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Identification of an anticoagulant peptide that inhibits both fXIa and fVIIa/tissue factor from the blood-feeding nematode *Ancylostoma caninum*

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ABSTRACT

Factor VIIa-tissue factor complex (fVIIa/TF) and factor XIa (fXIa) play important roles in the initiation and amplification of coagulation, respectively. They may be good targets for the development of novel anticoagulants to treat and prevent thromboembolic disease. In this study, we cloned, expressed and identified a novel anticoagulant peptide, AcaNAP10, from the blood-feeding nematode *Ancylostoma caninum*. AcaNAP10 showed potent anticoagulant activity and doubled the activated partial thromboplastin and prothrombin times at estimated concentrations of 92.9 nM and 28.8 nM, respectively. AcaNAP10 demonstrated distinct mechanisms of action compared with known anticoagulants. It inhibited fXIa and fVIIa/TF with IC_{50} values of 25.76 ± 1.06 nM and 123.9 ± 1.71 nM, respectively. This is the first report on an anticoagulant that can inhibit both fXIa and fVIIa/TF. This anticoagulant peptide may be an alternative molecule for the development of novel anticoagulants.

Introduction

The maintenance of hemostasis prevents blood loss from injured blood vessels and requires the generation of thrombin, which is responsible for converting fibrinogen to fibrin [1]. Factor VIIa–tissue factor complex (fVIIa/TF) and factor XIa (fXIa) play important roles in the initiation and amplification of coagulation, respectively [1–3]. Blood coagulation is triggered by fVIIa/TF, which activates factor IX (fIX) and factor X (fX), and thus leads to the formation of small amounts of thrombin [1–4]. However, once fXa is formed, fVIIa/TF is rapidly limited by the tissue factor pathway inhibitor (TFPI) [1,5,6] and an alternative pathway is thus required to maintain thrombin formation. Indeed, thrombin has been demonstrated to activate fXI to fXIa [7–9]. The thrombin initially generated by fVIIa/TF activates fXI, which encourages coagulation through activation of fIX, resulting in the formation of additional thrombin [7–9]. Furthermore, additional thrombin generation via this pathway can protect the fibrin clot from lysis by activation of thrombin activatable fibrinolysis inhibitor (TAFI) [10–13]. Therefore, fVIIa/TF and fXIa are promising targets for interfering with hemostasis and for the development of novel anticoagulant drugs to treat or prevent thromboembolic disease.

In addition to TFPI, the physiological inhibitor of fVIIa/TF, two main groups of exogenous fVIIa/TF inhibitors have been isolated from blood-feeding animals [14–17]. One group includes Ixolaris and Penthalaris isolated from the deer tick *Ixodes scapularis*, while the second group includes the nematode anticoagulant protein/peptide c2 (NAPc2) and its homologs NAPc3 and NAPc4 from the dog hookworm *Ancylostoma caninum*. NAPc2 and Ixolaris are currently undergoing clinical or preclinical trials aimed at the development of antithrombotic drugs to block the initiation of coagulation [18–20]. However, compared with fVIIa/TF inhibitors, very few exogenous fXIa inhibitors have been isolated.

Hookworms are blood-feeding intestinal nematodes that can cause chronic gastrointestinal blood loss of their hosts [21]. They secrete antihemostatic molecules to counteract host hemostatic responses and facilitate blood feeding [16,17,22–24]. To date, several anticoagulant peptides, also known as nematode anticoagulant proteins/peptides (NAPs), from hookworms have been characterized. The *A. caninum* peptide NAP5 and NAP6 are inhibitors of fXa [16], NAPc2, NAPc3 and NAPc4 are inhibitors of fVIIa/TF [16,17], while AceAP1, the only anticoagulant peptide from *A. ceylanicum*, is an inhibitor of both fXa and fVIIa/TF [23,24]. We re-

Abbreviations: AcAP, *Ancylostoma caninum* anticoagulant peptide; AceAP, *Ancylostoma ceylanicum* anticoagulant peptide; aPTT, activated partial thromboplastin time; EGR-fXa, active site-blocked factor Xa containing Glu-Gly-Arg chloromethyl ketone; fXI, factor XI; fXIa, activated factor XI; fVIIa/TF, factor VIIa/tissue factor complex; NAP, nematode anticoagulant protein/peptide; PT, prothrombin time; TAFI, thrombin activatable fibrinolysis inhibitor; TFPI, tissue factor pathway inhibitor

