

**Molecular characterization of *Ancylostoma* inhibitors of coagulation factor Xa: hookworm anticoagulant activity *in vitro* predicts parasite bloodfeeding *in vivo*.**

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## SUMMARY

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' RACE. This is the first anticoagulant cloned from a hookworm species for which humans are recognized permissive hosts. Despite approximately 50% amino acid similarity, *Ancylostoma ceylanicum* Anticoagulant Peptide 1 (AceAP1) is both immunologically and mechanistically distinct from AcAP5, its homologue isolated from the dog hookworm *A. 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.

## INTRODUCTION

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 hematophagous 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, *Ancylostoma caninum* Anticoagulant Peptide 5 (AcAP5) is a potent and specific inhibitor of coagulation factor Xa, while 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* (7,8,9)

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 *Ancylostoma 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 *Ancylostoma ceylanicum* hookworms was maintained as previously described (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 x g and supernatants 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 hours. The worms were then removed and the remaining ES products centrifuged at 3,300 x g to pellet debris.

*Cloning of rAceAPI-* *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 a related serine protease inhibitors from *Ascaris suum* and *Ancylostoma caninum* (6-8). The 5' primer (5' GT TTA ATT ACC CAA GTT TGA G 3') is a 22mer 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 sec, followed by 40 cycles of 94°C for 5 sec, 50°C annealing for 5 sec, 72°C extension for 30 sec and a final extension of 72°C for 2 minutes. The resulting PCR products were ligated into the pCR2.1 (Invitrogen) plasmid cloning vector, and One Shot INVαF' *E. coli* competent cells (Invitrogen) were transformed with the ligation product as per 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 approximately 250 base pairs in length was ligated into the pCR2.1 cloning vector as before and transformed into INV $\alpha$ F' 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 IPTG (final concentration=1 mM) and monitored using immunoblots with a HRP-labeled monoclonal antibody (Sigma) to the poly-histidine sequence present on the N-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 previously described (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 was determined using Electrospray Ionization Mass Spectrometry (ESMS) and quantitative amino acid analysis as previously described (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 hours at 4°C in PBS/5% milk/0.1% Tween-20 containing a 1:2000 dilution of pooled serum collected from 5 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 hour 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 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 sheep anti-rabbit IgG.

*Activated partial thromboplastin time assay-* An activated partial thromboplastin time (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  $\mu$ l of normal human plasma. The total volume per well was adjusted to 80  $\mu$ l with sterile PBS. After incubating for 15 minutes at 37°C, 20  $\mu$ l of 50 mM CaCl<sub>2</sub> was added to each well in order to initiate the clotting reaction. Optical density readings at 630 nm (OD<sub>630</sub>) were obtained every 7 seconds for 3 minutes using a Dynex MRX HD kinetic microplate reader (Dynex Laboratories, Chantilly, VA). Under these conditions, plots of OD<sub>630</sub> vs. time (sec) demonstrated a highly reproducible sigmoid shaped curve. Using the software provided with the microplate reader (Revelation 2.2), the aPTT clotting time result was defined as the time (sec) 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 previously been used to measure the potency of recombinant hookworm anticoagulants (5, 6, 17). The clotting times  $t_C$  vs. the corresponding inhibitor concentrations  $[I]$  were subjected to least-squares linear regression analysis according to equation [1] (below). The best-fit values of the slope  $a_1$  and intercept  $a_0$  were used to calculate the inhibitor concentration  $[I]_D$  required to double the clotting time compared to the control experiment, using equation [2].

