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Anticoagulant repertoire of the hookworm *Ancylostoma caninum*

(Ascaris-like inhibitors/blood coagulation/serine proteases/factors Xa and VIIa)

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ABSTRACT **Hookworms are hematophagous nematodes that infect a wide range of mammalian hosts, including humans. There has been speculation for nearly a century as to the identity of the anticoagulant substance(s) used by these organisms to subvert host hemostasis. Using molecular cloning, we describe a family of potent small protein (75–84 amino acids) anticoagulants from the hookworm *Ancylostoma caninum* termed AcAP (*A. caninum* anticoagulant protein). Two recombinant AcAP members (AcAP5 and AcAP6) directly inhibited the catalytic activity of blood coagulation factor Xa (fXa), while a third form (AcAPc2) predominantly inhibited the catalytic activity of a complex composed of blood coagulation factor VIIa and tissue factor (fVIIa/TF). The inhibition of fVIIa/TF was by a unique mechanism that required the initial formation of a binary complex of the inhibitor with fXa at a site on the enzyme that is distinct from the catalytic center (exo-site). The sequence of AcAPc2 as well as the utilization of an exo-site on fXa distinguishes this inhibitor from the mammalian anticoagulant TFPI (tissue factor pathway inhibitor), which is functionally equivalent with respect to fXa-dependent inhibition of fVIIa/TF. The relative sequence positions of the reactive site residues determined for AcAP5 with the homologous regions in AcAP6 and AcAPc2 as well as the pattern of 10 cysteine residues present in each of the inhibitors suggest that the AcAPs are distantly related to the family of small protein serine protease inhibitors found in the nonhematophagous nematode *Ascaris lumbricoides* var. *suum*.**

Hookworm infection is a leading cause of iron deficiency anemia in developing countries, affecting approximately one billion people worldwide. Hookworms cause anemia in their host by extracting their blood meal from lacerated capillaries in the mucosa of the small intestine over an extended period of time (1). Like other hematophagous invertebrates such as ticks (2, 3) and leeches (4, 5), hookworms have evolved highly effective anticoagulant strategies to facilitate the acquisition of their required blood meal. Although the anticoagulant properties of adult hookworm extracts have been the subject of scientific investigation for nearly a century (6–8), only recently has an attempt been made to characterize the molecular entities responsible for this function.

We have previously described the identification (9) and purification (10) of a potent inhibitor of blood coagulation factor Xa (fXa) (termed AcAP for *Ancylostoma caninum* anticoagulant peptide) that represents a major anticoagulant activity present in soluble protein extracts of adult *A. caninum* hookworms. Determination of the first 30 amino acids of purified AcAP revealed a unique partial sequence with heterogeneity at two distinct positions, suggesting that more than one protein may be responsible for its anticoagulant activity (10). We sought to determine the extent of molecular heterogeneity in the original AcAP preparation purified from *A. caninum*. Here, we report the cloning, recombinant expression, and characterization of three homologous small proteins that specifically inhibit different key blood coagulation serine proteases.‖ From these studies, it appears that the hookworm *A. caninum* has evolved an effective strategy for manipulation of the mammalian blood coagulation pathway that is mechanistically and structurally distinct from those strategies utilized by other hematophagous parasites or the mammalian host.

MATERIALS AND METHODS

cDNA Isolation and Sequencing. An oligo(dT)-primed cDNA library was constructed in the λgt11 Sfi-Not vector (Promega) and screened by described procedures (11). The mRNA used for cDNA synthesis was isolated from adult *A. caninum* hookworms (Antibody Systems, Bedford, TX). Recombinant phage ($\approx 10^6$) were probed by standard procedures (11) with the radiolabeled oligonucleotide 5′-AAR GCI TAY CCI GAR TGY GGI GAR AAY GAR TGG-3′ (where R = A or G and Y = T or C). The cDNA inserts of five candidate clones were subcloned on pGEM phagemids (Promega) and both strands were sequenced by using the Autoread kit and the A.L.F. sequencer (Pharmacia). The sequence data revealed two different types of cDNAs corresponding to AcAP5 and AcAP6. Restriction analysis of additional positive cDNA clones demonstrated that they all belong to one or the other type and that the two cDNAs are approximately equally represented in the library.

