

# Molecular Characterization of *Ancylostoma* Inhibitors of Coagulation Factor Xa

HOOKWORM ANTICOAGULANT ACTIVITY *IN VITRO* PREDICTS PARASITE BLOODFEEDING *IN VIVO*\*

Received for publication, October 12, 2001, and in revised form, November 15, 2001  
Published, JBC Papers in Press, December 7, 2001, DOI 10.1074/jbc.M109908200

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**Bloodfeeding hookworms, which currently infect over a billion people in the developing world, are a leading cause of gastrointestinal hemorrhage and iron deficiency anemia. The major anticoagulant inhibitor of coagulation factor Xa has been identified from the hookworm parasite *Ancylostoma ceylanicum* using reverse transcription PCR and 3'-rapid amplification of cDNA ends. This is the first anticoagulant cloned from a hookworm species for which humans are recognized permissive hosts. Despite ~50% amino acid similarity, *A. ceylanicum* anticoagulant peptide 1 (AceAP1) is both immunologically and mechanistically distinct from AcAP5, its homologue isolated from the dog hookworm *Ancylostoma caninum*. Studies using plasma clotting times and single stage chromogenic assays of factor Xa activity have demonstrated that the recombinant AceAP1 protein is substantially less potent than AcAP5 and that soluble whole worm protein extracts of adult *A. ceylanicum* possess less anticoagulant activity than extracts of *A. caninum*. These values correlate with previously reported differences in bloodfeeding capabilities between these two species of hookworm, suggesting that factor Xa inhibitory activity is predictive of hookworm bloodfeeding capabilities *in vivo*. These fundamental differences in the mechanism of action and immunoreactivity of the major anticoagulant virulence factors from related *Ancylostoma* hookworm species may have significant implications for human vaccine development.**

Bloodfeeding hookworms, parasitic nematodes of the gastrointestinal tract, remain a leading cause of anemia throughout much of the developing world (1–3). Although it has been recognized for nearly a century that adult hookworms produce potent inhibitors of mammalian thrombosis (4), only in the past decade have the molecular mechanisms by which these hema-

tophagous parasites block host coagulation been elucidated. To date, the two predominant anticoagulant serine protease inhibitors have been cloned from the dog hookworm *Ancylostoma caninum*. One of these, *A. caninum* anticoagulant peptide 5 (AcAP5)<sup>1</sup> is a potent and specific inhibitor of coagulation factor Xa, whereas the second, AcAPc2, blocks the activity of factor VIIa in complex with membrane-bound tissue factor (5, 6). Both of these hookworm proteins are members of a family of related serine protease inhibitors first identified in the non-bloodfeeding intestinal nematode *Ascaris suum* (7–9).

Although much is known about the anti-thrombotic mechanisms of *A. caninum*, none of the inhibitors of coagulation have been isolated from hookworm species for which humans are naturally permissive hosts. However, anticoagulant activities directed against factor Xa have been identified in soluble hookworm protein extracts (HEX) and excretory/secretory products (ES) from the human hookworms *Necator americanus* (10) and *Ancylostoma ceylanicum* (11), suggesting that secreting inhibitors of the blood coagulation cascade is a well conserved evolutionary bloodfeeding strategy. Interestingly, recent data suggest that there may be significant structural and mechanistic differences between the canine hookworm factor Xa inhibitor AcAP5 and its homologue from *A. ceylanicum* (12). We report here the cloning and characterization of the major inhibitor of coagulation factor Xa from the human hookworm parasite *A. ceylanicum*. Evidence from *in vitro* studies suggests that *A. ceylanicum* anticoagulant peptide 1 (AceAP1), despite significant amino acid sequence homology to its counterpart from the dog hookworm *A. caninum*, is both immunologically and mechanistically distinct.

## EXPERIMENTAL PROCEDURES

**Hookworm Life Cycle and Preparation of HEX and ES**—All animal studies were approved by the Yale University Animal Care and Use Committee. The life cycle of *A. ceylanicum* hookworms was maintained as described previously (13, 14). Soluble adult hookworm extracts (HEX) were prepared by manually homogenizing frozen adult worms in 50 mM Tris-HCl, pH 7.5, using a glass homogenizer (13, 14). Extracts were clarified by centrifugation at 10,000 × *g*, and supernatants were removed and clarified a second time prior to use. Adult hookworm ES products were prepared by incubating live, freshly harvested adult worms in filter-sterilized PBS at 37 °C for 6–8 h. The worms were then

\* This work was supported by National Institutes of Health Grants AI01299 (to M. C.) and AI07404 (to R. B.), a New Investigator award in Molecular Parasitology from the Burroughs Wellcome Fund (to M. C.), and a Hellman Family Fellowship from the Office of the President of Yale University (to M. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF399710.

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<sup>1</sup> The abbreviations used are: AcAP5, *A. caninum* anticoagulant peptide 5; HEX, hookworm protein extracts; ES, excretory/secretory products; AceAP1, *A. ceylanicum* anticoagulant peptide 1; PBS, phosphate-buffered saline; RT, reverse transcription; rpHPLC, reverse phase-high pressure liquid chromatography; ESMS, electrospray ionization mass spectrometry; aPTT, activated partial thromboplastin time; HRP, horseradish peroxidase; Tricine, *N*-tris(hydroxymethyl)methylglycine.

removed, and the remaining ES products were centrifuged at  $3,300 \times g$  to pellet debris.

**Cloning of rAceAP1**—*A. ceylanicum* cDNAs were amplified using RT-PCR of total RNA from adult worms. The 3' primer used for these reactions (5' GTC TCT GTA GAA TCC NTC NTC GCA TAC GCA 3') corresponds to a region of shared amino acid homology near the carboxyl terminus of related serine protease inhibitors from *A. suum* and *A. caninum* (6–8). The 5' primer (5' GT TTA ATT ACC CAA GTT TGA G 3') is a 22-mer that encodes the nematode spliced leader sequence, which has been identified at the 5' end of mRNAs from hookworms and other nematode species (15, 16). Cycles were as follows: 94 °C for 15 s, followed by 40 cycles of 94 °C for 5 s, 50 °C annealing for 5 s, 72 °C extension for 30 s, and a final extension of 72 °C for 2 min. The resulting PCR products were ligated into the pCR2.1 (Invitrogen) plasmid cloning vector, and One Shot INVαF' *Escherichia coli* competent cells (Invitrogen) were transformed with the ligation product as per the manufacturer's protocol. Plasmid DNA from transformed colonies was sent to the William Keck Biotechnology Laboratory at Yale School of Medicine for nucleotide sequencing. Gene sequences were analyzed for homology to other known sequences using the BLAST algorithm through the National Center for Biotechnology Information.