$$t_C = a_0 + a_1 [I] \quad [1]$$

$$[I]_D = a_0 / a_1 \quad [2]$$

*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  $\mu$ l of each enzyme for 15 minutes at 25°C. After

addition of the appropriate chromogenic substrate, the rate of substrate hydrolysis was measured at 405 nm (mOD/min) over 5 minutes using a kinetic microplate reader. The final concentrations (200  $\mu$ l total volume) of enzymes/substrates were as follows: human coagulation factor Xa (Enzyme Research Laboratories) 250 pM /S2765 (DiaPharma) 250  $\mu$ M; human  $\alpha$ -thrombin (Enzyme Research Laboratories) 1 nM /S2238 (DiaPharma) 250  $\mu$ M; human kallikrein (Haematologic Technologies, Burlington, VT) 5 nM / S2302 (Diapharma) 250  $\mu$ M; human factor XIIa (Haematologic Technologies) 5 nM/ S2302 (Diapharma) 250  $\mu$ M;  $\alpha$ -chymotrypsin (Sigma) 3 nM/Suc-Ala-Ala-Pro-Phe-pNA (Bachem) 200  $\mu$ M; pancreatic elastase (Sigma) 1.5 nM/Suc-Ala-Ala-Pro-Ala-pNA (Bachem) 250  $\mu$ M. Initial results were expressed as percent inhibition using the following formula: percent inhibitory activity =  $(1 - \text{inhibited rate}/\text{uninhibited rate}) \times 100$ . 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  $\mu$ M). The inhibitor concentration ranged between 0 and 300 nM for rAceAP1, or 0 and 2.5 nM for rAcAP5. Initial reaction velocities (mOD/min at 405nm) were determined using the plate-reader software, after which the experimental data (initial velocity vs. 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 in the RESULTS section.

## RESULTS

*Cloning of the rAceAPI cDNA*- 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 base pair partial cDNA was amplified from adult *A. ceylanicum* RNA using RT-PCR. The complete cDNA corresponding to AceAPI, which was obtained using a 3' RACE protocol, is 438 base pairs in length, and includes the putative signal peptide (54 nucleotides/18 amino acids), the mature protein (252 nucleotides/84 amino acids) and a 3' untranslated region (132 nucleotides) (Fig. 1). The predicted MW of the mature AceAPI 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 rAceAPI*- The *AceAPI* cDNA was cloned into the pET28a expression vector, and the orientation confirmed by sequencing of plasmid DNA purified from *E. coli* transformed with the *AceAPI*/pET28 construct. The soluble lysate from an induced cell pellet contained an 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 rAceAPI 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 in order 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 rAcAP5 demonstrated species specific neutralizing activity (12). This purified IgG, while 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 rAcAP5 and rAceAP1 with the  $\alpha$ -rAcAP5 or  $\alpha$ -rAceAP1 polyclonal IgG in order to determine the degree to which shared epitopes might be identified in these two functionally related proteins. As shown in Fig. 3, the polyclonal  $\alpha$ -rAcAP5 IgG failed to recognize rAceAP1. Likewise, the  $\alpha$ -rAceAP1 IgG appropriately recognized rAceAP1, but not rAcAP5. These data suggest that the two proteins share no prominent immunoreactive epitopes.

Pooled serum collected from hamsters 102 days following infection with 50 third stage larvae of *A. ceylanicum* (18) contains antibodies that recognize rAceAP1 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 MW of 8-10 kDa, which is the predicted size of the native AceAP1 protein (9,626 Da). Importantly, sera collected from animals prior to hookworm infection failed to recognize rAceAP1, nor 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.

*rAceAP1 prolongs the aPTT and inhibits coagulation factor Xa in vitro-* The anticoagulant activity of the purified rAceAP1 protein was characterized using a microtiter plate based aPTT clotting time assay. Increasing amounts of purified rAceAP1 caused a concentration-dependent prolongation in the time to clot formation, as measured by OD<sub>630</sub>. As shown in Fig. 5A, a plot of the aPTT clotting time vs rAceAP1 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 rAceAP1 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 rAceAP1 was estimated to be 73 nM. Similar analysis of the data shown in Fig. 5B reveals that the doubling time concentration of rAcAP5 in this assay is 560 pM, suggesting that the anticoagulant from *A. caninum* is more than 100 fold more potent (560 pM vs. 73 nM) in the aPTT assay than rAceAP1 from *A. ceylanicum*.

