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Ancylostoma ceylanicum anticoagulant peptide-1: role of the predicted reactive site amino acid in mediating inhibition of coagulation factors Xa and VIIa

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Abstract

Hookworm infection is a leading cause of gastrointestinal blood loss and iron deficiency anemia in developing countries. *Ancylostoma* hookworms secrete potent anticoagulants, which have been shown to target coagulation factors Xa and the factor VIIa/Tissue Factor complex, respectively. The goal of these experiments was to determine the mechanism of action of three recombinant hookworm anticoagulants using in vitro assays. Three hookworm coagulation inhibitors were expressed and purified, along with site directed mutants targeting each of the predicted P1 inhibitory reactive site amino acid residues. Using chromogenic assays, it has been confirmed that *Ancylostoma caninum* Anticoagulant Peptide 5 (AcAP5) inhibits coagulation factor Xa (fXa) by a canonical, substrate-like mechanism. In contrast, *Ancylostoma ceylanicum* Anticoagulant Peptide-1 (AceAP1) binds to and inhibits fXa by both active site and non-active site mediated interactions. Data from in vitro studies also demonstrates that AceAP1 inhibits the factor VIIa/Tissue complex (fVIIa/TF) and displays a distinct pattern of fXa binding. Together, these data suggest that the human hookworm *A. ceylanicum* has evolved a single anticoagulant that targets multiple components of the mammalian coagulation response, effectively mimicking the concerted action of the two related inhibitors from *A. caninum*. Despite the amino acid sequence similarity, AceAP1 appears to interact with coagulation proteases fXa and fVIIa by a novel mechanism, perhaps explaining its spectrum of inhibitory activity.

Keywords: Hookworm; *Ancylostoma*; Thrombosis; Factor Xa; Factor VIIa; Tissue factor; Coagulation

1. Introduction

Bloodfeeding hookworms infect nearly one billion people worldwide and are a leading cause of iron deficiency anemia in developing countries [1–3]. The pathogenesis of hookworm anemia is primarily attributable to gastrointestinal hemorrhage caused by the adult worm as it attaches to the intestinal mucosa and feeds on blood from lacerated capillaries [4,5]. It has been recognized for over a century that adult *Ancylostoma* hookworms produce potent inhibitors of mammalian coagulation [6–12]. Work over the past 10 years has led to the identification of novel anticoagulants from the dog hookworm *A. caninum*, as well as the related species *A. ceylanicum* [8,9,13,14]. Based on amino acid sequence analysis, these hookworm anticoagulant proteins belong to a family of serine protease inhibitors first identified from the non-bloodfeeding intestinal nematode *Ascaris suum* [15–19]. The dog hookworm *A. caninum* secretes multiple related anticoagulants with at least two distinct mechanisms of action. *A. caninum* Anticoagulant Peptide 5 (AcAP5) is a direct active site inhibitor of coagulation factor Xa (fXa) that acts by the canonical, substrate like mechanism previously described for tight binding serine protease inhibitors [8,9,13,20]. In contrast, AcAPc2 inhibits the factor VIIa-Tissue Factor complex (fVIIa/TF), but requires the presence of fXa (or the inactive zymogen) as part of a quaternary complex for full inhibitory activity [21,22]. Due to their potent activity in vitro, the hookworm

Abbreviations: rpHPLC, reverse phase high pressure liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MW, molecular weight; kDa, kilodaltons; TFA, trifluoroacetic acid; HRP, horseradish peroxidase; PBS, phosphate buffered saline

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anticoagulants AcAPc2 and AcAP5, also known as Nematode Anticoagulant Peptide c2 (NAPc2) and NAP5, have been developed and tested as agents for the prevention and treatment of thrombotic disease in humans [23–25]. Recently, two additional fVIIa/TF inhibitors with in vitro activities similar to AcAPc2, named AcAPc3 and AcAPc4, have been isolated from *A. caninum* [26].

A. ceylanicum Anticoagulant Peptide-1 (AceAP1) was the first anticoagulant isolated from a species of hookworm for which humans are fully permissive hosts [27,28]. Using in vitro and ex vivo clotting studies, it was determined that AceAP1 has substantially weaker affinity for fXa than AcAP5, and that the two inhibitors appear to be both immunologically and mechanistically distinct [13]. We report here results from studies utilizing site directed mutagenesis in order to characterize the mechanism of anticoagulant activity of the novel anticoagulant AceAP1. These data confirm that AceAP1 inhibits the catalytic activity of both fXa and the fVIIa/TF complex, yet does so by a mechanism that is distinct from its anticoagulant homologues (AcAP5 and AcAPc4) from *A. caninum*.

