UNDERSTANDING TICK SALIVARY SECRETIONS USING RNA INTERFERENCE (RNAI)

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December 2006

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USING RNA INTERFERENCE (RNAI)

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ACKNOWLEDGEMNTS

I thank God for giving me the perseverance, hard work and determination to pursue a Ph.D. I should thank my parents, brother, sister in law and other close relatives for their love, support and blessings without which I would not have been able to accomplish this. Over the years of my education, I have had the opportunity to learn from eminent teachers and scholars and I acknowledge each one of them for providing me the right platform to develop my research skills and subject knowledge. I thank all my friends who have been helpful and supportive during my graduate studies and for their best wishes and prayers for me to achieve success.

As a graduate student, I have had the privilege to work and learn from extraordinary mentors. First and foremost, I thank my thesis advisor, Dr. Richard C. Essenberg and co-advisor, Dr. John R. Sauer. I am in lack of words to express my gratitude to them. They have been great mentors, who have motivated and encouraged me constantly during the entire course of my Ph.D. Their support and mentorship have helped me a great deal to better understand tick biology and the process of secretion from tick salivary glands. The things that I have learned from them have motivated me to choose a career in the area of vector biology. I have also been touched by their kindness towards students, their colleagues and people in general.

I thank Dr. Robert L. Matts, Dr. Margaret K. Essenberg and Dr. Charlotte L. Ownby for agreeing to be in my committee and helping me constantly with their inputs and suggestions throughout my graduate studies. I thank Dr. Charlotte L. Ownby for helping

me a great deal with my confocal microscopy studies and enabling me to feel positive by her optimism during tough times of my research. I appreciate Dr. Margaret K. Essenberg for her enthusiastic comments and questions which have made me to better understand my research project. I thank her for making me more passionate towards my work and making me pay attention to detail by her regular conversations and questions.

I acknowledge Mr. James S. Tucker for helping me with my laboratory techniques and teaching me to dissect ticks and lot of practical tips on tick system. I thank Dr. Shahid Karim who was a post doctoral fellow in Dr. John R. Sauer's lab with whom I did majority of the regulated exocytosis work I thank him for teaching me certain laboratory techniques and helping me in my project. I also thank Dr. Majd Aljamali who was a graduate student in Dr. Richard C. Essenberg's lab for the discussions we have had which have helped me to gain a better scientific understanding. I also acknowledge him for standardizing the RNA interference protocol in ticks which was of great help for me to apply the technique to study tick salivary secretions.

I thank Dr. Margaret K. Essenberg, (Interim Head of the Department of Biochemistry and Molecular Biology 2001-2004) and Dr. Earl D. Mitchell (current Interim Head of the Department of Biochemistry and Molecular Biology) for providing me teaching and graduate research assistantships for a significant time period without which I would not have been able to complete my graduate studies.

Last, but not least, I dedicate this thesis to my grandfather Sri. K.P. Kailasm. I have been moved by his kindness and helping tendency towards people to go in addition with his good deeds. Though, he is no longer with us, I am sure his blessings have been an integral part of my growth as a student and as a person.

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
AQP	aquaporin
BT	bicarbonate transporters
ClC	chloride channel
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dsRNA	double stranded RNA
EDTA	ethylenediaminetetraacetic acid
KDa	kilo dalton
µl	micro liter
mg	milligram
miRNA	micro RNA
NSF	N-ethylmaleimide-sensitive factor protein
n-Sec1	neuronal Sec-1
RNA	ribonucleic acid
RNAi	rna interference
siRNA	small interfering RNA
SNAP	soluble N-ethylmaleimide-sensitive factor protein attachment Protein

SNARE	soluble N-ethylmaleimide-sensitive factor protein attachment protein receptor
NBC	sodium potassium co-transporter
Na K ATPase	sodium potassium ATPase
NKCC	sodium potassium chloride c-transporter
TSG	tick salivary gland
Vamp	vesicle associated membrane protein

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CHAPTER 1: INTRODUCTION

Ticks are obligate blood-sucking ectoparasites that feed on a variety of hosts including dogs, cattle, sheep, deer and humans. Ticks are second only to mosquitoes in spreading human diseases but are the first and foremost among arthropods in the variety of pathogens they spread to livestocks. Ticks belong to the class arachnids and subclass acari. Parasitiformes is an order under the subclass Acari. Parasitiformes has a suborder called Ixodida. The suborder Ixodida can be further classified into two major families of ticks, the Argasidae (soft ticks) and the Ixodidae (hard ticks). Nuttalliellidae, a third family of ticks, also exists but consists of just one species. Argasid ticks feed rapidly and do not grow to the extent of ixodid ticks. Hence, argasid ticks consume less blood and transmit no or few pathogens. Ixodid ticks on the other hand feed for prolonged periods on hosts and transmit several pathogens. Ixodidae ticks are known to vector the causative agent of several diseases, including Rocky Mountain spotted fever, Lyme disease and ehrlichiosis. Some of the prevalent diseases and the types of pathogens that cause those diseases that are transmitted through tick feeding are listed (Table 1).

