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Disruption of blood meal-responsive serpins prevents *Ixodes scapularis* from feeding to repletion

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ABSTRACT

Serine protease inhibitors (serpins) are thought to mediate the tick's evasion of the host's serine protease-mediated defense pathways such as inflammation and blood clotting. This study describes characterization and target validation of 11 blood meal-responsive serpins that are associated with nymph and adult *Ixodes scapularis* tick feeding as revealed by quantitative (q)RT-PCR and RNAi silencing analyses. Given the high number of targets, we used combinatorial (co) RNAi silencing to disrupt candidate serpins in two groups (G): seven highly identical and four non-identical serpins based on amino acid identities, here after called GI and GII respectively. We show that injection of both GI and GII co-dsRNA into unfed nymph and adult *I. scapularis* ticks triggered suppression of cognate serpin mRNA. We show that disruption of GII, but not GI serpins significantly reduced feeding efficiency of both nymph and adult *I. scapularis* ticks. Knockdown of GII serpin transcripts caused significant respective mortalities of ≤40 and 71% of nymphal and adult ticks that occurred within 24–48 h of attachment. This is significant, as the observed lethality preceded the tick feeding period when transmission of tick borne pathogens is predominant. We suspect that some of the GII serpins (S9, S17, S19 and S32) play roles in the tick detachment process in that upon detachment, mouthparts of GII co-dsRNA injected were covered with a whitish gel-like tissue that could be the tick cement cone. Normally, ticks do not retain tissue on their mouthparts upon detachment. Furthermore, disruption of GII serpins reduced tick blood meal sizes and the adult tick's ability to convert the blood meal to eggs. We discuss our data with reference to tick feeding physiology and conclude that some of the GII serpins are potential targets for anti-tick vaccine development.

1. Introduction

Ticks are among the most important ecto-parasites with global public health and veterinary health impact. In terms of the diversity of transmitted animal and human disease pathogens, ticks are the second most important vectors after mosquitoes (Jongejan and Uilenberg, 2004). In public health, the impact of tick-borne diseases (TBD) has been on the rise since the description of *Borrelia burgdorferi* as the causative agent of Lyme borreliosis vectored by *I. scapularis* (Burgdorfer et al., 1982, 1989; Burgdorfer, 1984). *I. scapularis* and its sister species, *I. ricinus, I. pacificus,* and *I. holocyclus* are major vectors of human TBD agents in North America, Europe and Australia (Davis et al., 2015; Piesman and Stone, 1991; Rizzoli et al., 2014). Currently, the US Centers for Disease Control (http://www.cdc.gov/ticks/diseases/) has listed 16 reportable human TBDs agents, six of which are transmitted by *I. scapularis.* Other *I. scapularis* vectored agents include the newly described Lyme disease agent, *B. mayonii* (Dolan et al., 2016, 2017a, 2017b; Pritt et al., 2016; Telford et al., 2015), *B. miyamotoi,* closely related to tick relapsing fever causative agents (Breuner et al., 2017), *Anaplasma phagocytophilum* (animal and human anaplasmosis, Levin and Ross, 2004), *Babesia microti* (Prusinski et al., 2014), Powassan encephalitis virus (Dupuis et al., 2013), and *B. odocoilei* (cervid babesiosis) (Steiner et al., 2006). On the west coast of the United States, *I. pacificus* is the primary vector for Lyme disease and human anaplasmosis agents (Burgdorfer et al., 1985; Dahlgren et al., 2015). The importance of *I. scapularis* in public health justified sequencing of the full genome (Gulia-Nuss et al., 2016; Hill and Wikel, 2005). Availability of the *I. scapularis* genome sequence data provided for opportunities for in depth studies to understand tick feeding physiology as means through which to find out ways to design better tick control strategies.

