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Ac-AP-12, a novel factor Xa anticoagulant peptide from the esophageal glands of adult *Ancylostoma caninum*☆

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ABSTRACT

Immunoscreening an *Ancylostoma caninum* cDNA library with canine hookworm-infected dog serum resulted in the isolation of a 461 bp cDNA encoding *Ac*-AP-12, a new 9.1 kDa anticoagulant peptide (100 amino acids) with 43–69% amino acid homology to other nematode anticoagulant peptides (NAPs) from *Ancylostoma* hookworms. Messenger RNA transcription and expression of *Ac*-AP-12 was unique to the adult stage of *A. caninum*. The yeast expressed recombinant *Ac*-AP-12 demonstrated potent anticoagulant activity on human blood plasma in a concentration dependent manner, and was shown to specifically inhibit human factor Xa activity. Immunolocalization with specific rabbit antiserum showed that *Ac*-AP-12 was exclusively located in the esophageal glands of adult hookworm. *Ac*-AP-12 is hypothesized to facilitate both parasite blood feeding and digestion.

1. Introduction

Hookworms are blood-feeding nematode parasites that currently infect an estimated 576–740 million people in developing countries [1,2]. The adult stage hookworms parasitize the small intestine of human and other mammalian hosts, where they attach to the intestinal mucosa and submucosa by using their buccal capsule and mechanically rupture capillaries and arterioles [3,4]. Hookworms then ingest the blood, rupture red blood cells, and catalyze the degradation of host hemoglobin using an ordered cascade of proteases [5]. Through these mechanisms hookworms cause intestinal blood loss leading to iron deficiency anemia [3,4].

In order to facilitate blood feeding, hookworms have evolved effective anticlotting strategies to interfere with host hemostasis [6–9], including the parasite release of anticoagulant peptides and anti-platelet aggregating agents into the attachment site [10–13]. To date, a number of structurally related potent anticoagulants termed nematode anticoagulant proteins (NAPs) have been isolated and characterized from canine hookworm *Ancylostoma caninum* [10,11,14–17] and its related species *Ancylostoma ceylanicum* [18,19]. In addition, an anticoagulant peptide called AduNAP4 was recently cloned and characterized from the major human hookworm *Ancylostoma* species, *Ancylostoma duodenale* [20]. Each of these NAPs is a protease inhibitor that targets one or more serine proteases comprising the mammalian coagulation cascade [21,22]. For instance, *Ac*AP5, as well as its homologous *Ac*AP6, is a specific inhibitor of active coagulation factor Xa (fXa) [10,11], a convergent point of the extrinsic and intrinsic coagulation pathways; whereas *Ac*APc2 and its homologues *Ac*APc3/*Ac*APc4 inhibit activities of factor VIIa/tissue factor complex (fVIIa/TF) [10,11,23]. Another fVIIa/TF inhibitor of *A. caninum* anticoagulant (*Aca*NAP10) also inhibits factor XIa [17]. *Ace*AP1 from *A. ceylanicum* inhibits both fXa and fVIIa/TF [19], while *Adu*NAP4 inhibits factors Xa and XIa [20]. By immunoscreening an *A. caninum* cDNA library with canine hookworm infected dog sera we identified and cloned a novel factor Xa inhibitor anticoagulant peptide designated *Ac*-AP-12. The recombinant *Ac*-AP-12 was biochemically characterized and the native *Ac*-AP-12 was localized to the parasite esophageal glands.

2. Materials and methods

2.1. Immunoscreening of A. caninum adult cDNA library

An *A. caninum* adult cDNA λZapII expression library constructed as reported previously [24] was immunoscreened with serum of dog infected with *A. caninum* [25] as described previously [26]. Briefly, approximately 5×10^4 plaques were plated on each LB agar

☆ *Note:* Nucleotide sequence data reported in this paper is available in the GenBank™ database under the accession number: HQ637266.

