAACTGGACAGACGTCTCTGGAGAGGAGAAGAAATT 400
 C  D  L  L  R  N  W  T  D  V  S  G  E  E  K  K  L 130
GCTCGGAACGTTCAAATGTGAAAATCAGTCATAAACGCCGATTATATATA 450
  L  G  T  F  K  C  E  N  Q  S  *                  140
ATTGAaAGAAGAGAAATGAACATTTTTCACGCGAAAAAAAAAAAAAAAAA 550

AAAAAAAAA                                          559
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FIGURE 1. Nucleotide and deduced amino acid sequence of Ac-TMP. The putative signal sequence is shaded.

DNA sequencing. The immunopositive clones were excised into pBluscript phage according to manufacturer's instructions (Stratagene). Phagemid DNA was extracted by means of the alkaline lysis method (Qiagen, Valencia, CA), and double-strand sequencing was performed via flanking vector primers (T_3 and T_7). Nucleotide and deduced amino acid sequences were compared with existing sequences in GenBank by BLAST searching. ESEE 3.1 software was used for sequence analysis. Nucleotide sequence data are available in the GenBank database under the accession number AF372651.

Reverse transcription-polymerase chain reaction (RT-PCR) amplification. We used RT-PCR to determine the developmental stage specificity of *Ac-tmp* mRNA transcription. *Ancylostoma caninum* eggs, first-stage (L_1) and second-stage (L_2) larval stages, and third-stage (L_3) infective larvae were obtained as described previously.[17] The total RNA was isolated from each life history stage with TRIzol reagent (Gibco BRL, Gaithersburg, MD). Single-strand cDNA was synthesized with oligo d(T) primer and MMLV-RT (Gibco BRL). Specific primers (TIMP3′-1HR: 5′-CCC AAG CTT CGG CGT TTA TGA CTG ATT TTC AC-3′, TIMP5′-2ER: 5′-CGG AAT TCT GCA AGT GCG AAA AGA AAC C-3′) on the basis of the sequence of *Ac-tmp* (see below) from 60 bp to 440 bp were used to amplify the *Ac-tmp* cDNA. The PCR reaction parameters comprised 94°C denaturing for 1 min, 55°C annealing for 1 min, and 72°C extension for 2 min. A total 30 cycles was performed.

Purification of Ac-TMP natural product. Optimization of semipreparative reverse-phase chromatographic conditions for the fractionations of *A. caninum* adult secretory products was carried out on a 510 high-performance liquid chromatography (HPLC) system (Waters Milford, MA), equipped with a 490 E multiwavelength detector with a semipreparative flow cell, set at 214, 280, 260, and 254 mm and a 250-mm × 4.6 mm inner diameter YMC-Pack protein-RP, 200 Å, 5 μm C_4 column (Waters). The adult *A. caninum* secretory products used