2. Materials and methods

2.1. Site directed mutagenesis and expression of recombinant hookworm anticoagulant peptides

Based on alignment with known members of the *Ascaris* protease inhibitor family, the predicted P1 inhibitory reactive site amino acids were determined for the hookworm anticoagulants AcAP5, AcAPc4, and AceAP1. The ExSite^{TM} PCR-Based Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was then utilized in order to modify the predicted P1 amino acid residues. The cDNAs corresponding to AcAP5, AcAPc4 and AceAP1 were cloned into the pET28a prokaryotic expression plasmid. Oligonucleotide primers were then constructed in order to substitute an Alanine (Ala) in place of the P1 Arginine (Arg) residues for each of the anticoagulants.

In order to express recombinant protein, BL21 *Escherichia coli* cells were transformed with pET28a (Novagen) expression vectors bearing the following cDNA sequences: AcAP5 WT (wild type), AcAP5 R40A (Arg to Ala at amino acid residue 40), AcAPc4 WT, AcAPc4 R41A, AceAP1 WT, AceAP1 R40A mutant using the standard protocol. Recombinant protein expression was induced in 1-l cultures by the addition of IPTG (final concentration = 1 mM). Recombinant proteins containing vector derived poly-histidine tags were purified from the soluble fraction of induced bacterial cultures using nickel resin affinity chromatography followed by C_{18} rpHPLC as described previously [13,20,29]. The relative purity and molar concentration of the recombinant proteins were determined using matrix assisted laser desorption ionization mass spectometry and quantitive amino acid analysis, respectively [13,20,29].

2.2. Single stage chromogenic substrate assays

A single stage in vitro assay [13] was used to characterize the direct inhibition of fXa activity by each recombinant hookworm anticoagulant and its corresponding site directed mutant. Ten microliters of each inhibitor were incubated with 100 μl of a 2 nM fXa (Enzyme Research Labs, South Bend, IN, USA) solution in PBS for 15 min at 25 °C. After addition of 50 μl of chromogenic substrate L-2115 (Bachem Biosciences, King of Prussia PA) (0.5 μM solution), the rate of hydrolysis was measured at 405 nm over 5 min (mOD/min) using a kinetic plate reader (Molecular Devices, Sunnyvale, CA, USA).

A chromogenic substrate cleavage assay was also utilized in order to characterize the inhibition of the fVIIa/TF complex by each recombinant anticoagulant and its respective site directed mutant [26]. Lox cells were used as the source of TF for these experiments [30]. Each recombinant inhibitor was incubated with a suspension of Lox cells (9.6×10^5) and purified fVIIa (Haematologic Technologies, Burlington, VT) (100 nM) for 20 min at 25 °C. In order to determine the degree to which fXa might be required as a protein scaffold for maximal inhibitory activity, as has previously been described for AcAPc4 [26], these experiments were conducted in the presence or absence of EGR-Xa, which is fXa (250 nM) that has been catalytically blocked by the tripeptide EGR [8,21,22] (Haematologic Technologies). The residual fVIIa activity was measured by adding chromogenic substrate S-2288 (250 μM) (D-Ile-Pro-Arg-*p*-nitroaniline; KabiPharmacia Heper, Franklin, OH, USA) and incubating for 45 min at 37 °C. At this point, the Lox cells were pelleted by centrifugation and 95 μl of supernatant from each reaction tube was transferred to wells of a microtiter plate in order to read the absorbance at 405 nm. Positive and negative control wells consisted of reaction mixtures lacking either recombinant inhibitor (positive) or fVIIa (negative), respectively.

2.3. Ex vivo clotting time assays

A modified activated partial thromboblastin time (aPTT) Assay was used to measure the anticoagulant activity of each recombinant protein and its respective mutant [13,26]. Increasing concentrations of each recombinant protein (in a volume of 10 μl) was incubated in wells of a microtiter plate with 20 μl aPTT Alexin reagent (Sigma) and 50 μl of pooled human plasma. After incubating for 15 min at 37 °C, 20 μl of 50 mM CaCl2 was added to each well to initiate the clotting reaction. The time to clot initiation was determined by measuring the light absorbance at 630 nm every 7 s over 3 min using a kinetic plate reader.

The effect of recombinant proteins on the prothrombin time (PT) of human plasma was also measured using a modified microtiter plate assay [26]. Increasing amounts of recombinant anticoagulant (10 μl) were added to 45 μl of pooled human plasma. After incubating for 15 min at 37 °C,

45 μl of PT reagent (ThromboMax with Calcium; Sigma) were added, and the time to clot initiation was determined by measuring the absorbance at 630 nm every 5 s over 3 min.

2.4. Binding of recombinant proteins to fXa

The degree to which each recombinant inhibitor, as well as the respective site directed mutants, effectively binds fXa was measured using an in vitro assay. Individual wells of a microtiter plate were incubated overnight at 4 °C with 100 μl of each recombinant protein at a concentration of 40 μg/ml in PBS. The wells were blocked with 200 μl of 5 mg/ml BSA in PBS for 1 h at room temp, washed three times with 200 μl of PBS, and incubated in triplicate with 100 μl of bXa in PBS for 4 h at 25 °C. Wells were washed 3 times with PBS, followed by the addition of HRP-labeled Streptavidin (Sigma) for 1 h. After washing, the HRP activity was detected by adding 100 μl of 1-StepTM Turbo TMB ELISA reagent (PIERCE). After 10 min, the reaction was stopped by adding 100 μl of 1N sulfuric acid, and the absorbance was measured at 405 nm. In order to account for non-specific binding, control wells were incubated with bXa and a 50-fold excess of non-biotinylated fXa.