A second PCR using an antisense 3' TTTT primer (5' GGC CAC GCG TCG ACT ACT TTT TTT TTT TTT TTT 3') and a 5' primer designed from known internal sequence (5' GTG GAA AAT CTG TGA AGA AAT GTG GTC TCA ATG AAA GG 3') was used to amplify the complete 3' end of the AceAP1 cDNA. A PCR product of ~380 bp in length was ligated into the pCR2.1 cloning vector as before and transformed into INVαF'-competent cells.

**Expression of rAceAP1 in *E. coli***—The coding sequence for the predicted mature AceAP1 protein was directionally cloned into the bacterial expression plasmid vector pET28a (Novagen), and the resulting ligation product was used to transform ultra-competent *E. coli* strain BL21 (DE3) cells (Stratagene, La Jolla, CA). Recombinant protein expression was induced in 2-liter cultures by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration = 1 mM) and monitored using immunoblots with an HRP-labeled monoclonal antibody (Sigma) to the polyhistidine sequence present on the amino-terminal of the recombinant AceAP1 fusion protein. The recombinant protein was purified from soluble lysates of induced bacterial pellets using a combination of nickel resin affinity chromatography and C<sub>18</sub> rpHPLC as described previously (13). Individual peaks of protein from the C<sub>18</sub> column were collected and assayed for inhibitory activity in the activated partial thromboplastin time (aPTT) and serine protease chromogenic assays as described below. The molar concentration and relative purity of the recombinant AceAP1 were determined using electrospray ionization mass spectrometry (ESMS) and quantitative amino acid analysis as described previously (13, 17).

**Immunoblots Using Day 102 Post-infection Serum or Polyclonal IgG**—Approximately 1 μg of purified recombinant protein (rAceAP-1 or rAcAP5 (17)), 1 μg of adult *A. ceylanicum* ES products, and/or 5 μg of adult *A. ceylanicum* HEX were subjected to SDS-PAGE using a 10% Tricine gel and transferred to nitrocellulose membrane. For detection of antibodies against hookworm proteins following natural infection, the membrane was incubated 16 h at 4 °C in PBS, 5% milk, 0.1% Tween 20 containing a 1:2000 dilution of pooled serum collected from five hamsters that had been followed for 102 days post-infection with 50 third stage (L3) *A. ceylanicum* larvae (18). The blot was washed with PBS, 0.1% Tween 20 and then incubated for 1 h at 25 °C in a 1:5000 dilution of goat anti-hamster HRP-labeled IgG (Sigma) in PBS, 5% milk, 0.1% Tween 20. The blots were washed as described, and the peroxidase label was detected using the West-Pico chemiluminescent substrate (Pierce) and exposure to autoradiography film. For experiments aimed at defining cross-reactive epitopes between the two *Ancylostoma* factor Xa inhibitors, blots of rAceAP1 and rAcAP5 were probed with purified rabbit polyclonal IgG raised against either rAcAP5 or rAceAP1 (12). After washing, the primary antibody was detected using a HRP-labeled goat anti-rabbit IgG.

**Activated Partial Thromboplastin Time Assay**—An aPTT assay (17, 19) was modified for use in a 96-well microtiter plate. Inhibitor (HEX, ES, or purified recombinant protein) was added in duplicate to wells containing 20 μl of aPTT FS reagent (Sigma) and 50 μl of normal human plasma. The total volume per well was adjusted to 80 μl with sterile PBS. After incubating for 15 min at 37 °C, 20 μl of 50 mM CaCl<sub>2</sub> was added to each well to initiate the clotting reaction. Absorbance readings at 630 nm (A<sub>630</sub>) were obtained every 7 s for 3 min using a Dynex MRX HD kinetic microplate reader (Dynex Laboratories, Chantilly, VA). Under these conditions, plots of A<sub>630</sub> versus time (in seconds) demonstrated a highly reproducible sigmoid-shaped curve. By using the

software provided with the microplate reader (Revelation 2.2), the aPTT clotting time result was defined as the time (in seconds) at which clot formation was initiated.

The linear increase in clotting time with inhibitor concentration was then used to derive the concentration of rAceAP1 that causes a doubling of the aPTT clotting time. This doubling time concentration has been used previously to measure the potency of recombinant hookworm anticoagulants (5, 6, 17). The clotting times  $t_C$  versus the corresponding inhibitor concentrations [I] were subjected to least squares linear regression analysis according to Equation 1. The best fit values of the slope  $\alpha_1$  and intercept  $\alpha_0$  were used to calculate the inhibitor concentration [I]<sub>D</sub> required to double the clotting time compared with the control experiment, using Equation 2.

$$t_C = \alpha_0 + \alpha_1[I] \quad (\text{Eq. 1})$$

$$[I]_D = \alpha_0/\alpha_1 \quad (\text{Eq. 2})$$

**In Vitro Assays of Protease Inhibition**—A single stage chromogenic assay was used to characterize the inhibitory activity of rAceAP1 against a panel of serine proteases (5, 13). Purified rAceAP1 was incubated with 100 μl of each enzyme for 15 min at 25 °C. After addition of the appropriate chromogenic substrate, the rate of substrate hydrolysis was measured at 405 nm (mAU/min) over 5 min using a kinetic microplate reader. The final concentrations (200 μl of total volume) of enzymes/substrates were as follows: human coagulation factor Xa (Enzyme Research Laboratories) 250 pM/S2765 (DiaPharma) 250 μM; human α-thrombin (Enzyme Research Laboratories) 1 nM/S2238 (DiaPharma) 250 μM; human kallikrein (Hematologic Technologies, Burlington, VT) 5 nM/S2302 (DiaPharma) 250 μM; human factor XIIa (Hematologic Technologies) 5 nM/S2302 (DiaPharma) 250 μM; α-chymotrypsin (Sigma) 3 nM/Suc-Ala-Ala-Pro-Phe-p-nitroanilide (Bachem) 200 μM; pancreatic elastase (Sigma) 1.5 nM/Suc-Ala-Ala-Pro-Ala-p-nitroanilide (Bachem) 250 μM. Initial results were expressed as percent inhibition using the following formula: percent inhibitory activity = (1 – inhibited rate/uninhibited rate) × 100. Inhibitory activity against the factor VIIa/tissue factor complex was measured using the Actichrome TF Activity Assay kit (American Diagnostica, Inc., Greenwich, CT) according to the manufacturer's instructions.