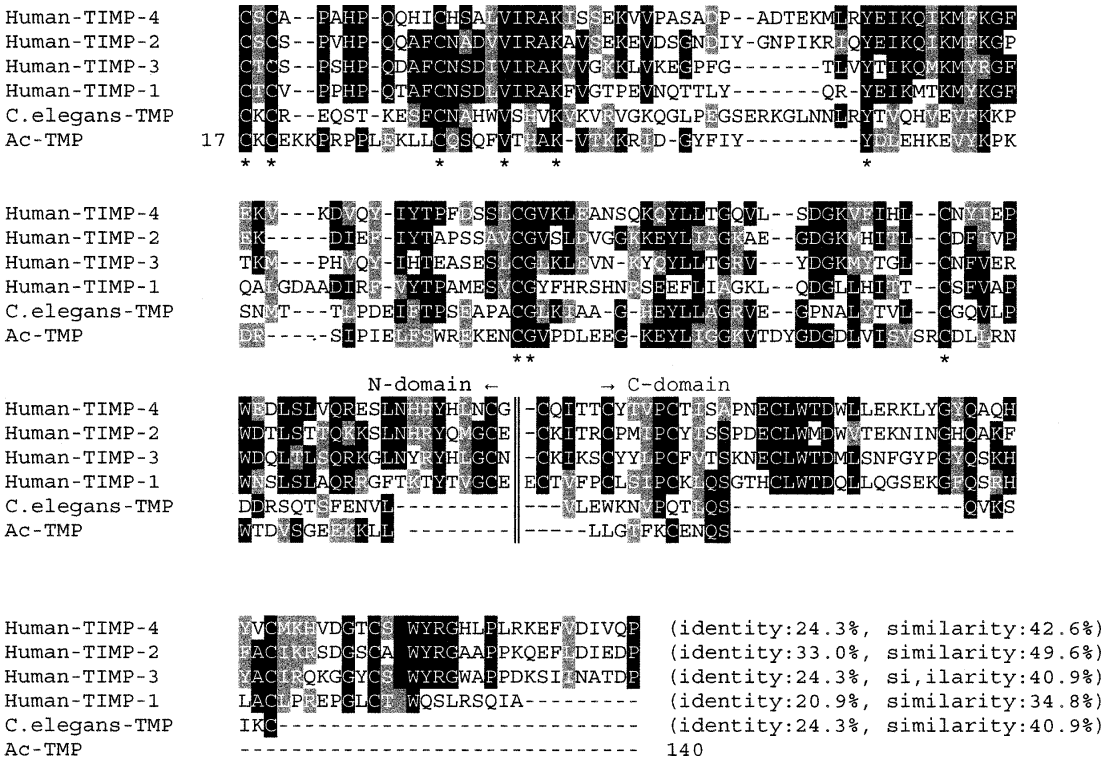

FIGURE 2. Alignment of Ac-TMP, human tissue inhibitor of metalloproteases (TIMP) 1–4, and TIMP from *Caenorhabditis elegans*. Indicated are residues identical to Ac-TMP **(black)** and conservative substitutions **(gray).** Amino acids common to every sequence are marked **(asterisk).** The sequences are Human-TIMP-4 (GenBank accession number Q99727); Human-TIMP-2 (G4929928); Human-TIMP-3 (P35625); Human-TIMP-1 (XP010392); and *C. elegans*-TMP (AAA96174). The alignment was performed with CLUSTAL W and prepared for display by BOXSHADE.

as starting material were collected over 15 hr from 1,260 adult hookworms in 15 mL RPMI 1640 containing 25 mM HEPES, 100 U/mL ampicillin, 100 μg/mL streptomycin, and 100 μg/mL gentamicin at 37°C. The supernatant was concentrated by ultrafiltration in a Centricon-3 microconcentrator (Amicon, Beverly, MA) to 0.3 volumes before centrifugation for 1 hr at 7,500 × g. Approximately 0.6 mg of the parasite secretory protein was chromatographed. Eluent A was 0.01% trifluoroacetic acid (TFA) in water, and Eluent B was 0.01% TFA in acetonitrile. A 40-min linear gradient from 0 to 80% Eluent B was run at a flow rate of 1 mL/min. Fractions of 0.5 min were collected and lyophilized, and they were used for further purification and analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[18] For SDS-PAGE, 2 μL of secretory products, 10 μL of HPLC column Fraction 51, and 12 μL of 2× SDS-PAGE sample buffer (4% SDS, 2.5% 2-mercaptoethanol, 15% glycerol) were mixed, boiled for 5 min, and run on a 4–20% gradient polyacrylamide gel at 100 V for 2 hr. The gel was stained with silver according to manufacturer's instruction (Bio-Rad, Hercules, CA).

The reverse-phase HPLC of Fraction 51, the fraction that contained the most abundant *A. caninum* secretory protein from the semipreparative separation, was carried out on a 510 HPLC system equipped as described above with a 250-mm × 3.0 mm inner diameter YMC-Pack protein-RP, 200 Å, 5-μm C4 column. Eluent A was 0.01% TFA in water, and Eluent B was 0.01% TFA in acetonitrile. A 30-min linear gradient from 0 to 60% Eluent B was run at a flow rate of 1 mL/min. Fractions of 0.5 min were collected and lyophilized. The major protein peak collected from this separation was subjected to amino acid sequence analysis and SDS-PAGE.[18] Amino acid sequence analysis based on the Edman degradation of protein was performed on Procise 494 model protein sequencer (Applied Biosystems, Foster City, CA) equipped with a 785A programmable detector and a 140°C pump system by ProSeq (Boxford, MA). The sequencer products were identified by standard Procise 610A software.