The purified rAceAP1 protein was then tested for inhibition of a panel of coagulation proteases using a single stage chromogenic assay. The purified rAceAP1 was found to inhibit the catalytic activity of human factor Xa, with no effect on thrombin, kallikrein or coagulation factor XIIIa. In addition, the rAceAP1 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 rAceAP1 has no inhibitory activity against chymotrypsin or

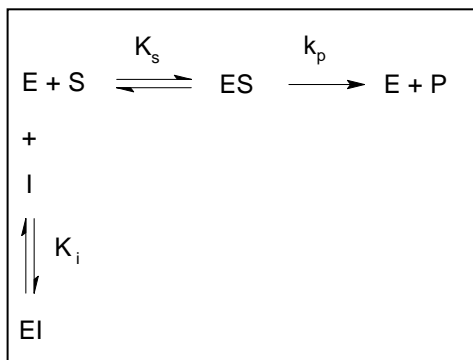
pancreatic elastase.

*rAceAP1 inhibits factor Xa by a novel mechanism-* The initial velocities from inhibition of factor Xa by purified rAceAP1 and rAcAP5 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 protease inhibition represented by reaction Scheme 1 below. Enzyme concentration was held at a constant value  $[E] = 0.5 \text{ nM}$ , whereas  $v_0$  and  $K_i^{\text{app}}$  were treated as adjustable parameters.

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

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

Scheme 1.

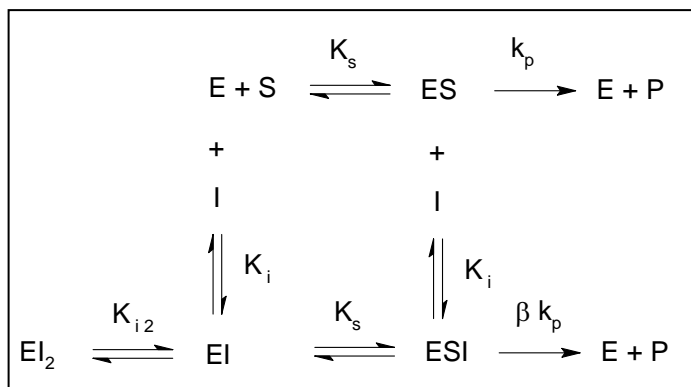


The least-squares fit of factor Xa inhibition data equation [3] is represented by the solid curve in Figure 6a (rAcAP5) and by the dashed curve in Figure 6b (rAceAP1). The solid curve in Fig. 6a represents the least-squares fit of rAcAP5/factor Xa data to equation [3], which yielded a best-fit value for the apparent inhibition constant ( $K_i^{\text{app}}$ ) of  $0.19 \pm 0.01 \text{ nM}$ . Since rAcAP5 is a competitive inhibitor of factor Xa (6, 17), the true inhibition constant  $K_i = K_i^{\text{app}} / (1 + [S] / K_s) = 0.1 \text{ nM}$ . This is comparable to previously reported values for the  $K_i$  of rAcAP5 against factor Xa (6,17). In Figure 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 line in Figure 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 Figure 6c (rAcAP5) and a dashed straight line in Figure 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"

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

(22), except for the additional term  $[I]^2/K_iK_{i2}$  in the denominator, which represents the additional binding site. The following parameters were held at constant or fixed values in the regression:  $[E] = 0.5 \text{ nM}$ ,  $[S] = 125 \text{ }\mu\text{M}$ ,  $K_s = 32.5 \text{ }\mu\text{M}$ . The best fit values and associated formal standard errors for optimized parameters were as follows:  $k_p = (43.2 \pm 0.6) \text{ min}^{-1}$ ;  $\beta = (0.38 \pm 0.01)$ ;  $K_i = (2.0 \pm 0.6) \text{ nM}$ ; and  $K_{i2} = (690 \pm 100) \text{ nM}$ . In other words, the ternary complex enzyme•substrate•inhibitor seems to retain approximately 40% reactivity compared to the binary Michaelis complex (enzyme•substrate). In terms of inhibition constants, the secondary binding site ( $K_{i2} = 0.7 \text{ }\mu\text{M}$ ) appears almost three orders of magnitude weaker than the main inhibitor-binding site ( $K_i = 2 \text{ nM}$ ).

