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RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding

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Abstract

In addition to being the conduit for pathogens into hosts, tick saliva contains a broad array of secretory products that facilitate prolonged tick attachment and blood feeding. Proteins found in tick saliva modulate host hemostasis and immune responses. However, it is not clear whether ticks manipulate the immune responses of their hosts by disrupting the antigen-processing pathways of the hosts. Protein secretion into tick saliva from the salivary glands is due to exocytosis of vesicular membrane-bound granular material regulated by SNARE complex proteins. Proteins associated with vesicles (v-SNAREs) are essential components of the exocytotic process. In this study, we assessed the functional significance of synaptobrevin, a SNARE protein, and cystatin, a cysteine protease inhibitor to blood feeding success, in the lone star tick, *Amblyomma americanum*, using in vivo RNA interference. In separate experiments, tick salivary cystatin and synpatobrevin genes were silenced by injecting adult ticks with 500 ng of dsRNA complementing each gene sequence. Silencing was demonstrated by reduced transcript in midguts and salivary glands. Additionally, disrupting expression of cystatin and synaptobrevin by RNAi reduced the ability of ticks to feed successfully, as demonstrated by feeding inhibition and reduced engorgement weights. Moreover, normal ticks exposed to a rabbit previously exposed to cystatin-silenced ticks exhibited significant resistance to tick feeding. Based on these findings, ticks appear to skillfully evade the host immune system by secreting cystatin, which disrupts normal antigen processing in antigen-presenting cells of hosts.

Keywords: Ticks; Cystatin; Synaptobrevin; Salivary glands; RNAi; SNARE; Exocytosis; *Amblyomma americanum*; APC (antigen-presenting cells); Blood feeding

Proteins and other molecules synthesized and secreted by ixodid tick salivary glands are crucial for these organisms' successful blood feeding on vertebrate hosts. As the feeding process progresses over the course of several days, the rate of salivary fluid secretion greatly increases, enabling the tick to concentrate the blood meal in its midgut by returning excess water and ions to the host. Since ticks feed exclusively on host blood in each active stage—larvae, nymph, and adult—active modulation of a host's immune response is a critical component of these ectoparasites' strategies for prolonged feeding. Tick salivary glands produce numerous bioactive proteins (e.g., anticoagulants, anti-inflammatory proteins, and immunosuppressants) and prostaglandins to modulate interactions of the tick with the host [1,2]. Identifying specific immunomodulatory or secretory machinery molecules represents a potential opportunity towards developing an anti-tick vaccine.

To adapt to and survive in or on hosts, many parasites have evolved clever mechanisms for modulating host defense systems [3]. Before protective immunity

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can develop, pathogens must be processed into antigenic subunits by a set of lysosomal proteases, such as cathepsins in antigen-presenting cells (APCs), and then the processed peptides must be presented by major histocompatibility complex molecules expressed on the APCs [4]. Studies have shown the ability of various intracellular parasites to block the antigen presentation pathway in host APCs, such as in hosts with leishmanial infections [3,5]. However, there is little evidence that ectoparasites, including ticks, modulate the antigen-presenting capacity of host APCs. The cystatins are low molecular mass cysteine protease inhibitors known from a range of arthropods and vertebrates [6–9]. Members of the cystatin family are secretion-type proteins having a single domain with a molecular mass of 13–15 kDa. Cystatins from nematodes are thought to suppress the proteases involved in antigen processing through their intrinsic functions, and the nematode *Nippostrongylus brasiliensis* utilizes this protease inhibitor to evade the host defense system [10]. Genes encoding cysteine protease inhibitors also have been found in the sialome of various ixodid ticks [11]. It may be that tick cysteine protease inhibitors facilitate tick blood feeding by suppressing proper antigen processing and immune recognition of other tick salivary molecules. However, the function of tick cystatins remains unknown.