Production of Recombinant (r)AcAPs. The corresponding proteins encoded by the AcAP cDNAs were produced in the yeast *Pichia pastoris* using the vector pYAM7SP8 as described (12). In each case, a His^+ transformant of strain GS115 (13) that secreted a high level of inhibitory activity (see below) upon induction and exhibited a wild-type, methanol utilization phenotype, was selected. All three recombinant proteins were purified from the yeast culture medium as follows. The pH of the culture supernatant was adjusted to pH 3.0 and the conductivity was brought to <10 mS/cm. The diluted supernatant was applied to a Poros20HS (Perseptive Biosystems, Framingham, MA) column preequilibrated with cation buffer (50 mM sodium citrate, pH 3). The column was washed with the same buffer and subsequently eluted with cation buffer containing 1 M NaCl. Material with inhibitory activity was

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Abbreviations: AcAP, *Ancylostoma caninum* anticoagulant peptide; f, human blood coagulation factor; PT, prothrombin time; rAcAP, recombinant AcAP; TF, human tissue factor; PLV, phospholipid vesicle.

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‖The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U30795 (AcAP5), U30794 (AcAP6), and U30793 (AcAPc2)].

pooled and fractionated on Superdex 30 PG equilibrated with 10 mM sodium phosphate, pH 7.4/150 mM NaCl. The pooled inhibitory activity was fractionated by reverse-phase HPLC using a C_{18} column (model 218TP54; Vydac, Hesperia, CA) developed with a linear gradient of 10–35% CH_3CN in 0.1% trifluoroacetic acid. Fractions containing inhibitory activity were pooled and lyophilized. Each rAcAP was judged >95% pure using analytical reverse-phase HPLC, electrospray mass spectrometry, and quantitative amino acid analysis and was shown to be correctly processed by N-terminal sequence analysis (data not shown). Each protein was quantified by amino acid analysis.

Prothrombin Time Clotting Assay. The anticoagulant properties of the rAcAP proteins were measured *ex vivo*, using the prothrombin time (PT) clotting assay. Dilutions of each rAcAP were made in freshly thawed normal human plasma (George King Biomedical, Overland Park, KS) and assayed using the Coag-A-Mate RA4 automated coagulometer (General Diagnostics, Organon Technika) and Simplastin Excel as the initiating reagent according to the manufacturer's instructions (Organon Technika).

Serine Protease Specificity. The selectivity profile of the rAcAP anticoagulants was examined against 11 serine proteases: human-derived proteins, fXa, α-thrombin, plasmin, fXIa, fXIIa, recombinant tissue plasminogen activator, activated protein C, urokinase, plasma kallikrein, and the bovine-derived proteins trypsin and chymotrypsin. The sources for the enzymes and the conditions for the chromogenic assays were as described (10), except that the final concentrations of enzyme and inhibitor were 0.75 and 75 nM, respectively. The results are expressed as percentage inhibition compared to the control (uninhibited) substrate hydrolysis rate.

fXa and Prothrombinase Inhibition Assays. The assays for inhibition of free fXa amidolytic activity and of thrombin generation by the preformed prothrombinase complex were performed as described (10). The velocity of substrate hydrolysis in the presence (V_i) and absence (V_o) of inhibitor was measured. Values for the apparent equilibrium dissociation constant for the EI complex (K_i^*) were determined from the ratios of V_i/V_o as described (10) using the algorithm derived for analysis of tight-binding inhibition (14).

fVIIa/TF Inhibition Assays. The enzymatic activity of a complex of recombinant fVIIa (Novo-Nordisk, Copenhagan) and recombinant full-length tissue factor (TF; Corvas International) was determined in the presence and absence of either fXa or the catalytically inactive derivative EGR-fXa (i.e., fXa treated with the irreversible active-site inhibitor Glu-Gly-Arg-chloromethylketone; Hematologic Technologies, Essex Junction, VT). EGR-fXa was determined to have <0.05% of the amidolytic activity of untreated fXa. TF was incorporated into phospholipid vesicles of uniform size (PLV; 120 nm average diameter) as described (15). A complex of TF/PLV and fVIIa was formed for 10 min in 10 mM Hepes, pH 7.5/150 mM NaCl (HBS) containing 0.1% bovine serum albumin and 3 mM $CaCl_2$ (HBSA) prior to the addition of inhibitor and/or substrate.