To confirm that the N-terminal sequence corresponded to Ac-TMP, degenerate oligonucleotide primers (ADES-F: TGC AAG TGY GAR AAR AAR CC and ADES-R: GGR TTR TTR TCY CAC TTG CA) were synthesized in both orientations that corresponding to the partial N-terminal peptides sequence of column Fraction 51. Paired flanking degenerate vector primers (T_3 and T_7) were used to amplify the product from DNA obtained from the adult cDNA library constructed in λZapII. The "hot-start" PCR conditions were 10 mM Tris-HCl (pH 8.5) containing 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 μL cDNA library, in 20 μL reaction. The reactions were heated at 94°C for 5 min, then lowered to 85°C for 5 min; next, 1 U of Taq DNA polymerase (Gibco BRL) was added. This was followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C. The PCR products were run on an agarose gel and stained with ethidium bromide. The PCR products were gel purified with the QIAEX II gel extraction kit (Qiagen) and sequenced.

RESULTS

***Ac-tmp* cDNA.** *Ac-tmp* cDNA was cloned from an adult hookworm cDNA library by immunoscreening with rabbit antibody directed against whole *A. caninum* adult secretory products. Two positive identical clones were isolated. The full-length cDNA consists of 559 bp encoding an open reading frame (ORF) of 140 amino acids and a poly-A tail at the 3′ end (Figure 1). The predicted ORF has a calculated molecular weight of 16,100 Da and a theoretical pI of 7.55. There is a hydrophobic signal peptide sequence with a signal peptidase cleavage site between amino acids 16 and 17. Ac-TMP has a signature N-terminal Cys-X-Cys sequence immediately after the signal peptide. One putative N-linked glycosylation site (N-X-T) exists between amino acids 119 and 122. The results of a GenBank database search revealed that the predicted amino acid sequence of this molecule shares 33% identity and 50% similarity to the N-terminal domain of human tissue inhibitor of metalloproteinase 2 (TIMP-2). Both Ac-TMP and a putative TIMP from the free-living nematode *Caenorhabditis elegans* contain a single domain but lack a second, C-terminal domain that is characteristic of vertebrate TIMPs (Figure 2).

Amplification via RT-PCR. To identify the life history, stage-specific expression of Ac-TMP, mRNAs were extracted