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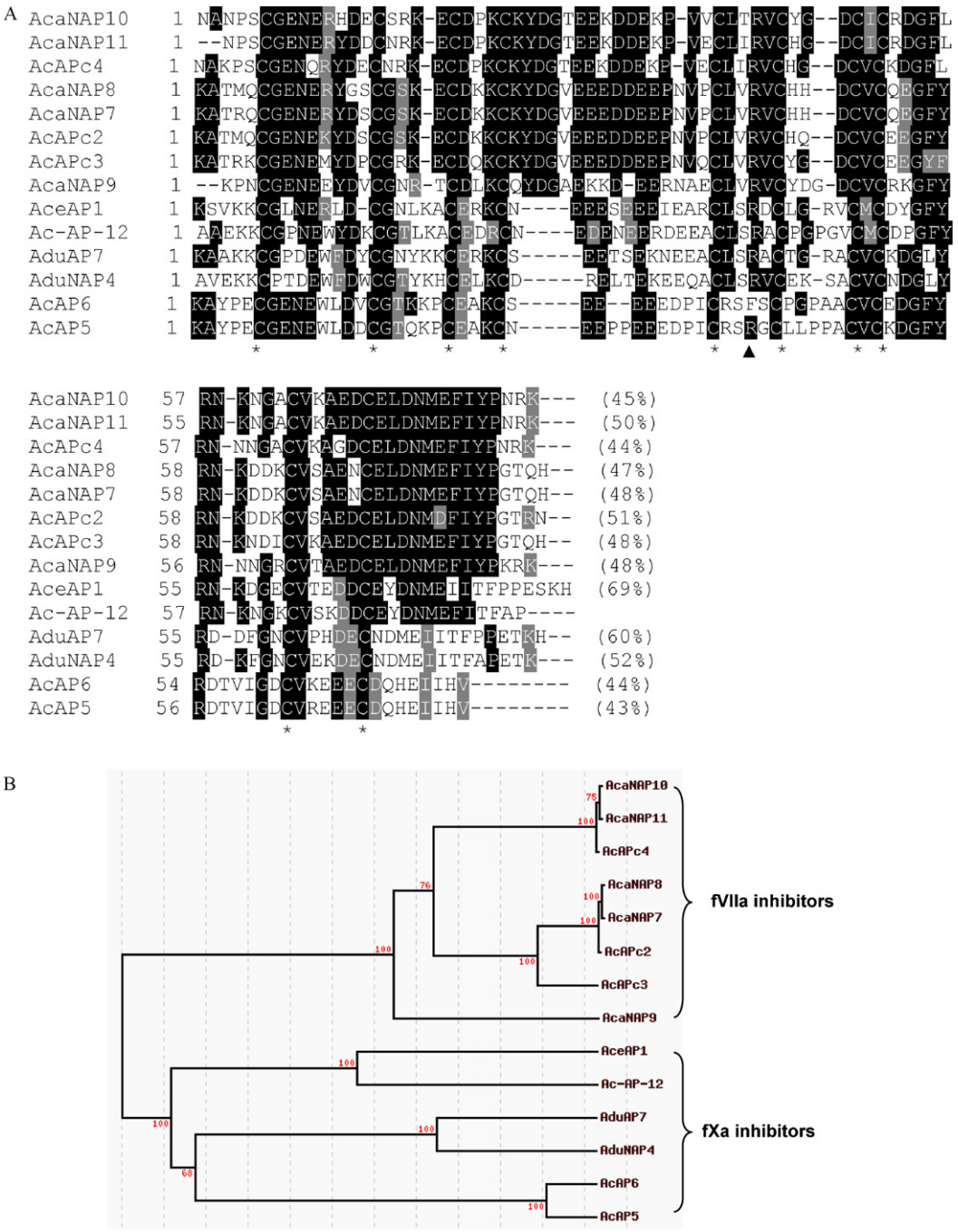

Fig. 1. (A) Alignment of the deduced amino acid sequences of identified hookworm NAPs. Sequences were aligned using CLUSTAL W and prepared for display using BOXSHADE. Identical amino acids are shaded in black and similar amino acids in gray. The conserved 10 cysteines are marked by asterisk below the alignment. The conserved Arg[40] residue at the predicted P1 inhibitory reactive site is marked by a solid triangle. The percentage of sequence identity to *Ac*-AP-12 is shown at the end of each sequence. (B) Neighbour-joining tree representing phylogenetic relationships between hookworm NAPs. The hookworm anticoagulant protein sequences (GenBank accession number) used in the alignment and the tree include AcaNAP10 (ABP88128); AcaNAP11 (ABP88129); AcAPc4 (AAP82926); AcaNAP8 (ABD98796); AcaNAP7 (ABD98795); AcAPc2 (AAC47080); AcAPc3 (AAP57305); AcaNAP9 (ABP88127); *Ace*AP1 (AAK81733); *Ac*-AP-12 (HQ637266); *Adu*AP7 (ABP88734); *Adu*NAP4 (ACD80355); *Ac*AP6 (AAC47081) and *Ac*AP5 (AAC47082).

plate and induced with 10 mM IPTG. Expressed antigens blocked on nitrocellulose membranes were incubated with 1:5000 dilutions of *A. caninum* infected dog serum. The positive clones were detected with ECL Western blotting detection reagent (GE Healthcare, UK).

2.2. DNA sequencing and analysis

The immuno-positive clones were *in vivo* excised into pBluscript phagemids according to manufacturer's instructions (Stratagene). Phagemid DNA was extracted using the alkaline lysis method (Qiagen) and double strand sequencing was performed using vector flanking primers, T_3 and T_7 promoter. Nucleotide and deduced amino acid sequences were compared to existing sequences in the GenBank by BLAST searching (http://www.ncbi.nlm.nih.gov).

2.3. Expression and purification of recombinant Ac-AP-12

DNA encoding the matured *Ac*-AP-12 without the signal peptide was amplified from the total first-strand cDNA of adult *A. caninum* with primers *Ac*-AP1-F1 (CGGAATTCGC AGAGAAGAAATGTGGTCC)