Activation of fIX. The fIX zymogen (Hematologic Technologies) was depleted of residual fVII and fX by sequential monoclonal antibody affinity chromatography prior to reductive tritiation (16). The resulting ^{3}H-fIX preparation had a specific activity of 2×10^8 dpm/mg and retained 97% of the original clotting activity measured in fIX-deficient plasma (George King Biomedical). To measure ^{3}H-fIX activation, preformed fVIIa/TF, inhibitor and fXa, or EGR-fXa were preincubated for 60 min prior to initiating the reaction by the addition of the ^{3}H-fIX substrate. The final concentration of reactants common to all the assays in a total vol of 420 μl of HBSA was as follows: fVIIa, 50 pM; TF, 2.7 nM; PLV, 6.4 μM; fXa or EGR-fXa, 0–500 pM; rAcAP inhibitors, 0–1 nM; and ^{3}H-fIX, 200 nM ($\approx$5 times K_m). The rate of ^{3}H-fIX activation was determined by linear regression analysis of the acid-soluble ^{3}H counts (i.e., activation peptide) released over time under steady-state conditions, where <5% of the radiolabeled substrate was consumed. The reaction was stopped at specific time points (8, 16, 24, 32, and 40 min) by diluting 80 μl of the reaction mixture with an equal vol of HBS containing 50 mM EDTA and 0.5% BSA followed by addition of an equal vol of 6% (wt/vol) trichloroacetic acid at 0°C. After centrifugation, the radioactivity contained in the resulting supernatant was quantitated. The background (<1.0% of uninhibited control velocity) was subtracted. K_i^* values were determined from the ratios of the inhibited/uninhibited initial velocities (V_i/V_o) as described above.

Amidolytic activity. Preformed fVIIa/TF was incubated with inhibitor and EGR-fXa for 30 min at ambient temperature prior to addition of the peptidyl chromogenic substrate S-2288 (D-Ile-Pro-Arg-*p*-nitroaniline; Kabi Pharmacia Hepar, Franklin, OH). The final concentration of reactants in a total vol of 200 μl of HBSA was as follows: fVIIa, 750 pM; TF, 3.0 nM; PLV, 6.4 μM; EGR-FXa, 2.5 nM; rAcAP, 0–25 nM; and S-2288, 3.0 mM ($\approx$3 times K_m). Initial velocities were measured as a linear increase in the absorbance at 405 nm over 10 min, using a Thermomax kinetic microplate reader (Molecular Devices), under steady-state conditions, where <5% of the substrate was consumed.

Cleavage of rAcAP5 by fXa. Purified rAcAP5 was incubated with fXa at a final molar ratio of 22:1 for 8 hr at 37°C in 0.1 M citrate buffer (pH 5.0). Following disulfide reduction with dithiothreitol and alkylation with iodoacetamide, the samples were evaluated by SDS/PAGE using a 16% Tricine gel (NOVEX, San Diego) and visualized by Coomassie blue staining. Identification of the fXa cleavage site in rAcAP5 was determined by N-terminal sequence analysis following purification of nonreduced material by reverse-phase HPLC on a C_{18} column (Vydac). Two sequences were detected, the first starting at the authentic N terminus and the second beginning with Gly-41.

RESULTS AND DISCUSSION

Cloning of AcAP-Related cDNA Sequences. AcAP-related cDNA clones were initially identified by hybridization, using an oligonucleotide probe specific for the predominant N-terminal sequence determined for purified AcAP (10). Sequence analysis of two representative isolates identified with this probe revealed unique open reading frames encoding highly homologous polypeptides, designated AcAP5 and AcAP6 (Fig. 1). The translated sequences were in agreement with the sequence for the first 30 amino acids determined for purified AcAP and confirmed that the heterogeneity observed at positions 14 and 18 was attributable to the presence of two distinct gene products (Fig. 1). Initial attempts to express the AcAP5/6 cDNAs utilized transient expression in COS cells and resulted in the secretion of proteins that carry an Arg-Thr-Val-Arg N-terminal extension when compared to *A. caninum*-derived AcAP (data not shown). This result suggests that the AcAP5/6 primary translation products contain a 19-residue secretion signal followed by a 4-residue prosequence, which is apparently not processed by COS cells (Fig. 1). The mature sequences for AcAP5 and AcAP6 contain 77 and 75 amino acids, respectively, and their calculated molecular sizes are similar to that determined for purified AcAP (10). The sequences of both mature proteins include 10 cysteine residues and predict an acidic pI.