This binding assay was utilized in order to define the equilibrium dissociation constants (K_d) for each of the recombinant hookworm anticoagulants and bXa. Increasing concentrations of bXa (0–10 nM) were added to wells coated with purified wild type or mutant rAcAP5, rAcAPc4 or rAceAP1, followed by washing and detection as described above. Data from these binding experiments were analyzed using Scatchard analysis, and the K_d for each recombinant inhibitor was calculated as previously described [30]. This binding assay was also utilized in order to determine the degree to which each recombinant inhibitor required a direct interaction with the enzyme's reactive site. For these experiments, wells coated with each recombinant wild type anticoagulant were incubated with bXa and a 50-fold excess of EGR-fXa. After washing, the bound bXa was detected as described above. For these experiments, 100% binding was defined as the OD_{450} measured in the absence of EGR-fXa. The percent inhibition was calculated by dividing this value by the OD_{450} obtained in the presence of a 50-fold excess of EGR-fXa.

3. Results

3.1. Expression and purification of site directed mutants

Mutant plasmids were generated using the ExSite™ PCR-based site directed mutagenesis kit, which allowed for the incorporation of a series of Arg to Ala mutations at the predicted P1 inhibitory reactive sites of the hookworm anticoagulants AcAP5, AcAPc4, and AceAP1 (Fig. 1). Each recombinant protein (rAcAP5, rAcAP5 R40A, rAcAPc4, rAcAPc4 R41A, rAceAP1 and rAceAP1 R40A) was expressed in *E. coli* and purified using affinity chromatography and HPLC. Each purified recombinant protein was estimated to be at least 85% pure by LDMS. The molar concentration of each protein was subsequently determined by quantitative amino acid analysis as previously described [9,13,20,31].

```
          P3' P2' P1' P1  P2  P3
AcAPc4:C---L---I---R---V---C
AceAP1:C---L---S---R---D---C
AcAP5: C---R---S---R---G---C
```

Fig. 1. Alignment of the inhibitory reactive site amino acid sequences of the hookworm anticoagulants AcAPc4, AceAP1, and AcAP5. Single letter amino acid code is used.

3.2. AcAP5 and AceAP1 inhibit fXa via interaction with the enzyme's reactive site

Consistent with prior reports, rAceAP1 and rAcAP5 inhibited the catalytic activity of fXa against a chromogenic substrate (Fig. 2). rAcAP5 also inhibited fXa, whereas rAceAP1 only achieved partial inhibition of fXa activity even at a substantially greater inhibitor:enzyme ratio [13]. The data from site directed mutants rAcAP5 R40A and rAceAP1 R40A confirmed that the predicted P1 amino acid residue is required for active site mediated inhibition of fXa (Fig. 2). Substitution of Arg to Ala at the predicted reactive site of both inhibitors resulted in loss of inhibitory activity against fXa as measured in the chromogenic assay. These data confirm that the predicted reactive site of AceAP1 (Arg^{40}), is involved in the interaction between the inhibitor and fXa in vitro. As expected, rAcAPc4 had no inhibitory activity against fXa in this chromogenic assay (Fig. 2) [26].

3.3. AcAP5 and AceAP1 inhibit the prothrombinase complex by distinct mechanisms

The aPTT clotting time assay was utilized in order to characterize the anticoagulant activity of each recombinant anticoagulant and P1 site directed mutants. Both rAcAP5 and rAceAP1 prolonged the aPTT of human plasma in a concentration dependent fashion (Fig. 3). The rAcAP5 Arg40Ala mutant was substantially less effective at prolonging the aPTT than the rAcAP5 WT protein, confirming the role of Arg^{40} in mediating inhibition of fXa by AcAP5. In contrast, the rAceAP1 R40A mutant was comparable to the wild type rAceAP1 in this assay, suggesting that additional, non-P1 amino acid interactions play a role in prothrombinase complex inhibition by rAceAP1. This finding confirms that the mechanism of inhibition of fXa by rAceAP1 is distinct from that of rAcAP5, and is consistent with previous data suggesting that rAceAP1 might interact with fXa at multiple sites [13]. As expected, rAcAPc4 also inhibited the prothrombinase complex, presumably via an exosite mediated interaction that partially interferes with