## 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 of documented *in vitro* resistance of human hookworm isolates to mebendazole and pyrantel pamoate suggests that mass chemotherapy programs may not be a viable long-term approach to the control of hookworm in endemic areas (26,27). 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-thrombotics 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. 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), suggest 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 *Ascaris 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 primarily inhibit intestinal serine proteases. These data suggest 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 recently demonstrated 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 rAceAP1 (Fig. 3). Similar experiments demonstrate that a polyclonal IgG directed against rAceAP1 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 MW 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 that passive transfer of this day 102 post-infection serum to naïve animals is associated with partial protection against anemia and growth delay following infection with *A. ceylanicum* L3 larvae (14). 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. Using the aPTT clotting time, rAceAP1 was found to be approximately 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 rAceAP1 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 rAceAP1 do not fit this inhibitory scheme (Fig. 6b). Ultimately, 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 rAceAP1 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 previously been used (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  $\gamma$ -glutamyl carboxylase 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, while the resulting  $E \bullet I_2$  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 [DAHPS-(Trp)] (42).

It is noteworthy that the EI<sub>2</sub> complex in Scheme 2 does not retain any catalytic activity, but the EI complex does. In structural terms, this raises the possibility that Factor Xa contains exactly two binding sites for the rAceAP1 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), while 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 rAceAP1, including site-directed mutagenesis and elucidation of the structure of rAceAP1/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 *Ascaris 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 rAceAP1 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 in order to characterize the specific epitopes of factor Xa that are targeted by rAceAP1, in order 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 vs. residual Factor Xa activity) exhibited distinctly different shapes. While 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 previously been reported, these *in vitro* findings are consistent with prior studies of adult hookworm bloodfeeding activity *in vivo*. Using direct measurements of blood expelled from adult hookworms attached to the intestine of anesthetized dogs, Wang et al estimated that each adult female *A. caninum* causes approximately 43.1 cc of blood loss per day. In contrast, it was demonstrated that *A. ceylanicum* adult females cause only 7.8 cc of blood loss per day (46). Further work by Rep (47) estimated a similar ratio of the relative amount of blood loss per worm between *A. caninum* (43  $\mu$ l) and *A. ceylanicum* (14  $\mu$ 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, while 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, while 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.

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## FIGURE LEGENDS

Figure 1. Nucleotide and translated amino acid sequence of *AceAP1* cDNA: The open reading frame of the *AceAP1* cDNA consists of 432 nucleotides from the initial ATG to the 3' polyadenylation tail. The translated amino acid sequence of AceAP1 includes a putative 18 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 weight of the mature AceAP1 protein is 9626 Da. These sequence data are available from GenBank/EMBL/DDBJ under accession number AF399710.

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

Figure 3. The anticoagulants rAceAP1 and rAcAP5 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 rAceAP1 (left panel) or rAcAP5 (center panel), followed by a HRP-labeled secondary antibody and detection with chemiluminescent substrate. Lane A: rAceAP1; Lane B: rAcAP5; Lane C: Molecular weight markers. Panel on right shows identical gel stained with Coomassie.

Figure 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 weight 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.

Figure 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 measured using a microtiter plate based method. The estimated doubling time concentration for AceAP1 is 73 nM, versus 540 pM for rAcAP5.

Figure 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 represent best least-squares fit to equation [3]. (b) Direct plot for rAceAP1; the solid and dashed curves represents the best least-squares fit to equation [4] and [3], respectively. (c), (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.