**Kinetic Analysis of Factor Xa Inhibition**—Factor Xa (final concentration 500 pM) activity was assayed using the substrate S2765 (DiaPharma, final concentration 125 μM). The inhibitor concentration ranged between 0 and 300 nM for rAceAP1 or 0 and 2.5 nM for rAcAP5. Initial reaction velocities (mAU/min at 405 nm) were determined using the platereader software, after which the experimental data (initial velocity versus inhibitor concentration) were subjected to nonlinear least squares regression using two independent software packages, SigmaPlot (SPSS Inc.) and DYNAFIT (BioKin Ltd., Pullman, WA). The mathematical models used in the statistical analysis are described under "Results."

## RESULTS

**Cloning of the rAceAP1 cDNA**—By using a 5'-oligonucleotide primer corresponding to the nematode spliced leader (16, 17) and a degenerate 3'-oligonucleotide primer based on a conserved amino acid sequence from the hookworm anticoagulants AcAP5 (17) and AcAPc2 (6), an initial 303-bp partial cDNA was amplified from adult *A. ceylanicum* RNA using RT-PCR. The complete cDNA corresponding to AceAP1, which was obtained using a 3'-rapid amplification of cDNA ends protocol, is 438 bp in length and includes the putative signal peptide (57 nucleotides/19 amino acids), the mature protein (252 nucleotides/84 amino acids), and a 3'-untranslated region (144 nucleotides) (Fig. 1). The predicted mass of the mature AceAP1 protein is 9626 Da. The site of cleavage of the signal peptide was predicted using the SignalP computer software program (13). An initial BLAST search confirmed amino acid sequence homology to other members of the *Ascaris* family of serine protease inhibitors (7, 8), including the AcAP5 and AcAPc2 anticoagulants from *A. caninum* (6, 17) (Fig. 2).

**Expression and Purification of rAceAP1**—The AceAP1 cDNA was cloned into the pET28a expression vector, and the orientation was confirmed by sequencing of plasmid DNA purified from *E. coli* transformed with the AceAP1/pET28 construct. The soluble lysate from an induced cell pellet contained an

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ATG AAA ACG CTC TAC TTG ATT CCT ATC TGG TTA TTC CTC ATT TCG CAA TGC AGT
Met Lys Thr Leu Tyr Leu Ile Pro Ile Trp Leu Phe Leu Ile Ser Gln Cys Ser

19 ↓ 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
GGA AAA TCT GTG RAG AAA TGT GGT CTC AAT GAA AGG TTG GAC TGT GGT AAT CTA
Gly Lys Ser Val Lys Lys Cys Gly Leu Asn Glu Arg Leu Asp Cys Gly Asn Leu

37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
AAG GCA TGT GAG CGC AAA TGC AAT GAG GAG GAA AGC GAG GAA GAA ATT GAG GCG
Lys Ala Cys Glu Arg Lys Cys Asn Glu Glu Ser Leu Met Cys Glu Ile Glu Ala

55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72
AGA TGC CTC TCA CGT GAT TGT CTT GGT CGT GTT TGC ATG TGC GAT TAC GGA TTC
Arg Cys Leu Ser Arg Asp Cys Leu Gly Arg Val Cys Met Cys Asp Tyr Gly Phe

73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90
TAC AGG AAC AAA GAC GGT GAA TGT GTG ACA GAA GAT GAC TGC GAG TAT GAT AAC
Tyr Arg Asn Lys Asp Gly Cys Val Thr Glu Asp Asp Cys Glu Tyr Asp Asn

91 92 93 94 95 96 97 98 99 100 101 102 *
ATG GAG ATT ACT TCC CCA CCA GAA TCC AAA CAC TGA CCG GTA ATT CCA ACT
Met Glu Ile Ile Thr Phe Pro Pro Glu Ser Lys His stop

CTC GAA CCC CAA TCG AAT GAT CCA ATG CTT CAC TGG CTT CCC TTC ATG TTA GTA
GCT TTG CTT GAT TCT GTG TAT TTG GGC ACT GTC TAG TGA TGA GGA AAG TAA AGC
ATT TCA ACA AAA AAA AAA AAA

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**FIG. 1. Nucleotide and translated amino acid sequence of *AceAP1* cDNA.** The open reading frame of the *AceAP1* cDNA consists of 453 nucleotides from the initial ATG to the 3'-polyadenylation tail. The translated amino acid sequence of *AceAP1* includes a putative 19-amino acid secretory signal sequence, followed by a mature protein of 84 amino acids. The arrow denotes the signal sequence cleavage site predicted using the computer software program SignalP. The 10 cysteine residues are in bold. The predicted molecular mass of the mature *AceAP1* protein is 9,626 Da. These sequence data are available from GenBank™/EMBL/DBJ under accession number AF399710.