In absence of effective vaccines against TBD agents, killing ticks by chemical acaricides is currently the only reliable alternative method to protect against animal and humans TBD infections (Cisak et al., 2012; Drummond, 1976). The limitations of the current acaricide-based tick control methods including development of resistance against these chemicals and environmental contaminations are known (Abbas et al.,

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2014; Coles and Dryden, 2014; George et al., 2004; Graf et al., 2004). Immunization against tick feeding is a validated sustainable alternative tick control method (de la Fuente et al., 2015; Mulenga et al., 2001; Willadsen et al., 1995; Willadsen, 2004). The major limitation toward the global adoption of anti-tick immunization as an alternative tick control method is the lack of effective tick vaccine antigens. Effective vaccine antigens could confer better anti-tick protection levels that are comparable to acaricide-based tick control, and this would result to broader acceptance of tick vaccines as a tick control strategy. As transmission of animal and human TBD infections primarily occur via tick feeding (Liu and Bonnet, 2014), understanding the molecular basis of tick feeding physiology is likely to lead to discovery of effective tick vaccine target antigens. We are interested in understanding the role(s) of tick serine protease inhibitors (serpins) in tick feeding physiology.

The tick feeding style of lacerating host tissue and sucking up blood that bleeds into the wounded area is expected to provoke host defenses such as coagulation, inflammation, complement activation, all of which are serine protease mediated pathways that are under tight control of serpins (Gettins, 2002; Huntington, 2011; Olson and Gettins, 2011). Serpin malfunctions that lead to human diseases, emphysema, thrombosis, and angioedema (Carrell and Lomas, 2002; Carrell and Travis, 1985) attest to the importance of serpins. It is possible that, similar to other organisms, serpins play important role(s) in tick feeding physiology and if disrupted could reduce tick-feeding efficiency. On this basis, ticks were suspected to inject serpins into the host to evade host defense to tick feeding and thus this family of proteins represent important candidate tick vaccine antigens (Mulenga et al., 2001). Several serpin encoding cDNAs have been cloned and characterized in several tick species (Imamura et al., 2005; Kaewhom et al., 2007; Leboulle et al., 2002; Mulenga et al., 2003; Tirloni et al., 2016; Toyomane et al., 2016 Yu et al., 2013) including at least 45 in *I. scapularis* (Mulenga et al., 2009a,b). Some of the tick serpins are functional inhibitors of protease mediators of host defense pathways including blood clotting, inflammation, and complement activation (Chmelar et al., 2011; Kim et al., 2015b; Ibelli et al., 2014; Mulenga et al., 2013a; Radulović and Mulenga, 2017; Prevot et al., 2006; Rodriguez-Valle et al., 2015). In other studies, immunization of animals with recombinant tick serpins reduced tick-feeding efficiency (Imamura et al., 2005; Imamura et al., 2006; Imamura et al., 2008; Jittapalapong et al., 2010; Kim et al., 2016; Prevot et al., 2007; Sugino et al., 2003)

The goal of this study was to characterize the roles of 11 blood meal-responsive serpins in *I. scapularis* tick feeding. These serpins were first described among 45 *I. scapularis* serpin sequences (Mulenga et al., 2009a,b). Semi-quantitative RT-PCR expression analysis showed that some of the 11 candidate serpins were apparently up regulated during adult *I. scapularis* tick feeding, suggesting a role in regulating tick feeding. In this study, we have used quantitative RT-PCR expression analysis to relate the transcriptional profiles of candidate serpins to different stages of nymph and adult *I. scapularis* tick feeding. Target validation using the combinatorial RNAi silencing approach was successfully used to validate that four of the 11 *I. scapularis* serpins are important to nymph and adult *I. scapularis* tick feeding and thus represent potential target antigens for anti-tick vaccine development.

2. Materials and methods

2.1. Ethics statement

All experiments were done according to the animal use protocol approved by Texas A&M University Institutional Animal Care and Use Committee (IACUC) (AUP 2011–207 and 2011–189, 2014-0310) that meets all federal requirements, as defined in the Animal Welfare Act (AWA), the Public Health Service Policy (PHS), and the Humane Care and Use of Laboratory Animals.