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cently demonstrated that the human hookworm *A. duodenale* peptide AduNAP4, acted as an inhibitor of both fXa and fXIa [25]. We report here on the identification and characterization of a novel *A. caninum* anticoagulant peptide, named *A. caninum* nematode anticoagulant peptide 10 (AcaNAP10), which is an inhibitor of both fXIa and fVIIa/TF and has a mechanism of action distinct from that of other known anticoagulants.

Materials and methods

Parasite materials. Adult *A. caninum* were collected from naturally infected dogs in Zhanjiang, Guangdong province, China, and stored at −70 °C until use.

RNA extraction and cDNA synthesis. Total RNA was extracted from 10 worms (approximately 30 mg) using an RNAfast 200 kit (FASTAgen, Shanghai, China), and first-strand cDNA was synthesized from total RNA with a 3′-Full RACE Core Set kit (TakaRa, Dalian, China), according to the manufacturer's instructions.

Cloning of AcaNAP10 cDNA. PCR products were amplified from 2 μl of first-strand adult *A. caninum* cDNA using the primer pair AP11–1e (5′ GAGGATCCAATCCAAGCTGTGGTGAG 3′) and AP11–2e (5′ CGAAGCTTGGTCATTTTCTGTTAGGG 3′), which was originally designed for the amplification of the cDNA sequences encoding AcaNAP11 (GenBank Accession No. EF495068). The PCR reaction consisted of an initial denaturation for 4 min at 94 °C, followed by 30 cycles at 94 °C for 15 s, 54 °C for 30 s, and 72 °C for 1 min, with a final extension for 10 min at 72 °C. The PCR products were purified with TIANquick Midi Purification Kit (Tiangen, Beijing, China) and cloned into pUCm-T vector (Bio Basic Inc, Canada), following the manufacturer's instructions. Positive clones were selected and sequenced using an ABI 377 automated sequencer (Applied Biosystems, United States) in Shanghai Generay, China. Thereafter, the forward primer SL1 (5′ GGTTTAATTACCCAAGTTTGAG 3′), which is the nematode spliced leader sequence of many nematode mRNAs, and a reverse primer NAP-2 (5′ CTGCTTTCACACAGGCG 3′), which was designed based on the nucleotide sequences amplified using the AP11–1e/AP11–2e primer pair, were used for the amplification of the 5′cDNA sequences from the first-strand cDNA template. PCR was performed as follows: an initial denaturation for 2 min at 94 °C, followed by 30 cycles at 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 90 s, with a final extension for 10 min at 72 °C. The resulting products were purified, cloned and sequenced as described above.

Sequence analysis. Similarity searches of nucleotide and deduced amino acid sequences were performed using BLAST programs on the NCBI web site (http://www.ncbi.nlm.nih.gov). A multiple sequence alignment of mature AcaNAP10 with that of NAPs studied to date was generated using the CLUSTAL W method in Lasergene software.

Expression and purification of recombinant AcaNAP10 (rAcaNAP10). The pUCm-T vector containing the nucleotide sequences encoding mature AcaNAP10, which was amplified using the AP11–1e and AP11–2e primer pair from the adult *A. caninum* cDNA template, was digested with BamHI/HindIII. The cleaved products encoding the mature AcaNAP10 were then ligated into the pET32a expression vector (Novagen, Germany). The plasmids were then transformed into *Escherichia coli* BL21 (DE3). Expression of the recombinant protein was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside in Luria–Bertani broth. The cells were harvested by centrifugation at 5000 rpm, followed by cell lysis and sonication. The recombinant fusion proteins in the supernatant were affinity-purified using Protino Ni-IDA resin (Macherey–Nagel, Germany), eluted with 10–250 mM imidazole gradient buffer and concentrated and desalted by ultrafiltration with Nanosep 3 K Omega (Pall Co., United States). The concentrated and desalted fusion proteins were cleaved using enterokinase (Biowisdom, Shanghai, China) to remove the N-terminal fusion tag. In brief, after incubation at 20 °C for up to 14 h with 3 units of enterokinase per 1 mg of recombinant protein, the cleaved fusion tags and undigested protein were removed with Protino Ni-IDA. The expressed, purified and cleaved protein was analyzed by 16% Tricine–SDS–PAGE, or 12% SDS–PAGE. The concentration of the recombinant protein was estimated using a Bradford procedure kit (Sangon, Shanghai, China) with bovine serum albumin as a standard.