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1 10 20 30 40
AceAP1 KSVKKECHNERLDCGNLKAERKNEESEEIEAR...CLSRDC
AcAP5 KAYPECCENELDDCGGKQPKERAKNEEPEEEDPI...CRSRGC
AcAPc2 KATMCCGNEKYSKCGSKECKKCKYDGVSEEDDEEPNVECLVRVC

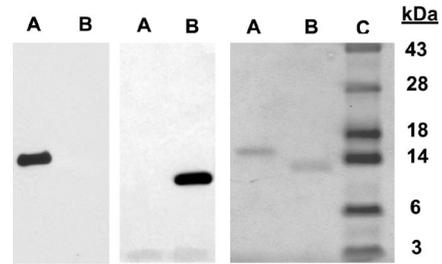
50 60 70 80
AceAP1 LGRVCMCDYGFYRNKD.GEVTEDDCE.YDNMEITFPPESKH
AcAP5 LLLPACVCKDGFYRDVTIGDVRREEDCDQH...EIHV...
AcAPc2 HQD...CVC.EGFYRNKD.DKCVSAEDCE.LDNMDFI.YPGTRN.

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**FIG. 2. Amino acid sequence comparison of hookworm anticoagulants *AceAP1*, *AcAP5*, and *AcAPc2*.** The shaded amino acid residues denote sequence identity, and the boxed residues designate conserved substitutions.

activity that prolonged the aPTT clotting time of human plasma and inhibited the catalytic activity of coagulation factor Xa using a single stage chromogenic activity. The r*AceAP1* protein was then purified to homogeneity using nickel resin affinity chromatography and rpHPLC. Individual protein fractions eluted from the C<sub>18</sub> rpHPLC column were tested for activity using the aPTT clotting time assay and single stage chromogenic assays of factor Xa inhibition. A single protein fraction contained both inhibitory activities, and this material was subjected to ESMS to determine its molecular mass and degree of purity. ESMS revealed a single major protein species with a mass of 13,311 Da, which corresponds to the predicted mass of the translated *AceAP1*/pET28 fusion protein cDNA (13,306 Da).

**The Anticoagulants from *A. ceylanicum* and *A. caninum* Are Immunologically Distinct**—Previous studies using a polyclonal IgG raised against the dog hookworm anticoagulant r*AcAP5* demonstrated species-specific neutralizing activity (12). This purified IgG, although effective at neutralizing the anticoagulant activity of soluble extracts from *A. caninum*, failed to neutralize the comparable activities from extracts of adult *A. ceylanicum*. In light of the significant amino acid sequence homology between the two anticoagulants, we probed immunoblots of both r*AcAP5* and r*AceAP1* with the  $\alpha$ -r*AcAP5* or  $\alpha$ -r*AceAP1* polyclonal IgG to determine the degree to which shared epitopes might be identified in these two functionally related proteins. As shown in Fig. 3, the polyclonal  $\alpha$ -r*AcAP5* IgG failed to recognize r*AceAP1*. Likewise, the  $\alpha$ -r*AceAP1* IgG appropriately recognized r*AceAP1* but not r*AcAP5*. These data suggest that the two proteins share no prominent immunoreactive epitopes.



**FIG. 3. The anticoagulants r*AceAP1* and r*AcAP5* are immunologically distinct.** Recombinant hookworm factor Xa inhibitors were subjected to SDS-PAGE, followed by transfer to nitrocellulose. The blots were probed with polyclonal rabbit IgG raised against r*AceAP1* (left panel) or r*AcAP5* (center panel), followed by an HRP-labeled secondary antibody and detection with chemiluminescent substrate. Lane A, r*AceAP1*; lane B, r*AcAP5*; lane C, molecular mass markers. Panel on right shows identical gel stained with Coomassie.

Pooled serum collected from hamsters 102 days following infection with 50 third stage larvae of *A. ceylanicum* (18) contains antibodies that recognize r*AceAP1* by immunoblot (Fig. 4). This pooled post-infection sera also recognized multiple protein bands present in adult *A. ceylanicum* ES products and HEX. Among those proteins recognized in ES and HEX is a prominent band with an estimated mass of 8–10 kDa, which is the predicted size of the native *AceAP1* protein (9626 Da). Importantly, sera collected from animals prior to hookworm infection failed to recognize r*AceAP1* or any of the hookworm proteins present in soluble extracts or ES products of *A. ceylanicum* (data not shown). These data suggest that hamsters are exposed to *AceAP1* during the course of natural infection and that the native protein is sufficiently immunogenic to stimulate a specific IgG response.

**r*AceAP1* Prolongs the aPTT and Inhibits Coagulation Factor Xa in Vitro**—The anticoagulant activity of the purified r*AceAP1* protein was characterized using a microtiter plate based aPTT clotting time assay. Increasing amounts of purified r*AceAP1* caused a concentration-dependent prolongation in the time to clot formation, as measured by A<sub>630</sub>. As shown in Fig. 5A, a plot of the aPTT clotting time versus r*AceAP1* concentration demonstrates that the anticoagulant effect of the recombinant protein is linear within a concentration range of 20–100 nM. Linear regression analysis was then used to derive the concentration of r*AceAP1* that causes a doubling of the aPTT clotting time (5, 6, 17). Based on extrapolation from the curve shown in Fig. 5A, the doubling time concentration of r*AceAP1* was estimated to be 73 nM. Similar analysis of the data shown in Fig. 5B reveals that the doubling time concentration of r*AcAP5* in this assay is 560  $\mu$ M, suggesting that the anticoagulant from *A. caninum* is more than 100-fold more potent (560  $\mu$ M versus 73 nM) in the aPTT assay than r*AceAP1* from *A. ceylanicum*.

The purified r*AceAP1* protein was then tested for inhibition of a panel of coagulation proteases using a single stage chromogenic assay. The purified r*AceAP1* was found to inhibit the catalytic activity of human factor Xa, with no effect on thrombin, kallikrein, or coagulation factor XIIIa. In addition, the r*AceAP1* protein also failed to inhibit factor VIIa/tissue factor using a commercially available kit assay. Single stage chromogenic assays using serine proteases not involved in coagulation revealed that r*AceAP1* has no inhibitory activity against chymotrypsin or pancreatic elastase.