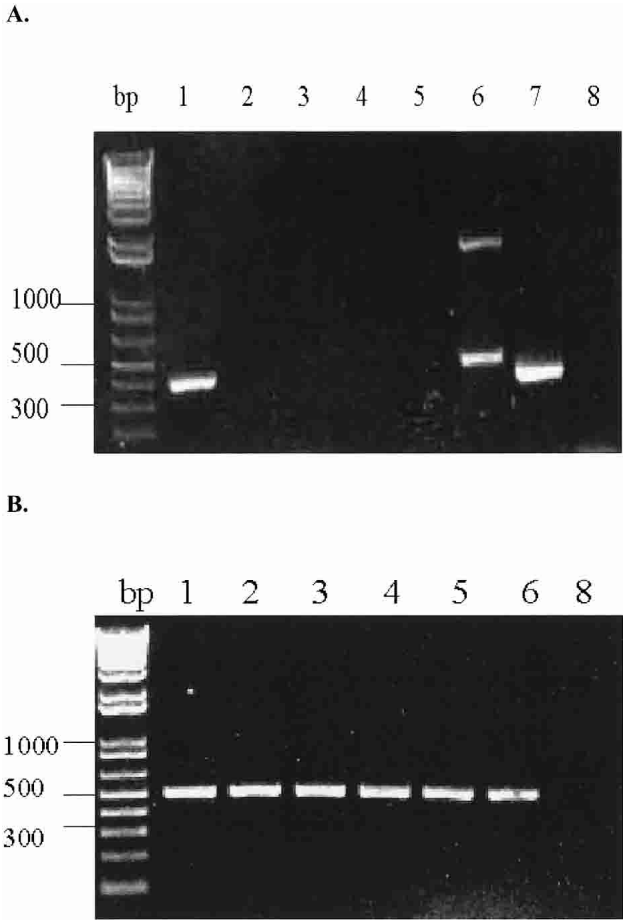

FIGURE 3. Developmental PCR (RT-PCR) of *Ac-TMP* transcription in different life history stages of *Ancylostoma caninum*. **(A)** Tissue inhibitor of metalloproteases (TIMP)-specific primers (TIMP3′-1HR: 5′-CCC AAG CTT CGG CGT TTA TGA CTG ATT TTC AC-3′, and TIMP5′-2ER: 5′-CGG AAT TCT GCA AGT GCG AAA AGA AAC C-3′). **(B)** Protein Kinase A (PKA) untranslated region primers (PKA3–1/5–4). Template DNAs: 1 = adult hookworm cDNA; 2 = L_3 activated cDNA; 3 = L_3 nonactivated cDNA; 4 = L_1-L_2 cDNA; 5 = egg cDNA; 6 = adult hookworm genomic DNA; 7 = *Ac-TMP*/pBluscript plasmid control; 8 = dH_2O.

from different developmental stages of *A. caninum* and reverse transcribed to cDNA with *Ac-tmp*-specific primers. We used RT-PCR to produce a 380-bp specific band that was only amplified from adult cDNA. No amplification was seen from the cDNA of eggs and from L_1-L_2 and L_3 life history stages (Figure 3). Amplification of *A. caninum* genomic DNA revealed 2 bands, which either suggests the possible existence of a second *Ac-tmp* gene that contains an intron, or results from nonspecific priming.