Anticoagulation assays. Anticoagulant activity was determined by measuring the activated partial thromboplastin time (aPTT) and prothrombin time (PT). For the aPTT assay [17,24], 10 μl recombinant protein at various concentrations was mixed with 20 μl aPTT reagent (MDC Hemostasis, Germany) and 50 μl fresh frozen normal human plasma in individual wells of a 96-well microtiter plate, and incubated for 15 min at 37 °C. Twenty microliters of 50 mM prewarmed $CaCl_2$ was then added to each well to initiate the clotting reaction. The clotting time was measured by reading the absorbance at 630 nm wavelength every 7 s for 6 min using an Elx808 kinetic microtiter reader (BioTek, United States). For the PT assay, which was modified from Mieszczanek et al. [17,24], 10 μl protein at various concentrations was mixed with 30 μl normal human plasma in individual microtiter wells and incubated for 15 min at 37 °C. Sixty microliters of prewarmed PT reagent (MDC Hemostasis) was then added and the clotting time was measured by reading the changes in absorbance at 630 nm every 5 s for 6 min using an Elx808 kinetic microtiter reader. The clotting times at various concentrations were analyzed using Excel software.

Serine protease inhibition assays. The activity of rAcaNAP10 against serine proteases was investigated by chromogenic assays carried out in a total reaction volume of 100 μl in individual wells of a 96-well microtiter plate. To determine the inhibitory activity against fVIIa/TF, 10 μl recombinant protein to a final concentration of 1 μM was placed in a microtiter plate well with 50 μl soluble TF (sTF) (final concentration: 1 μM) (Protgen, Beijing, China), recombinant fVIIa (2 nM) (Novo-Nordisk, Denmark), and with or without active site-blocked factor Xa containing Glu-Gly-Arg chloromethyl ketone (EGR-fXa) (500 nM) (Haematologic Technologies Inc., United States) in 2× HBSAC buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 10 mM $CaCl_2$, 0.2% bovine serum albumin and 0.1% NaN_3) for 15 min at 25 °C. After the addition of 40 μl of prewarmed chromogenic substrate S2288, the residual fVIIa activity was monitored by recording the absorbance at 405 nm using an Elx808 kinetic microtiter reader. For other serine proteases, 10 μl recombinant protein to a final concentration of 1 μM was incubated with 50 μl of each serine protease (Haematologic Technologies Inc.) in 2× HBSAC buffer for 15 min at 25 °C. After the addition of 40 μl of prewarmed chromogenic substrate (Chromogenix, Italy) to a final concentration of 800 μM, the changes in absorbance at 405 nm were monitored using an Elx808 kinetic microtiter reader. The following chromogenic substrates were used for these serine proteases assays: S2765 for human fXa (final concentration: 1 nM), S2288 for human α-thrombin (1 nM) and human plasmin (3 nM), and S2366 for human fXIa (1 nM), human fXIIa (5 nM) and human activated protein C (2 nM), respectively.

Kinetic analysis of inhibitory activities against fXIa and fVIIa/TF. To investigate the inhibitory kinetics of rAcaNAP10 against fXIa, 10 μl of rAcaNAP10 at various concentrations was incubated with 50 μl fXIa (1 nM) in individual microtiter plate wells for 15 min at 25 °C. Forty microliters of prewarmed S2366 to a final concentration ranging from 50–800 μM was added and the changes in absorbance at 405 nm (m*A*/min at 405 nm) due to substrate hydrolysis in the presence and absence of rAcaNAP10 were measured using an Elx808 kinetic microtiter reader. Nonlinear regression analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) to determine the IC_{50} and K_i.