**r*AceAP1* Inhibits Factor Xa by a Novel Mechanism**—The initial velocities from inhibition of factor Xa by purified r*AceAP1* and r*AcAP5* are shown in Fig. 6. In the first round of statistical analyses, the initial velocities from factor Xa inhibition were fit to the Morrison equation (3, 20) for tight-binding enzyme inhibition, corresponding to a molecular mechanism of

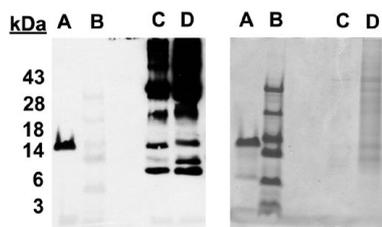


FIG. 4. Serum from hookworm-infected hamsters recognizes rAceAP1. Left panel, following SDS-PAGE and transfer to nitrocellulose, the blot was probed with pooled serum from hamsters at day 102 post-infection with *A. ceylanicum*. Lane A, rAceAP1; lane B, molecular mass markers; lane C, ES products harvested from live adult *A. ceylanicum* hookworms; lane D, soluble protein extracts made from homogenates of adult *A. ceylanicum*. Right panel, identical SDS-PAGE gel stained with Coomassie.

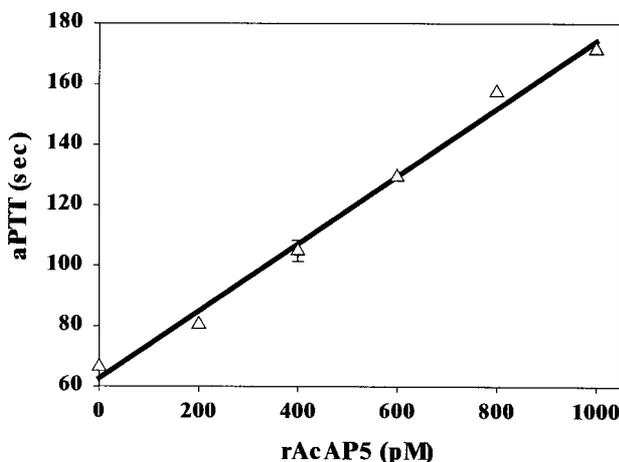
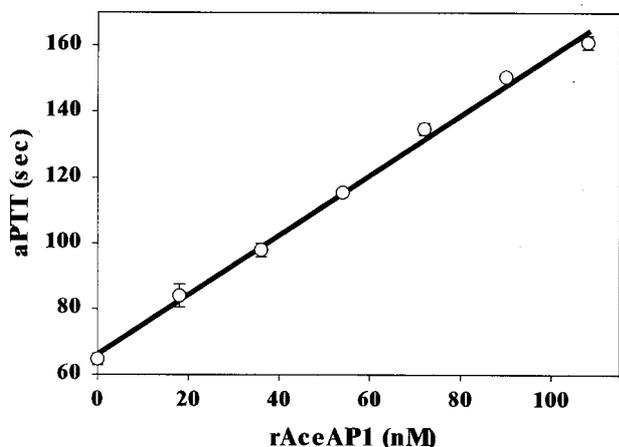
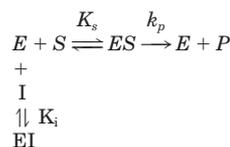


FIG. 5. rAceAP1 and rAcAP5 prolong the aPTT clotting time. Increasing amounts of purified recombinant inhibitor was added to pooled human plasma, and the aPTT clotting time was measured using a microtiter plate-based method. The estimated doubling time concentration for AceAP1 is 73 nM, versus 540 pM for rAcAP5.

protease inhibition represented by reaction Scheme 1 below. Enzyme concentration was held at a constant value  $[E] = 0.5$  nM, whereas  $v_0$  and  $K_{i(\text{app})}$  were treated as adjustable parameters.

$$v = \frac{v_0}{2[E]} \left[ [E] - [I] - K_i^{\text{app}} + \sqrt{([E] - [I] - K_i^{\text{app}})^2 + 4[E]K_i^{\text{app}}} \right] \quad (\text{Eq. 3})$$

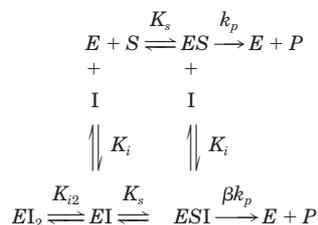
Equation 3 corresponds to a molecular mechanism of protease inhibition represented by reaction Scheme 1.



SCHEME 1

The least squares fit of factor Xa inhibition data Equation 3 is represented by the *solid curve* in Fig. 6a (rAcAP5) and by the *dashed curve* in Fig. 6b (rAceAP1). The *solid curve* in Fig. 6a represents the least squares fit of rAcAP5/factor Xa data to Equation 3, which yielded a best fit value for the apparent inhibition constant ( $K_{i(\text{app})}$ ) of  $0.19 \pm 0.01$  nM. Because rAcAP5 is a competitive inhibitor of factor Xa (6, 17), the true inhibition constant  $K_i = K_{i(\text{app})}/(1 + [S]/K_s) = 0.1$  nM. This is comparable to previously reported values for the  $K_i$  value of rAcAP5 against factor Xa (6, 17). In Fig. 6a, the experimental data and the theoretical curve, based on Equation 3 and Scheme 1, are in close agreement.

In contrast, the least squares fit of inhibitory data for rAceAP1 to Equation 3, represented by the *dashed curve* in Fig. 6b, suggests that the mechanistic Scheme 1 on which Equation 3 is based does not fit the experimental data. A corresponding linearized plot (a modification of the Dixon plot) is shown as a *solid straight line* in Fig. 6c (rAcAP5) and a *dashed straight line* in Fig. 6d (rAceAP1). For rAcAP5 against factor Xa, the modified Dixon plot is a straight line corresponding to reaction Scheme 1. However, the data for rAceAP1 do not fit a straight line. Ultimately, however, an appropriate reaction mechanism that fit the experimental data (Scheme 2) was delineated by using the software DYNAFIT (21).



SCHEME 2

The kinetic mechanism in Scheme 2 is very similar to a mechanism customarily identified in the classic literature (22) as "simple intersecting hyperbolic noncompetitive inhibition." As an added step, Scheme 2 introduces a second binding site for the inhibitor.