Tick salivary molecules, including cystatin, are synthesized and secreted by salivary gland cells. Many, if not all, of the protein and other molecules produced by tick salivary glands are released into the salivary gland lumen by exocytosis. Exocytosis is a highly orchestrated process involving docking and fusion of secretory vesicles with the plasma membrane [12,13]. According to the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) hypothesis of vesicle fusion, vesicles dock at target membranes through interaction of complementary sets of vesicular (v-SNARE) and target (t-SNARE) membrane proteins. SNARE proteins assemble in tight core complexes, which promote fusion of carrier vesicles with target compartments. Assembly of cognate v- and t-SNARE proteins promotes formation of extremely stable core complexes which serve as functional receptors for cytosolic factors implicated in transport and vesicle fusion, soluble NSF attachment proteins (SNAPs), and the *N*-ethylmaleimide-sensitive factor (NSF) [14]. Besides SNAPs and NSF, various other molecules are known to regulate SNARE protein interactions and activities [15–18]. Among these molecules, Synaptobrevin is a v-SNARE protein thought to play a key role in the process of exocytotic protein secretion in secretory cells [19]. Synaptobrevin is present in the salivary glands of partially fed female lone star ticks (*Amblyomma americanum*), and is important in the process of exocytotic protein secretion in this tick's salivary glands [20,21]. Its role in tick blood feeding success has not been evaluated in vivo.

RNA interference (RNAi) is the phenomenon in which long double-stranded (ds) RNA is able to silence cognate gene expression [22]. First, the dsRNA must be processed into small interfering RNA (siRNA) by a Dicer enzyme [23–26]. The Dicer and associated proteins are able to facilitate loading of siRNA onto a complex known as the RNA-induced silencing complex (RISC) [23]. The RISC-bound siRNA strand acts as a guide to identify mRNA targets with complementary sequence for nucleolytic cleavage [27]. RISC activity is defined simply as siRNA-guided, site-specific cleavage of an mRNA target. RNAi is possible through injection of sequence-specific dsRNA into an organism or incubation of tissues with the dsRNA. In organisms and tissues possessing Dicer, RNAi offers a method of specifically inactivating expression (gene silencing) of the corresponding gene, thereby providing an opportunity to investigate the corresponding protein's function [27]. In the present study, we used long double-stranded RNA to disrupt expression of a tick salivary secretory protein as well as a protein component of exocytotic machinery. Specifically, we used dsRNA to silence genes for cystatin and a tick homolog of the v-SNARE protein synaptobrevin to examine the significance of these molecules to tick blood feeding success.

Materials and methods

Restriction enzymes, *Taq* DNA polymerase, plasmid DNA, and polymerase chain reaction (PCR) product purification kits were purchased from Invitrogen (Carlsbad, CA, USA) and Qiagen (Valencia, CA, USA). All RNA extraction reagents and kits were bought from Ambion (Austin, TX, USA).

Ticks. Amblyomma americanum (L.) ticks were obtained from Oklahoma State University's tick rearing facility, where they are raised according to the methods of Patrick and Hair [28]. Briefly, all immature ticks were fed on NZ white rabbits and adult ticks on sheep. All unfed ticks were maintained at 27–28 °C and 90% relative humidity under 14 h light/10 h dark photoperiod before infestation on the hosts. To obtain partially fed adult ticks, females and an equal number of males were allowed to attach to the ears of NZ white rabbits for anywhere from 1 to 9 days. Ticks were removed using forceps and were dissected within 4 h of being removed from the host.

Salivary glands and midguts. Tick salivary glands and midguts were dissected in ice-cold 100 mM 3-(*N*-morpholino)-propanesulfonic acid (Mops) buffer containing 20 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH 6.8. After removal, glands were washed gently in the same ice-cold buffer. The dissected tissues were immediately stored in RNAlater (Ambion, Austin, TX, USA) prior to isolating total RNA. Tissues were used immediately after dissection or stored at −70 °C in 0.5 M piperazine *N*,*N*-bis-2-ethane sulfonic acid, pH 6.8, containing 20 mM EGTA, 1× Complete Mini Protease inhibitor cocktail (Roche, Indianapolis, IN, USA), and 40% glycerol for Western blotting. All other manipulations were carried out at 4 °C.

Synthesis of tick salivary gland cDNA and RT-PCR. Full-length (from NH2 terminus to stop codon) cystatin was amplified from *A. americanum* cDNA by PCR using High-Fidelity platinum Taq