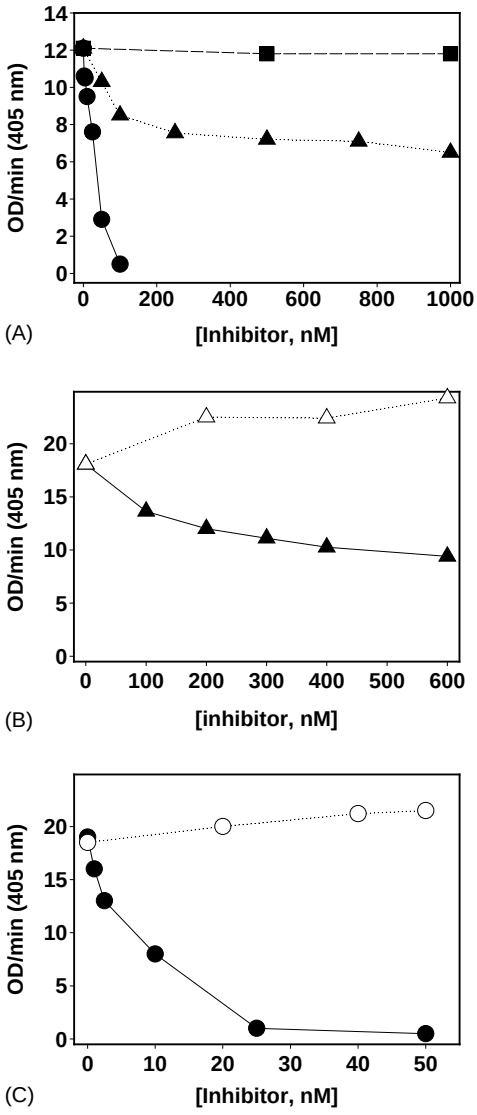

Fig. 2. Inhibition of coagulation factor Xa by recombinant hookworm anticoagulants. FXa activity (*Y*-axis) defined as the rate of chromogenic substrate hydrolysis in the presence of increasing concentration of recombinant inhibitor (*X*-axis). (A) rAcAPc4 (■), rAcAP5 (●), rAceAP1 (▲); (B) inhibition of fXa by rAceAP1 WT (▲) or rAceAP1 R40A mutant (△); (C) inhibition of fXa by rAcAP5 WT (●) or rAcAP5 R40A mutant (○).

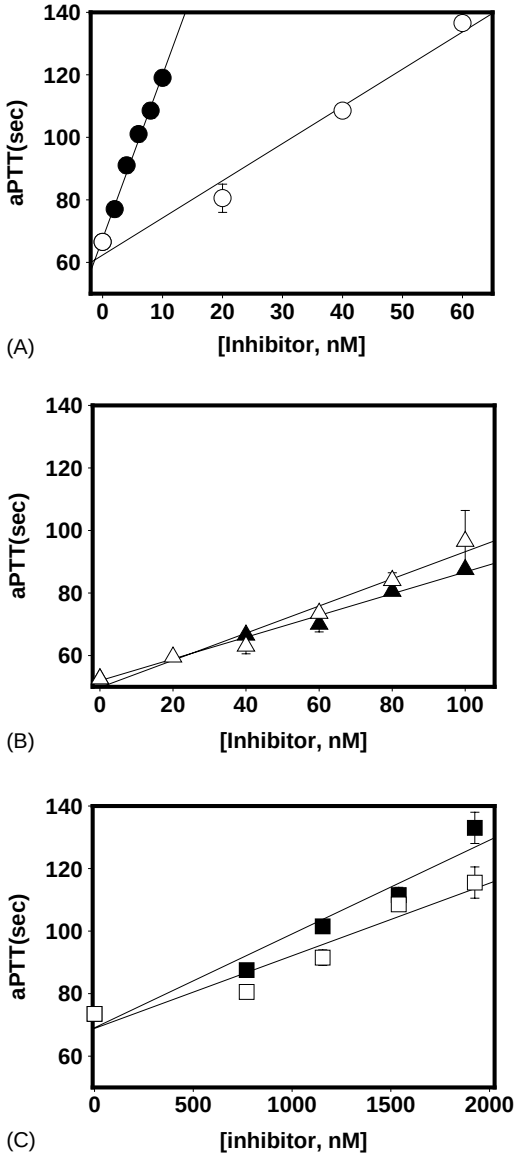

Fig. 3. Prolongation of aPTT clotting time by recombinant hookworm anticoagulants and P1 site directed mutants. Concentration of each inhibitor is shown on *X*-axis. (A) rAcAP5 (●), rAcAP5 R40A (○); (B) rAceAP1 (▲), rAceAP1 R40A (△); (C) rAcAPc4 (■), rAcAPc4 R41A (□).

the enzyme's ability to cleave its physiologic substrate prothrombin.