The *solid curve* in Fig. 6b shows the best least squares fit to the rate Equation 4. Equation 4 is identical to Segel's mathematical model for "noncompetitive partial inhibition" (22), except for the additional term  $[I]^2/K_i K_{i2}$  in the denominator, which represents the additional binding site.

$$v = [E] \frac{k_p \frac{[S]}{K_s} + \beta k_p \frac{[S][I]}{K_i K_{i2}}}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} + \frac{[S][I]}{K_s K_i} + \frac{[I]^2}{K_i K_{i2}}} \quad (\text{Eq. 4})$$

The following parameters were held at constant or fixed values in the regression:  $[E] = 0.5$  nM,  $[S] = 125$   $\mu$ M, and  $K_s = 32.5$   $\mu$ M. The best fit values and associated formal standard errors for optimized parameters are as follows:  $k_p = (43.2 \pm 0.6)$   $\text{min}^{-1}$ ;  $\beta = (0.38 \pm 0.01)$ ;  $K_i = (2.0 \pm 0.6)$  nM; and  $K_{i2} = (690 \pm 100)$  nM. In other words, the ternary complex enzyme-substrate-inhibitor seems to retain  $\sim 40\%$  reactivity compared with the binary Michaelis complex (enzyme-substrate). In terms of inhibition constants, the secondary binding site ( $K_{i2} = 0.7$   $\mu$ M) appears

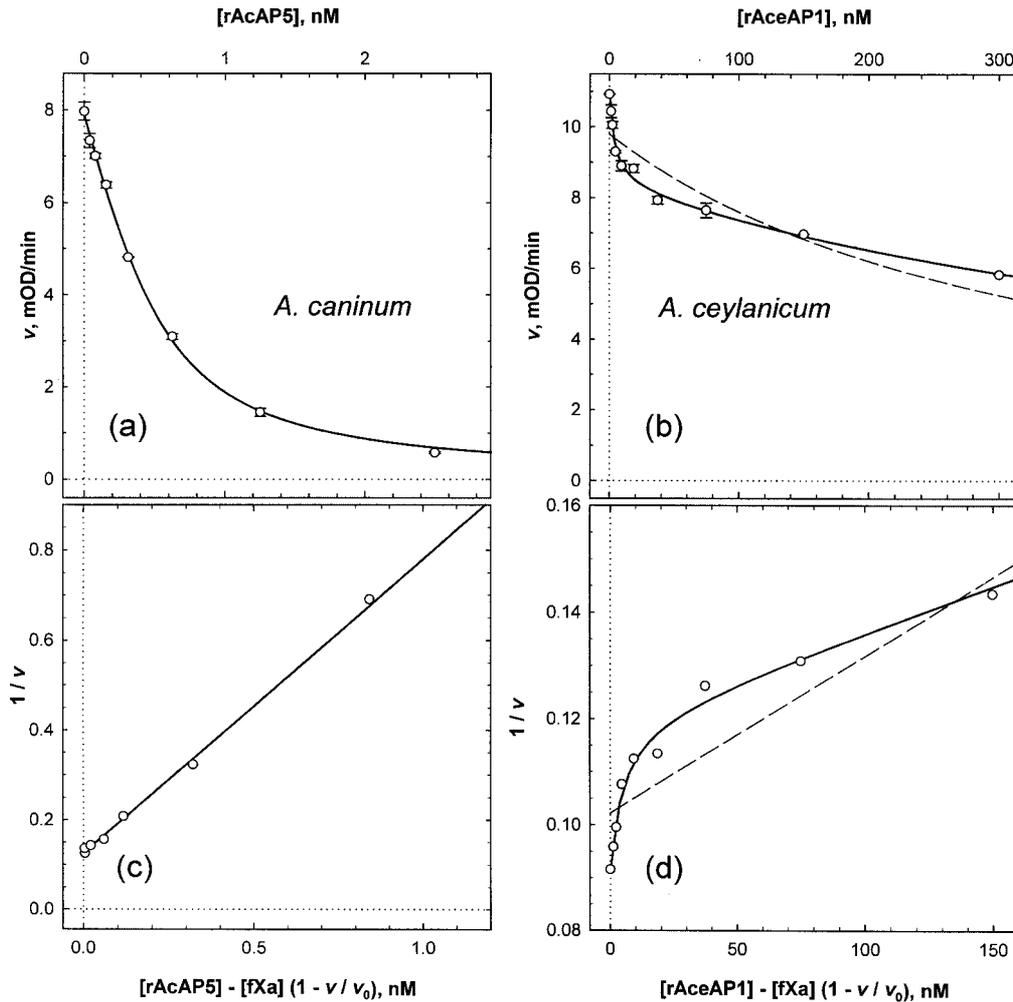


FIG. 6. **rAceAP1 inhibits factor Xa by a novel mechanism.** Factor Xa-mediated cleavage of chromogenic substrate (500 pM total enzyme; 125  $\mu$ M substrate) was measured in the presence of increasing amounts of the purified inhibitors. *a*, direct plot of rate data for rAcAP5; the *solid curve* represents best least squares fit to Equation 3. *b*, direct plot for rAceAP1; the *solid and dashed curves* represent the best least squares fit to Equations 4 and 3, respectively. *c* and *d*, transformed diagnostic plots corresponding to panels *a* and *b*. For a simple competitive inhibition mechanism, the diagnostic plot is expected to be strictly linear.

almost 3 orders of magnitude weaker than the main inhibitor-binding site ( $K_i = 2$  nM).

#### DISCUSSION

Despite the availability of broad spectrum anthelmintic agents with activity against intestinal nematodes, hookworm infection remains a leading cause of anemia and growth delay in much of the developing world (23). Although community-based interventions utilizing chemotherapy have been shown to improve weight gain and anemia, these benefits appear to be short lived unless anthelmintics are administered repeatedly (24, 25). Moreover, recent reports (26, 27) of documented *in vitro* resistance of human hookworm isolates to mebendazole and pyrantel pamoate suggest that mass chemotherapy programs may not be a viable long term approach to the control of hookworm in endemic areas. As a result, there has been renewed interest in the development of vaccines as a strategy for controlling hookworm infection and disease worldwide (28). In an effort to identify potential targets for hookworm vaccine development, much of our work has focused on identifying the major parasite-derived factors involved in the hookworm pathogenesis, including anticoagulants and inhibitors of platelet function (29). Because the major clinical sequela of hookworm infection is iron-deficiency anemia, which occurs as a direct effect of parasite bloodfeeding, the major anti-thrombot-

ics secreted by the adult stage of the parasite represent important targets for potential vaccine development.