Using a filimentous phage display, we previously selected an *A. caninum* cDNA based on the binding of the encoded product (fused to the C terminus of coat protein VI) to fXa (see ref. 19). Based on a comparison with AcAP5/6, the cDNA translation product is predicted to contain the complete mature sequence of a third AcAP-like protein with a partial

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                                  1
AcAP5   MKMLYAIAIMFLLVSLCSA RTVR KAYPEC-GENEWLDDCGTQKPCEAKC----NEEPP
AcAP6   MKMLYAIAIMFLLVSLCST RTVR KAYPEC-GENEWLDVCGTKKPCEAKC----SEE--
AcAPc2  ............LVSYCSG      KATMQC-GENEKYDSCGSKE-CDKKCKYDGVEE-E

ATI                               EAEKCTKPNEQWTKCG---GCEGTCAQK------

AcAP5   EEEDPI--CRSRGCLLPPACVCKD--GFYRDTVIGDCVREEEC--DQHEIIHV 77
AcAP6   EEEDPI--CRSFSCPGPAACVCED--GFYRDTVIGDCVKEEEC--DQHEIIHV 75
AcAPc2  DDEEPNVPCLVRVCHQD--CVCEE--GFYRNK-DDKCVSAEDCELDNMDFIYPGTRN 84

ATI     -----IVP-CTRECK-PPRCECIASAGFVRDA-QGNCIKFEDCPK 62
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FIG. 1. Deduced amino acid sequences of AcAP5, AcAP6, and AcAPc2. Translation of the AcAP5 and AcAP6 cDNAs was based on the first ATG codon. Underlined residues match the N-terminal sequence obtained for purified authentic AcAP (10) and were used to design a reverse-translated oligonucleotide probe. Putative secretion signal (open bar) and prosequence (solid bar) are indicated. The sequence of an *A. suum* trypsin inhibitor (ATI; refs. 17 and 18) is shown aligned with the deduced AcAP sequences. Common residues between the proteins based on this alignment are shaded. Number of residues contained in the mature proteins is indicated.

secretion signal and no putative proregion. The encoded protein designated AcAPc2 is slightly larger than the other two related sequences (84 residues). In addition, alignment of the 10 cysteines revealed significant sequence divergence despite the conservation of an overall acidic pI (Fig. 1).

Anticoagulant Activities of rAcAPs. To directly characterize the anticoagulant and inhibitory profiles of the proteins encoded by the AcAP5, AcAP6, and AcAPc2 cDNAs, recombinant forms of each protein were produced in the yeast *Pichia pastoris* using an expression vector that was engineered to yield the mature sequences of each protein (12) and purified to homogeneity. The anticoagulant properties of each of the rAcAP proteins were measured in the *ex vivo* PT clotting assay. The measurement of PT is particularly relevant since the series of biochemical events required to cause clotting in this assay are similar to those that must be overcome by the hookworm to facilitate feeding. In both cases, coagulation is initiated by formation of the fVIIa/TF binary complex, resulting in generation of fXa. The subsequent assembly of fXa into the prothrombinase complex is the key event responsible for the formation of thrombin and eventual clot formation. As shown in Fig. 2, all three of the recombinant proteins caused a dose-dependent prolongation of the PT similar to that observed for the authentic material (10). By comparing the concentration of each inhibitor that caused a doubling of the PT as a measure of potency in this assay, it appeared that rAcAPc2 was the most effective anticoagulant (Fig. 2).

Inhibition of Free fXa and the Prothrombinase Complex. Previous studies demonstrated that the anticoagulant effect of the authentic AcAP preparation was a result of the potent inhibition of fXa (10). To determine the inhibitory profiles of the three rAcAP anticoagulants, their selectivity was first examined against 11 serine proteases including several of those involved in hemostasis and fibrinolysis. At a 100-fold molar excess of inhibitor to enzyme, rAcAP5 and rAcAP6 inhibited fXa amidolytic activity by 100% and 87%, respectively. Surprisingly, there was only 18% inhibition of fXa by rAcAPc2 under these conditions. While there was no significant inhibition of any other serine protease by rAcAP6 and rAcAPc2, it appears that rAcAP5 was responsible for the observed inhibition of fXIa by the purified, authentic AcAP preparation (10) since it inhibited this enzyme by 50% at the concentration tested.