3.4. rAceAP1 binds to fXa via at least two distinct sites

The binding of bXa to each recombinant anticoagulant was characterized using a microtiter plate based biotinylated fXa (bXa) binding assay [30]. While all three wild type recombinant proteins bound bXa in vitro, they did so with varying affinities (Fig. 4A). The binding of bXa was greatest for rAcAP5, with a K_d of 0.9 nM as determined by Scatchard analysis. In contrast, the bXa bound rAcAPc4 and rAceAP1with substantially weaker affinities, with calculated K_d values of 1.93 and 1.96, respectively. The site directed mutants rAcAP5 R40A and rAceAP1 R40A failed to bind bXa with a great enough affinity to allow for an estimate of the K_d using this assay (not shown). In contrast, the K_d derived for rAcAPc4 R41A mutant was unchanged from that measured for the wild type inhibitor. These data suggest that the predicted P1 reactive site amino acid mediates the predominant interaction between fXa and AceAP1 or AcAP5, but not AcAPc4.

In order to further distinguish the mechanism of action of each of the hookworm anticoagulants, the binding of bXa was measured in the presence of an excess of EGR-Xa, which has a covalently blocked proteolytic site. As expected, the addition of a 50-fold excess of EGR-Xa failed to com-

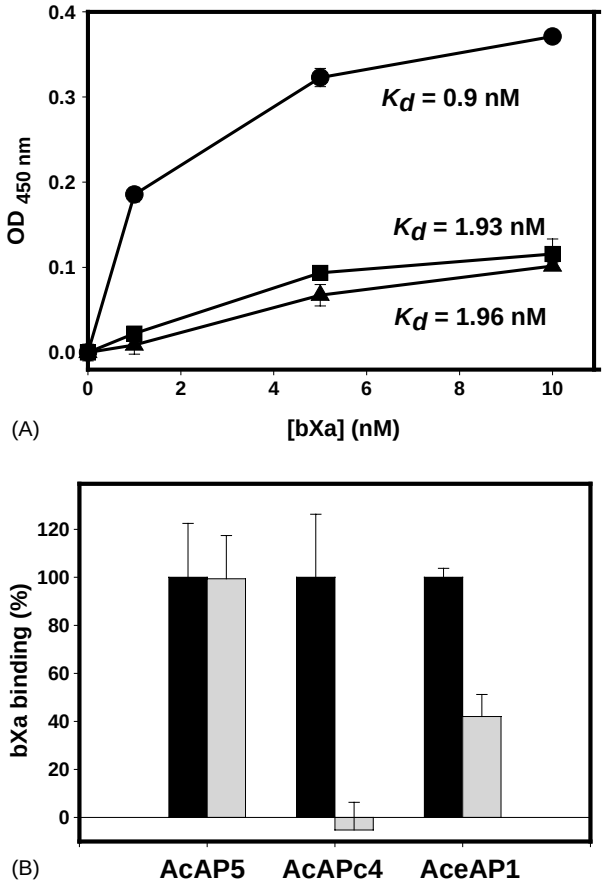

Fig. 4. (A) Concentration dependent binding of bXa to immobilized hookworm anticoagulants (rAcAPc4 (■), rAcAP5 (●), rAceAP1 (▲)). Data from binding curves were analyzed by Scatchard analysis in order to derive the equilibrium dissociation constants (K_d) for each inhibitor:enzyme interaction. (B) Active site dependence of binding of bXa to immobilized recombinant hookworm anticoagulants. Binding was measured in the presence of a 50-fold molar excess of EGR-fXa (gray bars), and the results are expressed as a percentage of the OD values obtained for binding to each recombinant protein in the absence of EGR-fXa (black bars).