We report here for the first time the cloning of an anti-thrombotic from a bloodfeeding hookworm species for which humans are naturally permissive hosts (30–32). Isolation of the factor Xa inhibitor from *A. ceylanicum* was accomplished using a molecular approach that took advantage of a region of conserved amino acid sequence from previously identified members of the *Ascaris* family of serine protease inhibitors. By using RT-PCR, we successfully amplified a cDNA whose translated amino acid sequence showed limited homology to the anticoagulants from *A. caninum*, AcAP5 and AcAPc2. The 40–44% amino acid sequence identity (52–55% similarity) to the two AcAP sequences, in addition to the conservation and alignment of the 10 cysteine residues, which are known to play a critical role in defining the tertiary molecular structure of the *Ascaris* type inhibitors (9, 33, 34), suggests that AceAP1 is, in fact, a member of this family of nematode proteins. In addition to those from *Ancylostoma* hookworms, *Ascaris*-type inhibitors have also been identified other nematode species, including *A. suum* (7, 8), *Anisakis simplex* (35), and *Trichuris muris* (36). In contrast to the hookworm anticoagulants, the *Ascaris* inhibitors from these non-bloodfeeding nematodes have been found to inhibit primarily intestinal serine proteases. These data sug-

gest that divergent nematode species have taken advantage of the fundamental protein backbone of the *Ascaris* inhibitor family to evolve specific molecules with inhibitory activities that suit their particular ecological niche.

We have demonstrated recently that a polyclonal IgG raised against the recombinant AcAP5 anticoagulant from the dog hookworm *A. caninum* effectively neutralized the anticoagulant activity of adult hookworm extracts *in vitro* (12). Interestingly, however, this antibody failed to neutralize the similar anti-factor Xa activity present in soluble extracts of the human parasite *A. ceylanicum*. Data from immunoblot experiments reported here are consistent with this previous observation, in that the  $\alpha$ -rAcAP5 IgG fails to recognize rAcAP1 (Fig. 3). Similar experiments demonstrate that a polyclonal IgG directed against rAcAP1 does not recognize rAcAP5, confirming the lack of shared immunoreactive epitopes between the two anticoagulants from *Ancylostoma* hookworms. This is particularly interesting in light of the degree of sequence homology and presumed similarity in tertiary protein structure, in that it suggests that there are few if any shared immunodominant epitopes between the two *Ancylostoma* anticoagulants. Moreover, it suggests that this lack of immunoreactivity may form the basis for the inability of the polyclonal  $\alpha$ -rAcAP5 IgG to neutralize the anticoagulant activity from *A. ceylanicum* (12).

We have demonstrated that by day 102 post-infection with *A. ceylanicum* L3 larvae, hamsters develop antibodies that recognize the recombinant protein by immunoblot (Fig. 3). Interestingly, when this same serum is used to probe soluble protein extracts or ES products from adult *A. ceylanicum*, a band of 8–10 kDa, which approximates the predicted mass of the native AceAP1 protein (9,626 Da), is also recognized (Fig. 4). In light of the fact that serum from infected animals clearly recognizes the recombinant protein, it is certainly possible that at least one component of this 9-kDa band represents the native anticoagulant. This observation is of particular significance in light of our recent report (14) that passive transfer of this day 102 post-infection serum to naive animals is associated with partial protection against anemia and growth delay following infection with *A. ceylanicum* L3 larvae. Because AceAP1 constitutes one of the antigens recognized by this serum, it will be important to determine whether antibodies raised against the recombinant anticoagulant alone will also confer resistance to hookworm disease.

In addition to differences in immunoreactivity, the two hookworm inhibitors of coagulation factor Xa also differ mechanistically. By using the aPTT clotting time, rAcAP1 was found to be ~100-fold less potent than AcAP5 from *A. caninum* (Fig. 5). This difference in anticoagulant activity was corroborated in studies using a single stage chromogenic assay of purified coagulation factor Xa activity. Detailed analysis of the inhibitory kinetics of these two hookworm anticoagulants demonstrates that the mechanisms of action of rAcAP5 and rAcAP1 are most certainly distinct. While single stage chromogenic assay data using the dog hookworm anticoagulant rAcAP5 are consistent with a competitive, active site-mediated inhibitory mechanism (Fig. 6a), the data for rAcAP1 do not fit this inhibitory scheme (Fig. 6b). Ultimately, by using the computer software program DYNAFIT (21), a more complex equation was derived that satisfactorily fits the inhibitory data. The results are strongly suggestive of the presence of at least two enzyme inhibitory sites on the rAcAP1 molecule, each with distinct affinities for factor Xa (2 nM and 0.7  $\mu$ M, respectively). It is important to note that the inhibition mechanism proposed here represents a working hypothesis consistent with the experimental data available at this time and does not exclude the

possibility that other inhibitory mechanisms may ultimately be identified.

The DYNAFIT method has been used previously (37, 38) to characterize complex inhibitory mechanisms. In the example perhaps most relevant to the mechanism proposed for AceAP1, Stanley *et al.* (39) used DYNAFIT to study the inhibition of vitamin K-dependent  $\gamma$ -glutamylcarboxylase by various polypeptides important in the blood coagulation cascade. The DYNAFIT method for model discrimination predicted a complex mechanism, in which the inhibitors bind at two separate binding sites, whereas the resulting  $E\cdot I_2$  complex retains catalytic activity. Similar inhibitory mechanisms have been described for other hydrolytic enzymes, including D-fructose-1,6-bisphosphate 1-phosphohydrolase (40), ribosomal peptidyl-transferase (41), and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (42).