The inhibition of fXa amidolytic activity by rAcAP5 and rAcAP6 was further characterized by determining the apparent dissociation constant of the EI complex (K_i*). As shown in Fig. 3*A*, rAcAP5 inhibited the amidolytic activity of fXa with a K_i* of 43 ± 5 pM, while rAcAP6 was a weaker, but effective inhibitor with a K_i* of 996 ± 65 pM. The partial inhibition of fXa amidolytic activity by rAcAPc2 was unexpected since fXa binding was initially used to identify the cDNA encoding this inhibitor and it was the most potent anticoagulant in the PT assay (Fig. 2). We therefore investigated the effects of rAcAPc2 as well as the two other recombinant proteins on thrombin generation mediated by fXa assembled in the prothrombinase complex, since this is a more physiologically relevant reflection of the role fXa plays in the coagulation process (20). In contrast to the partial inhibition of amidolytic activity observed for uncomplexed fXa, rAcAPc2 could completely inhibit the formation of thrombin by fXa in the prothrombinase assay (K_i* = 2385 ± 283 pM), although it was significantly less potent than either rAcAP5 (K_i* = 144 ± 15 pM) or rAcAP6 (K_i* = 207 ± 40 pM) (Fig. 3*B*).

Inhibition of the fVIIa/TF Complex. The relatively weak inhibition of prothrombinase activity by rAcAPc2, compared to rAcAP5/6, contrasted with its more potent anticoagulant effect in the PT assay. This paradoxical finding led us to investigate whether rAcAPc2 could inhibit the catalytic complex of fVIIa/TF, which plays the primary role in initiation of the coagulation response in the PT assay. These studies were performed by measuring the fVIIa/TF-mediated release of the tritiated activation peptide from the radiolabeled macromolecular substrate, ^{3}H-fIX (Fig. 4). None of the rAcAP inhibitors directly inhibited the catalytic activity of fVIIa/TF in this

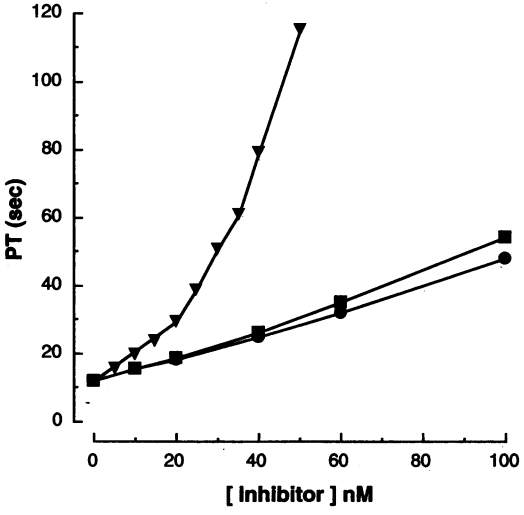

FIG. 2. Effect of rAcAP proteins on the PT *ex vivo* clotting time of normal human plasma. Clotting time in the absence of any added inhibitor was 12.1 sec. Concentrations of rAcAP5 (●), rAcAP6 (■), and rAcAPc2 (▼) required to double the clotting time in this assay were 43 ± 8, 37 ± 3, and 15 ± 1 nM, respectively (means ± SD). Results shown are representative of those obtained in three independent experiments.