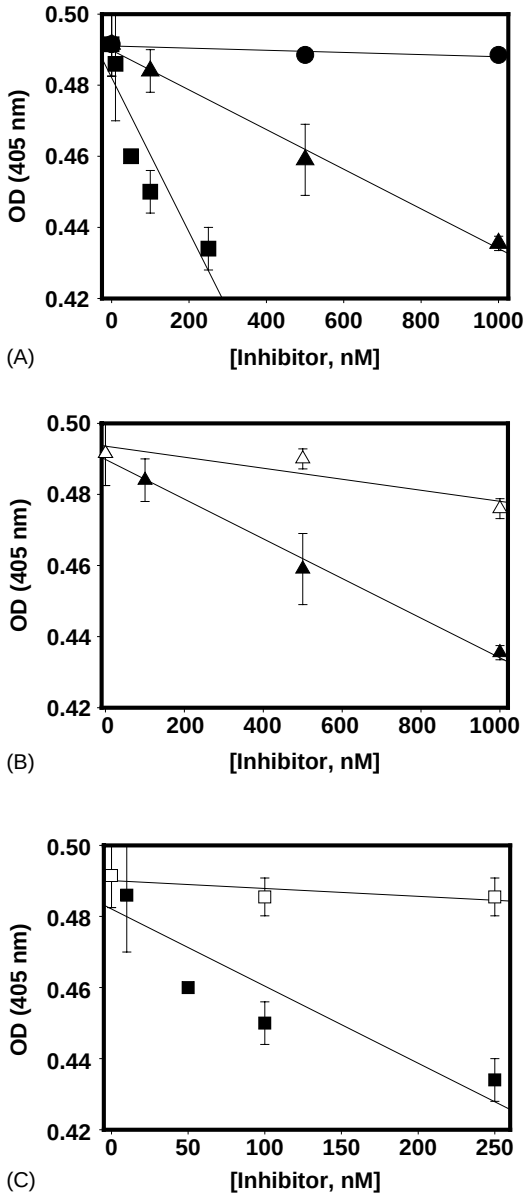

Fig. 5. (A) Inhibition of coagulation fVIIa/TF by recombinant hookworm anticoagulants. fVIIaTF activity (*Y*-axis) defined as the rate of chromogenic substrate hydrolysis in the presence of increasing concentration of recombinant inhibitor (*X*-axis): rAcAPc4 (■), rAcAP5 (●), rAceAP1 (▲). (B) Inhibition of fVIIaTF by rAceAP1 WT (▲) or rAceAP1 R40A mutant (△). (C) Inhibition of fVIIa/TF by rAcAPc4 (■) or rAcAPc4 R41A (□).

pete for binding to rAcAP5 (Fig. 4B), consistent with the active site mediated interaction hypothesized for this inhibitor. Also as expected, an excess of EGR-Xa effectively competed with the bXa for binding to rAcAPc4, since this inhibitor is known to interact with the enzyme via an exosite. However, EGR-Xa only partially competed with bXa for binding to rAceAP1, suggesting that there is an additional site that does not require a direct interaction with the enzyme's reactive site. Together, these data demonstrate three distinct patterns of bXa binding for AcAP5, AceAP1, and AcAPc4.

3.5. AceAP1 inhibits the fVIIa/TF complex via a mechanism that requires fXa and Arg^{40}

In order to determine whether rAceAP1 requires fXa for full inhibitory activity, as has previously been described for rAcAPc2, rAcAPc3, and rAcAPc4 [8,21,26], we examined each recombinant protein for inhibition of the fVIIa/TF complex using an in vitro chromogenic substrate assay [26]. As shown in Fig. 5, rAceAP1 and rAcAPc4 both effectively inhibited fVIIa/TF in vitro, while rAcAP5 did not. Moreover, the inhibition of fVIIa/TF by rAceAP1 and rAcAPc4 were both dependent on the P1 reactive site amino acid residue, as the mutants rAceAP1 R40A and rAcAPc4 R41A showed no inhibitory activity. Both rAceAP1 and rAcAPc4 required fXa for full inhibition of the complex (not shown), confirming that the likely mechanism of fVIIa/TF inhibition re-

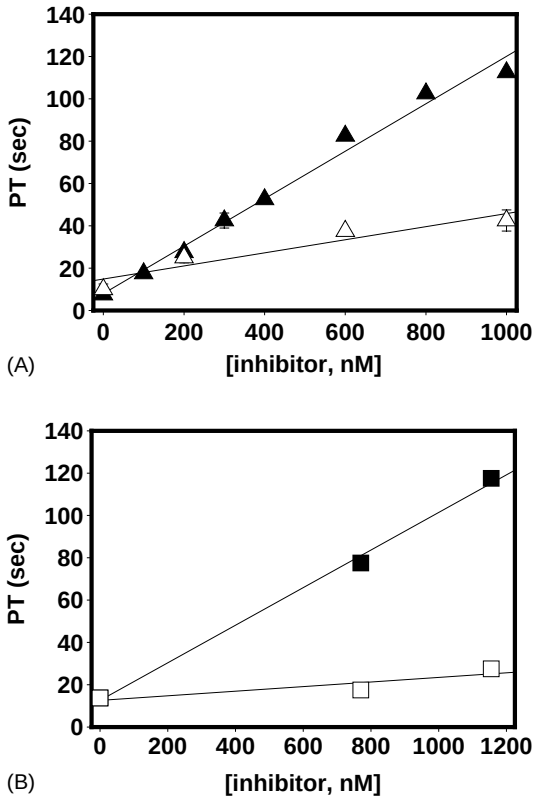

Fig. 6. Prolongation of the prothrombin time (PT) by recombinant hookworm anticoagulants and respective P1 site directed mutants. Concentration of each inhibitor is shown on *X*-axis: (A) rAceAP1 (▲), rAceAP1 R40A (△); (B) rAcAPc4 (■), rAcAPc4 R41A (□).

quires assembly of a quaternary macromolecular complex [21,22,26].

In order to validate the observed inhibitory activity against physiologic substrates, both fVIIa/TF inhibitors were tested for the ability to prolong the PT of human plasma. As shown in Fig. 6, rAceAP1 and rAcAPc4 both prolonged the PT. In contrast to the data from the aPTT assay, the rAceAP1 R40A mutant was substantially less effective at prolonging the PT than the wild type inhibitor, confirming that the anticoagulant mechanisms mediating inhibition of fVIIa/TF and fXa by rAceAP1 are, in fact, distinct.