**Figure 1.**

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	AAG	AAA	TGT	GGT	CTC	AAT	GAA	AGG	TTG	GAC	TGT	GGT	AAT	CTA	
Gly	Lys	Ser	Val	Lys	Lys	<b>Cys</b>	Gly	Leu	Asn	Glu	Arg	Leu	Asp	<b>Cys</b>	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	<b>Cys</b>	Glu	Arg	Lys	<b>Cys</b>	Asn	Glu	Glu	Glu	Ser	Glu	Glu	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	<b>Cys</b>	Leu	Ser	Arg	Asp	<b>Cys</b>	Leu	Gly	Arg	Val	<b>Cys</b>	Met	<b>Cys</b>	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	Glu	<b>Cys</b>	Val	Thr	Glu	Asp	Asp	<b>Cys</b>	Glu	Tyr	Asp	Asn	
91	92	93	94	95	96	97	98	99	100	101	102	*						
ATG	GAG	ATT	ATT	ACT	TTC	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	AAA											

Figure 2.

	1	10	20	30	40																
<b>AceAP1</b>	K	SVKK	CG	LN	ERL.D	CG	NL	KA	CE	RK	CNE	E	ES	EEE	I	EAR....	CL	SR	DC		
<b>AcAP5</b>	K	AYPE	CG	EN	EWLDD	CG	TQ	KP	CE	AK	CNE	E	PP	EEE	D	PI....	CR	SR	GC		
<b>AcAPc2</b>	K	ATMQ	CG	EN	EKYDS	CG	S.	KE	CD	KK	CKY	D	GV	EEE	D	DEEPNVP	CL	V	RV		
	50	60	70	80																	
<b>AceAP1</b>	L	GRV	CM	CDY	GF	YR	NKD	GE	CV	TE	DD	CE	.	YDNM	E	I	I	TF	PP	ES	KH
<b>AcAP5</b>	LL	PPA	CV	CKD	GF	YR	DTVIGD	CV	RE	EE	CD	QH...	E	I	I	HV.....					
<b>AcAPc2</b>	HQD..	CVC	.	E	GF	YR	NKD	DK	CV	SA	ED	CE	.	LDNM	D	F	I	.	YP	G	TRN.

**Figure 3.**

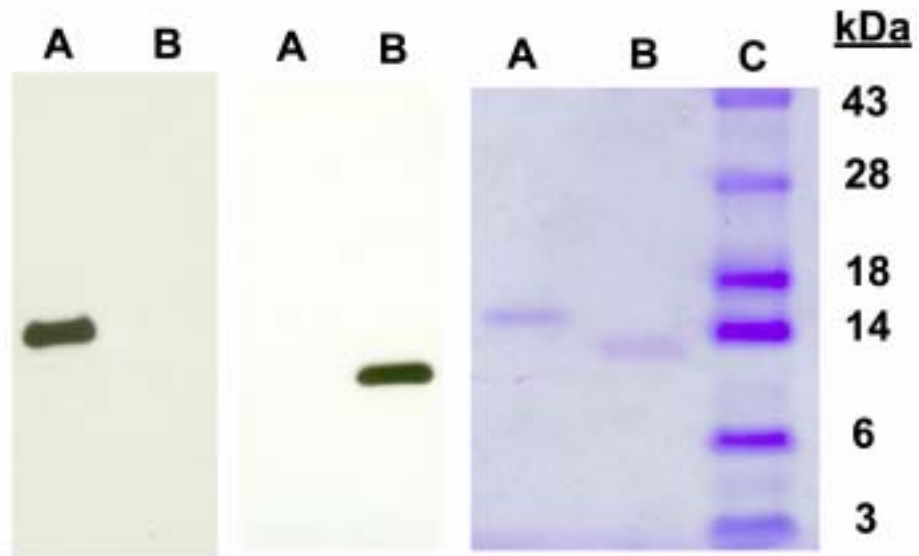


Figure 4.