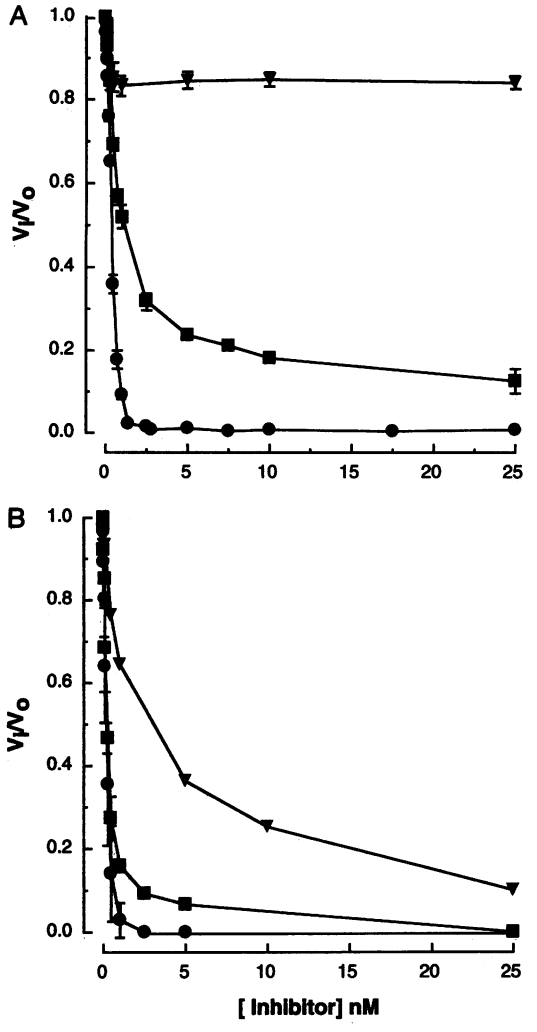

FIG. 3. Inhibition of uncomplexed fXa amidolytic activity (*A*) and prothrombin activation by fXa assembled in the prothrombinase complex (*B*) by rAcAP5 (●), rAcAP6 (■), and rAcAP2 (▼). Results represent means ± SD of three independent experiments.

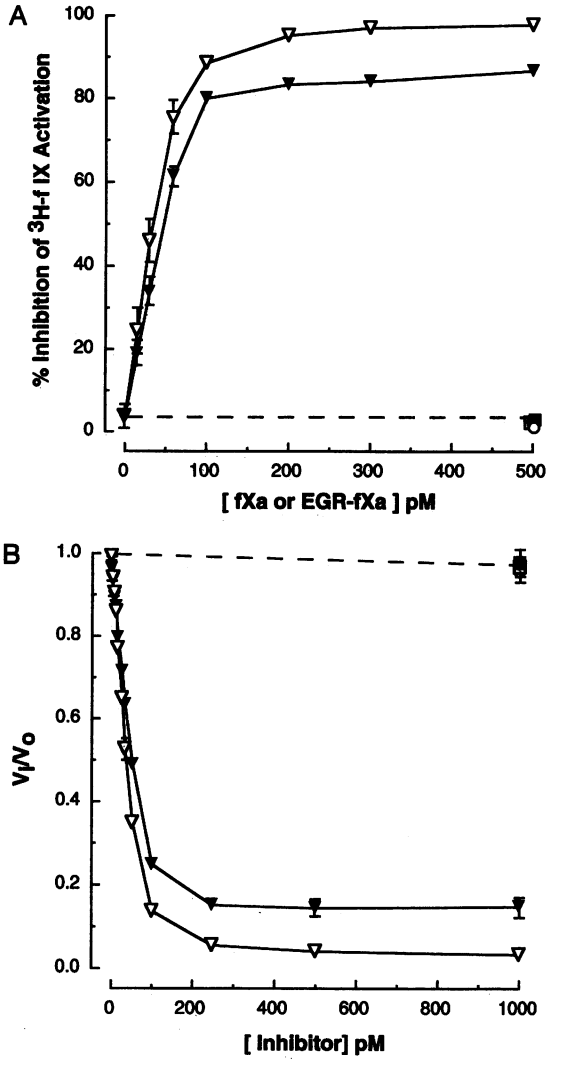

FIG. 4. Inhibition of fVIIa/TF-mediated ^{3}H-fIX activation by rAcAP inhibitors. rAcAP5 (●, ○), rAcAP6 (■, □), and rAcAP2 (▼, ▽) were assayed in the presence of either fXa (solid symbols) or EGR-fXa (open symbols). (*A*) Effect of increasing concentrations fXa or EGR-fXa on inhibition by a fixed concentration of each rAcAP (1 nM). (*B*) Effect of increasing concentrations of rAcAP in the presence of a fixed saturable concentration (300 pM) of either fXa or EGR-fXa. There was no inhibition of fVIIa/TF by rAcAPc2 in this assay in the absence of either fXa or EGR-fXa up to the highest concentration tested (100 nM). In addition, there was no significant inhibition with either rAcAP5 or rAcAP6 up to 100 nM under any condition tested. Results represent means ± SD of three independent experiments.

assay when added alone. However, in the presence of increasing concentrations of added fXa, there was a saturable and concentration-dependent increase in the inhibition of ^{3}H-fIX activation by rAcAPc2 (Fig. 4*A*). In contrast, there was no effect on the activation of ^{3}H-fIX by fXa alone or in complex with either rAcAP5 or rAcAP6 (Fig. 4*A*). rAcAPc2 inhibited fVIIa/TF-mediated ^{3}H-fIX activation in the presence of a fixed, saturable concentration of fXa with a K_i* of 35 ± 5 pM (Fig. 4*B*).