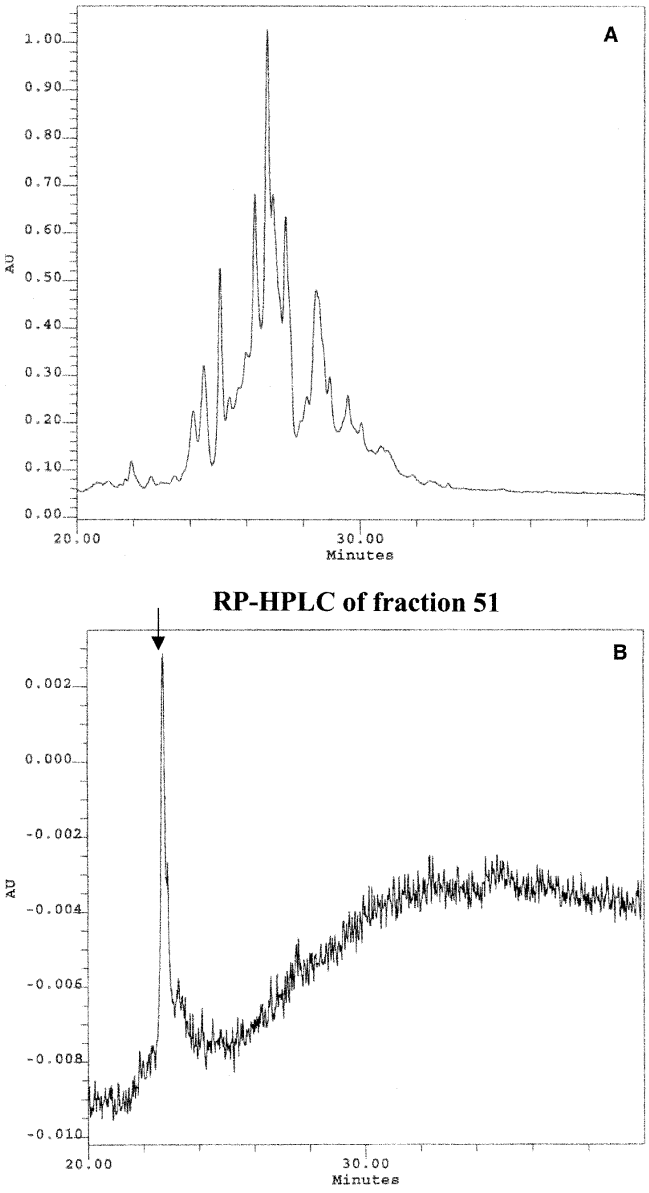

FIGURE 4. **(A)** Reverse-phase high-performance liquid chromatography (HPLC) separation of adult *Ancylostoma caninum* secretory protein (0.6 mg total protein) on a 250 mm × 4.6 mm inner diameter YMC-Pack protein-RP, 200 Å, 5-μm C4 column. Eluent A was 0.01% TFA in water, and Eluent B was 0.01% TFA in acetonitrile. A 40-min linear gradient from 0 to 80% Eluent B was run at a flow rate of 1 mL/min. Protein was detected at 214 nm. Fraction 51 was worked up for further analysis **(arrow). (B)** Reverse-phase HPLC separation of *A. caninum* secretory protein Fraction 51 on a 250-mm × 3.0-mm inner diameter YMC-Pack protein-RP, 200 Å, 5-μm C4 column. Eluent A was 0.01% TFA in water, and Eluent B was 0.01% TFA in acetonitrile. Separation of Fraction 51 was achieved by a 30-min linear gradient to 60% B. The flow rate was 1 mL/min; detection was at 214 nm.

Identification of Ac-TMP in secretory products of *A. caninum* adult worm. To confirm that Ac-TMP is released by adult *A. caninum* hookworms, the protein was identified in and purified from parasite secretory products. Figure 4A shows the column profile from a separation of *A. caninum* secretory products on reverse-phase HPLC. Each of the major peaks was subjected to amino acid sequence analysis as part of a larger *A. caninum* proteomics study (data not shown). The peak of protein corresponding to column Fraction 51 was selected for further study and were again chromatographed (Figure 4B). As shown in Figure 5, column Fraction 51 was composed of a predominant band after silver staining that migrated with an apparent molecular weight of M_r = 16,000. The N-terminal peptide sequence of this fraction was CKCEKKPRPPLEKLLCQSQF, which was an identical match with the sequence of the predicted ORF of Ac-TMP after the predicted signal peptidase cleavage site. On the basis of the calculated area under the curve of HPLC column Fraction 51 relative to the total area of the entire secretory product profile, Ac-TMP was determined to comprise ~ 6.3%

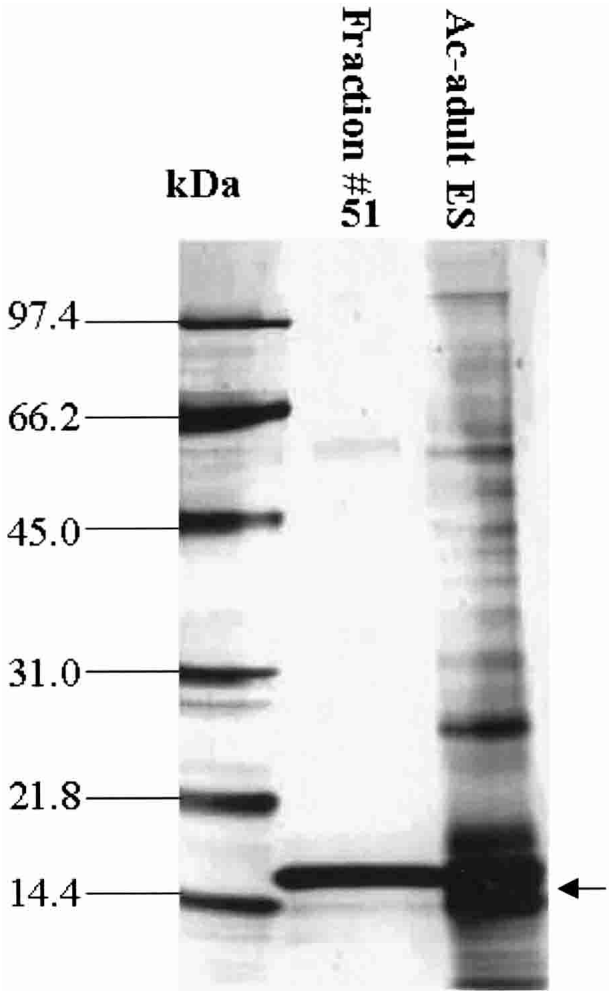

FIGURE 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4–20% gradient gel) of *Ancylostoma caninum* hookworm secretory products and isolated Fraction 51 **(arrow)** after silver staining.

of the total *A. caninum* secretory products. This identified the molecule as one of the most abundant proteins released by adult *A. caninum*. The abundance of Ac-TMP in hookworm secretory products was confirmed by visual inspection on SDS-PAGE (Figure 5). Paired degenerate primers based on the sequence of the first 7 amino acids were used to construct PCR products from the adult hookworm cDNA library. The DNA sequence of the PCR products confirmed the identity to *Ac-tmp* cDNA (data not shown).