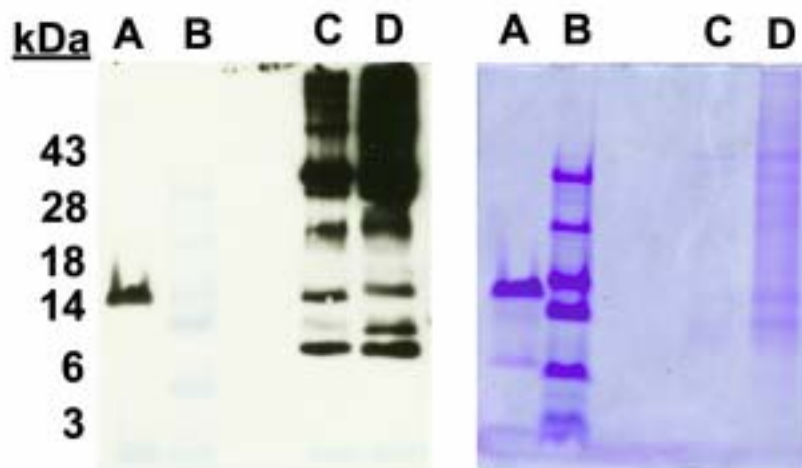


Figure 5.

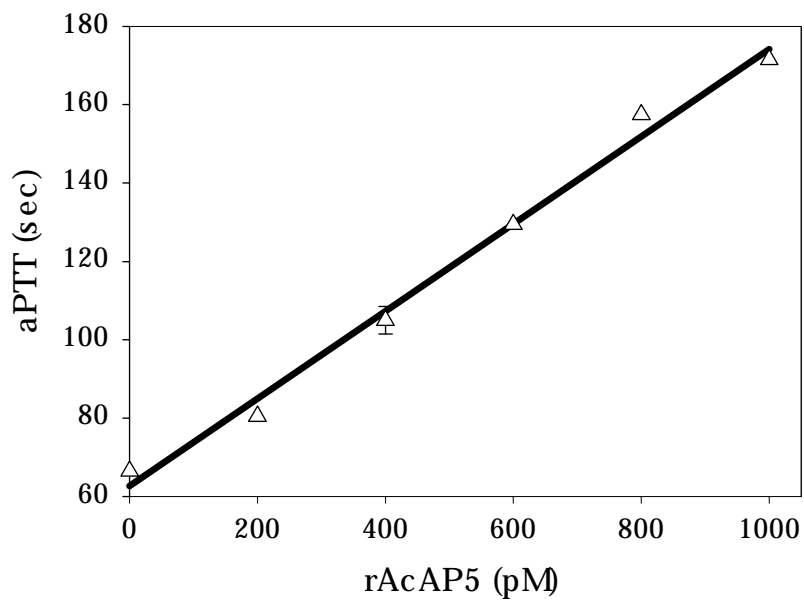
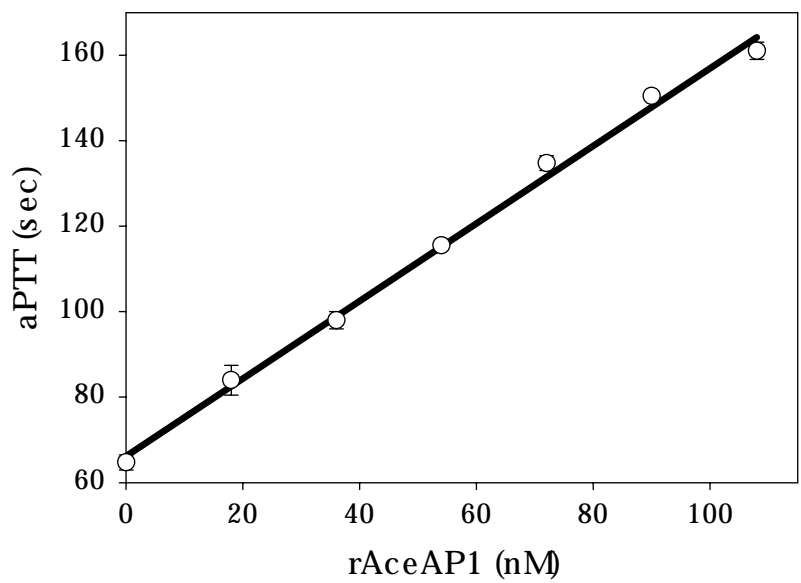


Figure 6.