Based on the differential effects of rAcAPc2 on the inhibition of fXa amidolytic and prothrombinase activity, we sought to determine whether the active site of fXa was required for the inhibitory cofactor function of this enzyme. As shown in Fig. 4*A*, there was a saturable increase in the inhibition of fVIIa/TF-mediated ^{3}H-fIX activation by rAcAPc2 in the presence of increasing concentrations of active-site blocked fXa (EGR-fXa). rAcAPc2 inhibited fVIIa/TF in the presence of a fixed concentration of EGR-fXa with a K_i* of 8.4 ± 1.5 pM (Fig. 4*B*). The EGR-fXa-dependent inhibition of fVIIa/TF activity by rAcAPc2 was also observed when a peptidyl chromogenic substrate was used in place of ^{3}H-fIX (K_i* = 35 ± 20 pM; data not shown).

The Reactive Sites of the AcAP Inhibitors. The pattern of the 10 cysteine residues suggested that the AcAPs may constitute a unique subset of the serine protease inhibitor family originally identified in the nonhematophagous, nematode parasite *Ascaris lumbricoides* var. *suum* (Ascaris; refs. 17, 18, 21, and 22) despite the lack of significant sequence homology (Fig. 1). Partial proteolytic cleavage at the reactive site by the cognate protease has defined the P1-P1′ residues (notation according to ref. 23) for several members of the Ascaris inhibitor family (17, 18). Similarly, we have found that incubation of rAcAP5 with its target enzyme fXa resulted in partial cleavage between Arg-40 and Gly-41 (Fig. 5*A*), which corresponds to the same relative sequence position of the P1-P1′ residues in the Ascaris inhibitors. This supports the alignment of AcAP5 and, by analogy, AcAP6 and AcAPc2, with an Ascaris trypsin inhibitor, even though the spacing of the cysteines in the reactive site sequences differs by one residue (Figs. 1 and 5*B*). It should be noted that the positioning of cysteine residues within the reactive site of many classes of small protein serine protease inhibitors may vary without altering the canonical substrate mode of binding to the catalytic site of the cognate enzyme (24, 25).

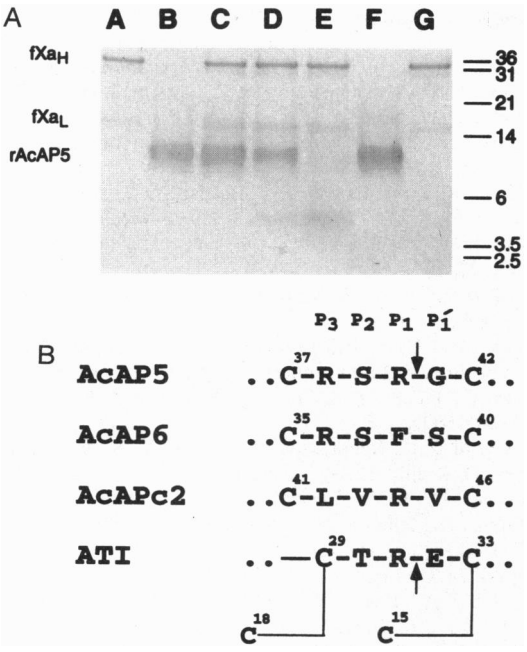

FIG. 5. Localization of reactive site sequence in rAcAP5. (*A*) Electrophoretic analysis following incubation of rAcAP5 with fXa. Samples taken prior to incubation are shown in lanes A (fXa), B (rAcAP5), and C (mixture). A sample of the mixture taken after 0.5 hr of incubation is shown in lane D, while samples taken after 8 hr of incubation are shown in lanes E (mixture), F (rAcAP5), and G (fXa). Positions of the fXa heavy (fXa$_H$) and light (fXa$_L$) chains and of uncleaved rAcAP5 are indicated on the left. The two polypeptides resulting from cleavage of rAcAP5 by fXa comigrate in this electrophoretic system. (*B*) Reactive sequences of rAcAP5 and the Ascaris trypsin inhibitor (ATI) are shown aligned with the corresponding regions of AcAP6 and AcAPc2. The P1-P1′ scissile bond is designated by an arrow. Disulfide bonds depicted for ATI in this region of the protein are based on the solution structure determined for this inhibitor (21).