4. Discussion

It has been recognized for more than a century that adult hookworms produce potent anticoagulant molecules that presumably function to facilitate the taking of a blood meal. With the isolation of a family of anticoagulant serine protease inhibitors from *Ancylostoma* hookworms, the molecular mechanisms by which these intestinal nematodes inhibit specific components of the mammalian coagulation cascade have recently been elucidated [8,9,13,26]. The *A. caninum* Anticoagulant Peptide family (also known as the Nematode Anticoagulant Peptides) consists of at least 5 distinct proteins ranging in length from 75–84 amino acids. These anticoagulants inhibit either coagulation fXa (AcAP5, AcAP6) or the factor VIIa/Tissue Factor complex (AcAPc2, AcAPc3, AcAPc4). Although AcAPc2, AcAPc3 and AcAPc4 do not inhibit fXa directly, the enzyme or its inactive zymogen (factor X) is required for full inhibition of fVIIa/TF, presumably via interaction with a non-catalytic site on the fXa molecule [8,21,22,26].

A. ceylanicum Anticoagulant Peptide-1 (AceAP1) is the only hookworm antithrombotic molecule isolated from a species for which humans are fully permissive hosts. Initial studies demonstrated that rAceAP1 is a partial competitive inhibitor of fXa, with submaximal inhibition of its proteolytic activity even at very high inhibitor:enzyme ratios [13]. Kinetic analysis suggested the presence of additional binding sites on fXa for rAceAP1, presumably distinct from those at or near the enzyme's catalytic site. These data raised the possibility that these two closely related hookworm species might have evolved distinct bloodfeeding strategies. In order to define the mechanism of anticoagulant activity of AceAP1, we expressed recombinant anticoagulant with a site directed mutation at the inhibitor's predicted P1 inhibitory reactive site amino acid. Among tight binding serine protease inhibitors, the P1 peptide bond is slowly cleaved via interaction with the enzyme's proteolytic site, thus creating a non-covalent but highly stable enzyme:inhibitor intermediate complex [32–34]. As shown in Fig. 2, the predicted P1 amino acid residue mediates inhibition of fXa against a chromogenic substrate for rAcAP5 (Arg^{40}) and rAceAP1 (Arg^{40}). These data confirm that AceAP1 inhibits fXa's catalytic site by the canonical substrate like mechanism, albeit with much lower affinity than rAcAP5.

In order to characterize the role of the predicted P1 amino acid residue in mediating inhibition of fXa against its physiologic substrate prothrombin, both wild type and site directed mutants were analyzed for anticoagulant activity using the aPTT ex vivo clotting time assay. As shown in Fig. 3, the rAcAP5 R40A mutant was substantially less potent at prolonging the aPTT compared to the wild type recombinant protein, confirming that its mechanism of inhibition of fXa is mediated primarily through interaction with the enzyme's reactive site. There was no difference in anticoagulant activity between the rAceAP1 R40A mutant and wild type protein in the aPTT assay (Fig. 3), which is consistent with data suggesting that the anticoagulant effect of rAceAP1 is mediated via interactions with fXa that do not involve the active site of the enzyme. These data thus define a potential mechanistic distinction between AcAP5 and AceAP1.

In order to define the interaction between fXa and each hookworm anticoagulant, binding studies were performed using immobilized inhibitor and bXa. As shown in Fig. 4A, all three wild type inhibitors (rAcAP5, rAceAP1, and rAcAPc4) were capable of binding bXa in vitro. Using Scatchard analysis, it was determined that bXa bound rAcAP5 with greater affinity ($K_d = 0.9$ nM) than AcAPc4 (1.93 nM) or rAceAP1 (1.96 nM). This higher affinity of

rAcAP5 for fXa compared to rAceAP1 roughly correlates with data from previous studies demonstrating a difference in the inhibitory constants (K_i) for these two hookworm anticoagulants [13]. In the case of rAceAP1, the K_d derived using the bXa binding assay (1.96 nM) is virtually identical to the K_i (2 nM) obtained using the single stage chromogenic assay of fXa inhibition [13]. Moreover, since we were unable to detect significant levels of binding of bXa to the AceAP1 R40A mutant, these data suggest that the predominant interaction between the two molecules requires the inhibitor's active site. Of note, the K_d derived for rAcAPc4 is comparable to that previously measured for the related compound rNAPc2, which inhibits fVIIa/TF by a similar mechanism [21].