It is noteworthy that the  $E\cdot I_2$  complex in Scheme 2 does not retain any catalytic activity, but the  $E\cdot I$  complex does. In structural terms, this raises the possibility that factor Xa contains exactly two binding sites for the rAcAP1 inhibitor. When only one site is fully saturated, the enzyme still retains at least a partial catalytic activity. However, when both sites are fully occupied, factor Xa activity is neutralized. This may signify that one of the inhibitor binding sites on factor Xa recognizes its natural physiologic substrate (prothrombin), whereas the other site does not. Ongoing studies will ultimately allow for the complete characterization of the molecular basis for the two-site partial non-competitive inhibition of factor Xa by rAcAP1, including site-directed mutagenesis and elucidation of the structure of rAcAP1-factor Xa complex using x-ray crystallography.

To date, the molecular structures of five members of the *Ascaris* family of serine protease inhibitors have been elucidated. Evidence suggests that four of these (9, 34, 43–44), including two from the intestinal nematode *A. suum*, interact with their target proteases in a canonical substrate-like fashion. According to this model, the inhibitor's P1 reactive site amino acid interacts directly with the catalytic site of the protease, leading to formation of a stable enzyme-inhibitor complex that dissociates slowly upon cleavage of the P1 peptide bond. In contrast, the structure of rAcAPc2, the inhibitor of the factor VIIa-tissue factor complex from *A. caninum*, reveals significant flexibility at both the amino- and carboxyl-terminal segments of the molecule, allowing for possible secondary interactions (9). These and other functional studies suggest that rAcAPc2 first interacts with factor Xa via an exosite and then binds to the factor VIIa-tissue factor complex at its catalytic domain (45).

Data presented here and elsewhere (6) demonstrate that rAcAP5 most likely inhibits human coagulation factor Xa by a mechanism similar to that of the *Ascaris* trypsin and chymotrypsin/elastase inhibitors. In contrast, however, it is clear that the inhibitory mechanism of rAcAP1 involves non-active site-mediated interactions with factor Xa, raising the possibility that its interaction with the enzyme may be similar to AcAPc2. Work is currently underway to characterize the specific epitopes of factor Xa that are targeted by rAcAP1, to more clearly elucidate its potentially novel mechanism of anticoagulant activity.

We have attempted to correlate the mechanistic differences between AceAP1 and ACAP5, clearly observed in the kinetic assays of purified inhibitors, with an analogous experiment involving soluble extracts from the corresponding worm species. Data from these kinetic experiments revealed that factor Xa was inhibited by soluble extracts both from *A. caninum* and from *A. ceylanicum* (not shown). Importantly, however, the two

dose-response curves ( $\mu\text{g}$  of worm extract *versus* residual factor Xa activity) exhibited distinctly different shapes. Although the dose-response curve for *A. caninum* HEX decreased asymptotically to zero, the dose-response curve for *A. ceylanicum* leveled off at about 50% residual activity. The shape of these curves closely resembled those constructed using the purified recombinant inhibitors (Fig. 6). This qualitative observation suggests that AceAP1 is the predominant if not only factor Xa inhibitor present in *A. ceylanicum* HEX. However, a truly quantitative interpretation of these data is not possible due to the fact that exact relative concentrations of native AceAP1 and rACAP5 peptides in the two extracts cannot be measured at this time.

Although the difference in anticoagulant activity between *A. caninum* and *A. ceylanicum* extracts has not been reported previously, these *in vitro* findings are consistent with prior studies of adult hookworm bloodfeeding activity *in vivo*. By using direct measurements of blood expelled from adult hookworms attached to the intestine of anesthetized dogs, Wang *et al.* (46) estimated that each adult female *A. caninum* causes ~43.1 ml of blood loss per day. In contrast, it was demonstrated that *A. ceylanicum* adult females cause only 7.8 ml of blood loss per day. Further work by Rep (47) estimated a similar ratio of the relative amount of blood loss per worm between *A. caninum* (43  $\mu\text{l}$ ) and *A. ceylanicum* (14  $\mu\text{l}$ ). In light of the previously reported differences, these data constitute the first experimental evidence that differences in hookworm bloodfeeding *in vivo* may be explained by differences in the relative *in vitro* activity of specific anticoagulants.

One potential limitation of these studies is the fact that only human plasma and coagulation factors were evaluated, raising the possibility that there might be differences in anticoagulant activity and mechanism of action of rACAP5 and rAceAP1 based on the species of enzyme used. However, although previously identified inhibitors of factor Xa have been shown to demonstrate species variability in anticoagulant activity (48), these differences are generally modest and have not been shown to correlate with any alteration in the mechanism of inhibition. Moreover, if such an effect were to be present, one would expect that the dog hookworm anticoagulant AcAP5 would be more adapted to canine factor Xa, whereas rAceAP1 would have greater affinity for human factor Xa. In contrast, our data demonstrate that rACAP5 is substantially more active against human factor Xa than rAceAP1. This suggests that the dramatic difference in activity and inhibitory mechanism of the two hookworm anticoagulants likely has little to do with the species of factor Xa examined.

The studies presented here confirm that the hookworm anticoagulants AceAP1 and AcAP5 are distinct in terms of affinity for factor Xa, anticoagulant activity using the aPTT clotting time, and immunoreactivity. If such differences exist between the hookworms of the closely related species *A. caninum* and *A. ceylanicum* (49), then it is quite possible that the differences between anticoagulants from *Ancylostoma* and *Necator* will be even less closely related. As a result, effective recombinant human vaccines may need to include antigens that specifically target those species known to be endemic to certain geographic regions. Otherwise, the use of species-limited vaccines may not confer significant protection in all endemic areas. Additional work on other adult hookworm secretory products from various *Ancylostoma* species (19), including anti-platelet agents (29) and intestinal serine protease inhibitors (13), will ultimately add to our understanding of the evolutionary divergence of specific hookworm virulence factors.

*Acknowledgments*—The Millennium Institute for Fundamental and Applied Biology is financed in part by MIDEPLAN (Chile). We thank

Eva Campodonico for technical assistance with clotting time assays and Sarah McCord (Health Sciences Library, Washington State University) for helpful discussions and a careful reading of the manuscript.

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