DISCUSSION

Ac-TMP from the parasitic nematode *A. caninum* exhibits a number of structural similarities to the N-terminal domains of vertebrate TIMPs, as well as 2 full-length invertebrate putative TIMPs from the free-living nematode *C. elegans*. The 2 *C. elegans* homologs (GenBank numbers AAA96174 and AAA96176) are derived from sequencing of the organism's genome.[19] All 3 nematode TIMPs (from *A. caninum* and *C. elegans*) each lack a region corresponding to the C-terminal domain of the vertebrate TIMPs. This is in contrast to another invertebrate TIMP from *Drosophila* that has both N- and C-terminal domains.[19,20] Mutagenesis studies with recombinant vertebrate TIMPs have identified the N-terminal domain as critical for retaining metalloproteinase inhibitory activity.[21] The N-terminal domain is a discrete protein domain that folds independently of the C-terminal domain and is sufficient for metalloproteinase inhibition; it contains the major sites of interaction with the catalytic N-terminal domain of active matrix metalloproteinases (MMPs).[22] In contrast, the C-terminal domain confers a higher level of regulation or specificity for its MMP target enzyme.[23] On the basis of its single domain structure, Ac-TMP, like the *C. elegans* proteins, may represent a single domain ancestral variant of modern TIMPs.[19]

Ac-TMP is one of the most abundant proteins secreted by adult hookworms. It was estimated that Ac-TMP comprises 6.3% of the total secretory protein. On the basis of our calculation that each adult hookworm secretes 0.63 μg total protein per hour, a single adult hookworm secretes an estimated 40 ng of Ac-TMP per hour.

The function of Ac-TMP released at the site of host attachment in the intestinal mucosa and submucosa is not known. No direct evidence is provided here that Ac-TMP has metalloproteinase inhibitory activity. Although there are strong amino acid sequence homologies between Ac-TMP and the N-terminal domain of human TIMPs, a large number of conserved amino acids are located in regions of the molecule that are not predicted to directly interact with MMPs.[19–23] As shown in Figure 6, with the exception of a critical N-terminal Cys-X-Cys moiety at the N terminus, the major regions of homology between Ac-TMP and human TIMPs 1–4 are located away from the predicted regions of contact with target MMPs. Recent studies suggest that vertebrate TIMPs have functions that are independent of or extend beyond proteinase inhibition including erythroid potentiation,[24] cell growth