We also utilized the bXa binding assay in order to characterize the role of the enzyme's reactive site in binding to each wild type recombinant anticoagulant. As expected, bXa binding to rAcAP5 was unchanged in the presence of an excess of EGR-Xa, which has a blocked active site and hence fails to compete for binding. Also as expected, binding to rAcAPc4 was fully inhibited in the presence of EGR-Xa, since this inhibitor interacts with fXa exclusively via an exosite [26]. In contrast, EGR-Xa was only partially effective at inhibiting the binding of bXa to rAceAP1, confirming the presence of both an active and non-active site interaction between the enzyme and inhibitor. Although we were unable to detect binding of bXa to the AceAP1 R40A mutant, data from previous studies using the chromogenic assay of fXa activity suggested the presence of a second, low affinity ($K_{i_2} = 700$ nM) inhibitory site for rAceAP1 [13]. It is possible that our inability to detect the binding of bXa to the AceAP1 R40A mutant might be due to the weak affinity of the interaction and inadequate sensitivity of the assay. Nonetheless, despite the fact that AceAP1 and AcAPc4 bind fXa with comparable affinities (see above), the demonstration that EGR-Xa competes effectively (but not fully) for binding to AceAP1 provides additional support for our hypothesis that these inhibitors act via distinct mechanisms.

We then characterized rAceAP1 for inhibition of the fVIIa/TF complex, in order to determine its full spectrum of anticoagulant activity. As shown in Fig. 5, both rAceAP1 and rAcAPc4 inhibit fVIIa/TF against a chromogenic substrate. This inhibition was dependent on the presence of fXa, as has previously been described for rAcAPc4 and the related hookworm inhibitors rAcAPc2 and rAcAPc3 [21,22,26]. Moreover, the inhibition of fVIIa/TF against the chromogenic substrate was only observed in the wild type anticoagulants, as the P1 mutants showed no significant inhibitory activity (Fig. 5). This finding was confirmed using the PT assay (Fig. 6). Thus, while rAceAP1 only partially inhibits fXa via interaction with its P1 reactive site, this residue appears to be absolutely required for any inhibitory activity against fVIIa/TF in vitro.

Taken together these data suggest that rAceAP1 may act via a mechanism that is distinct from the related compounds rAcAP5 and rAcAPc4. Whereas the canine hookworm anticoagulants each appear relatively specific for either fXa or fVIIa/TF, the inhibitor from *A. ceylanicum* seems to have evolved a less restrictive anticoagulant spectrum. While the exact structural explanation for this observation remains unknown, examination of the full reactive site amino acid loop of the AceAP1 protein may offer insight into its unique spectrum of anticoagulant activity. While it is generally acknowledged that the P1 amino acid has the greatest impact on target enzyme specificity, the surrounding amino acids also play a role in mediating inhibitory activity [35–39]. Inspection of the amino acid sequence of the AceAP1 reactive site loop reveals features that are similar to both AcAP5 and AcAPc4 (Fig. 1). Work is currently underway aimed at elucidating the role of the amino acid residues surrounding the P1 site in mediating the interaction between AceAP1 and its targets, fXa and fVIIa/TF. It has previously been suggested that protease inhibitors are particularly susceptible to evolutionary pressure, as evidenced by a high rate of spontaneous mutations in and around predicted reactive sites [40,41].

Interestingly, we have recently demonstrated that the source of production of the fXa and fVIIa/TF inhibitors in *A. caninum* are distinct, suggesting unique roles in the bloodfeeding process [26]. We have also demonstrated that the degree of anticoagulant activity found in soluble extracts of adult worms roughly correlates with prior estimates of bloodfeeding activity in vivo [13]. To date, AceAP1 appears to be the only major anticoagulant produced by adult *A. ceylanicum* hookworms. This contrasts markedly with *A. caninum*, from which multiple native anticoagulant proteins and corresponding cDNAs have been isolated [8,26]. It is interesting to speculate that the presence of a single anticoagulant with moderately less activity in vitro may in part explain why *A. ceylanicum* is associated with less severe intestinal blood loss the canine hookworm [42,43]. Further study will now be focused on isolating functionally related molecules produced by other human parasites, including *Necator americanus* and *A. duodenale*. Ultimately, a vaccine based approach aimed at impairing the ability of adult hookworms to feed by neutralizing the effect of these anticoagulants represents a novel strategy for reducing the devastating effects of hookworm anemia in the one billion people at risk worldwide [44]. The potential for inhibiting the function of specific hookworm secretory proteins in vivo has recently been validated, as it has been demonstrated that immunization with single recombinant antigens confers partial protection against clinical disease (anemia or growth delay) following challenge infection [45,46].

The hookworm anticoagulants rAcAP5 (rNAP5) and rAcAPc2 (rNAPc2) are currently in advanced stages of development as agents for the treatment or prevention of thrombosis in humans [24,25,47–49]. These compounds may also represent novel treatments for tumor metastasis [30,50–52], as well as conditions that are associated with tissue factor mediated inflammatory cytokine release, including sepsis syndrome and Ebola virus infection [47,53–56]. Based on the data presented here, it is possible

that rAceAP1, due to its distinct spectrum of anticoagulant activity and likely novel mechanism, may demonstrate more favorable activity in vivo than rAcAP5 and rAcAPc2, neither of which has yet been approved for use in humans. Most importantly, however, as these studies of parasitic hookworms demonstrate, intensive study of the antithrombotic mechanisms of hematophagous invertebrates holds great potential for identifying novel compounds with clinical application to a variety of human diseases.

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