The effectiveness of rAcAP6 as fXa inhibitor is surprising in view its putative reactive site sequence (Fig. 5*B*). In contrast to AcAP5, which contains arginine residues at the P1 and P3 positions similar to the fXa inhibitor antistasin (26), AcAP6 has Phe-38 at the putative P1 position, a residue typically found in inhibitors of chymotrypsin-like proteases (24). However, rAcAP6 did not inhibit chymotrypsin and was only ≈20-fold less potent than rAcAP5 with respect to the inhibition of uncomplexed fXa amidolytic activity (Fig. 3*A*). Moreover, substitution of Phe-38 in AcAP6 with arginine resulted in a mutant that inhibited fXa with a potency similar to rAcAP5 (Y.L., unpublished data). This result strongly suggests that the difference in inhibitory potency between rAcAP5 and rAcAP6 is principally due to the different P1 residues given the high degree of homology outside of this site. In addition, inhibition of fXa by rAcAP6 demonstrates that this enzyme is somewhat flexible in accommodating nonarginine side chains at its S1 specificity site, which has been observed previously for another potent inhibitor of fXa, Ecotin (27). It should be noted that while rAcAP6 is a potent anticoagulant and inhibitor of both free and prothrombinase-assembled fXa activity, the presence of an unconventional P1 reactive site phenylalanine residue suggests that fXa may not be the only physiological target for this inhibitor even though there was no inhibition of either trypsin or chymotrypsin, which have broad substrate specificity. It remains to be seen whether other proteolytic enzymes involved in the host-defense response to helminthic infection are inhibited by this protein.

The putative reactive site of AcAPc2 differs considerably from the homologous regions of AcAP5 and AcAP6 in the corresponding P3 and P2 sites where leucine (Leu-42) and valine (Val-43) residues are found, respectively (Fig. 5*B*). This sequence of residues resembles the recognition sites of the fVIIa substrates fX and fIX (Leu-Thr-Arg) (28). It would appear that the reactive site sequences of the AcAP inhibitors are a major determinant in defining their specificity. It remains to be seen what portions of the proteins outside this region also play a role in mediating the interaction of the AcAP inhibitors with their cognate proteases.

Proposed Mechanism of Enzyme Inhibition. The following working hypothesis has been developed to understand the mechanism of enzyme inhibition by the AcAP inhibitors. The differential effects of the three recombinant proteins on the inhibition of fXa and the comparison of the homologous reactive site residues suggests that rAcAP5 and rAcAP6 interact directly with the catalytic center of the fXa enzyme. In contrast, rAcAPc2 appears to bind to fXa at a site that is likely distinct from the catalytic center (exo-site) that interferes with the macromolecular interactions of this enzyme with either the substrate (prothrombin) and/or cofactor (fVa) while not completely inhibiting the catalytic turnover of the peptidyl substrate. The partial inhibition of fXa amidolytic activity by rAcAPc2 is not inconsistent with this proposal since it is known that exclusive binding at an exo-site can influence the catalytic activity of the related serine protease, thrombin (29, 30). The binding of AcAPc2 to the exo-site of fXa is also thought to precede the formation of a quaternary inhibitory complex between fXa/AcAPc2 and fVIIa/TF that results in the potent, fXa-dependent inhibition of both peptidyl and macromolecular substrate hydrolysis. Consistent with this hypothesis is that EGR-fXa can fully support the inhibition of fVIIa/TF by rAcAPc2 despite covalent occupancy of the catalytic site of fXa by the tripeptidyl chloromethylketone. The inhibition of fVIIa by rAcAPc2 may involve the occupation of the catalytic center based on the complete inhibition of fVIIa/TF amidolytic activity and the resemblance of the putative reactive site with fVIIa substrates.

The proposed utilization of an exo-site on fXa distinguishes rAcAPc2 from the mammalian protein anticoagulant tissue factor pathway inhibitor, which is functionally equivalent to AcAPc2 with respect to the fXa-dependent inhibition of fVIIa/TF activity (31, 32). Tissue factor pathway inhibitor uses separate Kunitz domains that sequentially bind to the catalytic sites of fXa and the fVIIa/TF complex (31, 32), while AcAPc2 is thought to bind both enzymes with a single, non-Kunitz-like Ascaris inhibitor motif. Despite these significant differences, however, it is remarkable that both inhibitors have evolved to utilize fXa to form the final inhibitory complex with fVIIa/TF, which may reflect the role of fXa in localizing the inhibitors to the membrane surface in close proximity of the fVIIa catalytic center (33).

The discovery of anticoagulants that are mechanistically and structurally distinct from those utilized by other hematophagous parasites or the mammalian host may provide new opportunities for development of a new generation of therapeutic antithrombotic agents.

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