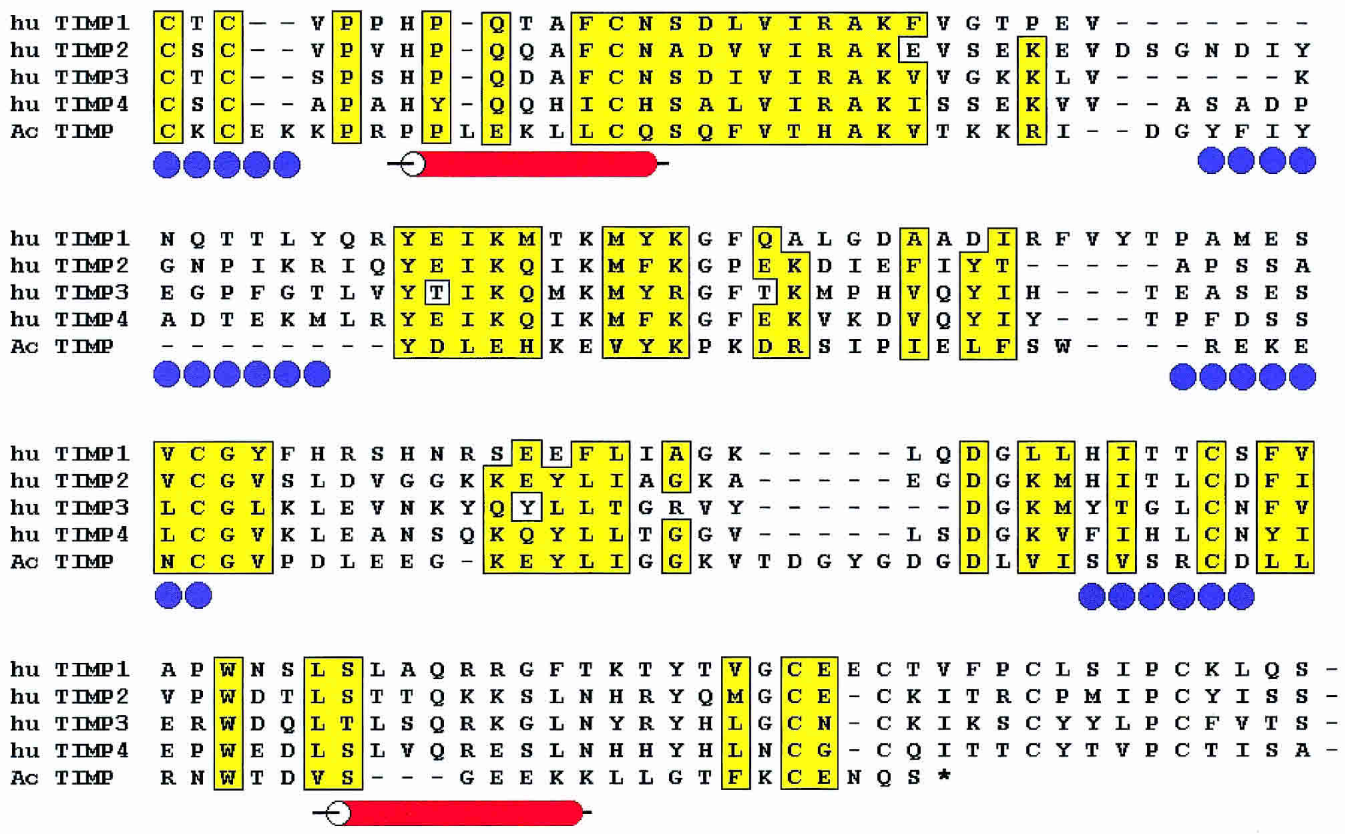

FIGURE 6. Major conserved domains between Ac-TMP and human tissue inhibitor of metalloproteases (TIMPs). Peptide sequences with strong homology are indicated **(highlighted boxes).** Regions identified in the published literature as those that interact directly with matrix metalloproteinases (MMPs) are also indicated **(solid circles).** The cylinders denote predicted regions of alpha helixes.

stimulation,[25] steroidogenesis,[26] and anti-apoptosis.[27] One or more of these roles might ultimately be ascribed to Ac-TMP.

Should Ac-TMP contain metalloproteinase inhibitory activity, it may function as an endogenous inhibitor of the parasite's own 98-kDa metalloendopeptidase (Ac-MEP) that lines the brush border membrane of its alimentary canal. Alternatively, it has been shown previously that host intestinal epithelial cells are rich in matrix metalloprotease 7 (MMP-7), otherwise known as matrilysin.[28] Matrilysin is the predominant MMP used in intestinal tissue injury repair.[28,29] Conceivably, active Ac-TMP secretion may help to modulate the ulcer caused by the parasite at the site of attachment. In addition, MMP-1 and MMP-3 may have roles in intestinal ulceration. It is also possible that Ac-TMP may inhibit neutrophil collagenase or MMP-8.[30] In this way, the hookworm-derived peptide could modulate host inflammation at the site of attachment, just like parasite-derived Mac-1 ligand known as neutrophil inhibitory factor, or NIF.[7] Bioactive Ac-TMP is being expressed in order to ascertain its function *in vivo*. Because Ac-TMP may have a critical role in the host-parasite relationship, the molecule is being evaluated as a possible anti-hookworm vaccine target.[31]

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