Table 1
Tick serpin sequences used for construction of phylogenetic tree.

Source sequence	Accession numbers in public databases
Ixodes scapularis Serpin 12	EW892008
Ixodes scapularis Serpin 14	ISCW015204
Ixodes scapularis Serpin 18	ISCW024013
Ixodes scapularis Serpin 21	EW881762
Ixodes scapularis Serpin 22	EW949864
Ixodes scapularis Serpin 10	ISCW023617
Ixodes scapularis Serpin 25	ISCW014257
Ixodes scapularis Serpin 9	B7QL31
Ixodes scapularis Serpin 17	B78JF1
Ixodes scapularis Serpin 19	B7PH24
Ixodes scapularis Serpin 32	B7Q0E8
Ixodes ricinus Serpin	AOA131XZ77
Ixodes ricinus Serpin	Q06B72
Amblyomma maculatum Serpin	G3ML50
Amblyomma triste Serpin	A0A023GPF9
Amblyomma cajennense Serpin	A0A023FM57
Amblyomma americanum Serpin	A0A0E9Y2F
Rhiphicephalus pulchellus Serpin	L7LRY7
Rhiphicephalus appendiculatus Serpin	A0A131YYG3
Rhiphicephalus microplus Serpin	A0A0R6CDI0
Hyalomma excavatum Serpin	A0A131XKU1
Ixodes ricinus Serpin	Q06B75
Ixodes ricinus Serpin	V5HBT7
Amblyomma cajennense Serpin	A0A023FQ10
Amblyomma americanum Serpin	A0A0E9Y314
Ixodes ricinus Serpin	A0A147BTY8

2.2. Phylogeny and comparative sequence analyses

The Neighbor joining method in MacVector (MacVector Inc., Cary, North Carolina) was used to construct the phylogeny tree of the 11 candidate *I. scapularis* serpins (Mulenga et al., 2009a,b) and 15 other tick serpin amino acid sequences that were downloaded from GenBank and Uniprot databases (Table 1). Specifications were set to the Poisson correction of amino acid substitution model and calculation of bootstrap values was set at a 1000 replications. Human alpha-anti trypsin (Accession number: AAB59495) was used as outgroup in the phylogeny tree. Pairwise sequence alignments to determine amino acid identity levels between sequences that segregated together in the phylogeny tree was done using ClustalW in MacVector software (MacVector Inc., Cary, North Carolina).

2.3. Tick feeding, dissections, total RNA extraction, and cDNA synthesis

scapularis nymph and adult ticks used in this study were obtained from BEI resources or purchased from the tick laboratory at Oklahoma State University (Stillwater, OK). Ticks were fed on New Zealand white rabbits. Ticks were restricted to feed on top of the rabbit ear using the two-inch cotton stockinet tick containment cells that were glued onto rabbit ears using the Kamar adhesive (Kamar Products Inc., Zionsville, IN) as described (Kim et al., 2016). To feed nymphs, the inside of the stockinet was lined with pantyhose material to prevent nymphal ticks from escaping. For adult ticks, females were pre-mated prior to feeding. To mate, ticks were placed into a petridish on ice and we visually monitored for paired male and female ticks, which were subsequently placed into a separate container to complete mating.

Dissections of adult tick salivary glands (SG), midgut (MG) and carcass (tick remnant after removal of SG and MG) of unfed and ticks that were partially fed for 24, 48, 72, 96 and 120 h was done as described (Kim et al., 2015b). Both unfed and partially fed ticks were washed in diethylpyrocarbonate water prior to dissection. Total RNA extraction was done using the TRIzol total RNA extraction reagent according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Approximately 200 µL Chloroform was added per 1 mL of TRIzol and aqueous phase was separated by centrifugation at 12000 x *g*