Amblyomma americanum, the lone star tick is an ixodid tick that feeds for long durations on the host. A typical lone star female tick increases from about 4 mg (unfed) to about 40 mg after 6 days of feeding. After 12 days of feeding it weighs approximately 150 mg. Then it enters a fast feeding stage, engorges to repletion within 24-48 h, attains a weight of approximately 600 mg, and then falls off the host (Figure 1).

Tick salivary glands (TSGs) play a central and crucial role in the success of tick feeding on the host [1]. A tick has a pair of acinous salivary glands located in the anterior end that look like clusters of grapes (Figure 2). TSGs are the major route of secretion of several factors, which challenge the host immune, inflammatory, blood clotting and pain producing responses by counteracting host platelet aggregation, coagulation and vasoconstriction [2, 3]. This enables ticks to feed for long periods on the host with no or minimal host grooming. As ticks feed, TSGs undergo enormous growth and differentiation without actually increasing in cell number [1]. TSGs in females consist of three types of acini, namely, acini I, II and III. Acinus type I is agranular and does not undergo much growth. Acinus I does not undergo marked growth during tick feeding. Acinus I is thought to secrete a hygroscopic material that aids in the absorption of water vapor from unsaturated air during non-feeding stages of tick life cycle. Both acini types II and III, especially acinus III, on the other hand, grow substantially in size and protein content. Acini II and III are granular and are active in secretion from TSGs. Acinus III is the most abundant among the three acini present in the salivary glands. Acinus III can be differentiated from acinus II by the presence of a large lumen when compared to acinus II. In addition to acini I, II and III the males have a fourth type of acinus, namely acinus IV, which is also granular.

Secretion by the TSGs is controlled by nerves [1]. Dopamine, a neurotransmitter is a potent stimulator of fluid secretion. Dopamine binds to a D_1-type G protein coupled receptor [4, 5] and stimulates adenylate cyclase (AC), which leads to an increase in the level of cAMP. cAMP stimulates the phosphorylation of several proteins, which regulate the fluid secretion. Dopamine, in addition to activating AC, also increases the level of

Ca^{2+} within the cell by opening a voltage gated Ca^{2+} channel [6]. This increase in Ca^{2+} activates phospholipase A_2 (PLA_2) that leads to an increase in free arachidonic acid (AA) levels. AA is converted to prostaglandin E_2 (PGE_2) by the cyclo-oxygenase pathway. PGE_2 then is secreted into the saliva. PGE_2, in addition, has an autocrine or paracrine role [7]. It binds to a specific PGE_2 receptor and stimulates PLC to form inositol triphosphate (IP_3), which binds to an IP_3 receptor on the endoplasmic reticulum. This causes an efflux of Ca^{2+} into the cytosol, which then leads to vesicle mediated exocytosis or regulated exocytosis, which results in membrane fusion and subsequent protein secretion (Fig: 3).

It has now been well established in the majority of secretory tissues of various organisms that the vesicle mediated protein secretion occurs with the help of proteins called Soluble-N-ethylmaleimide sensitive factor Attachment protein Receptors (SNAREs) [8]. Recently, it was shown that SNARE proteins are being expressed in the TSGs of the female *A.americanum* ticks [9]. In addition to the protein release, ticks, as mentioned above, secrete large volumes of fluid and ions back into the host thereby concentrating the blood meal. This means there should be key ion and water transporting channel proteins present on the TSG membrane that are functionally important in maintaining the membrane potential and controlling the cell volume. Thus, it is evident that both the SNARE proteins and ion transporting enzymes must be functionally active for the ticks to salivate and feed normally on the host. Most of the proteins involved in this process are yet to be identified. Cloning the genes encoding these key proteins and analyzing the functions performed by these proteins in TSGs might be of great help in better understanding the process of tick feeding and growth. If these proteins are found to be important for tick feeding, they could potentially serve as targets for vaccine

development against ticks, thereby blocking or inhibiting the pathogenic effects of tick feeding.

RNA interference:

RNAi is a gene silencing process that occurs in a wide range of organisms including fungi, insects, worms and mammals. In fungi the term quelling often denotes this process. RNAi is similar to the Post Transcriptional Gene Silencing [PTGS] process occurring in plants [10]. It has been shown that both in RNAi and PTGS the inducer is the same molecule, double stranded RNA (dsRNA). Moreover both these processes share key components in their mechanisms of action [11, 12]. The term RNAi was coined after studies in *C. elegans* showed that injection of double stranded RNA (dsRNA) led to specific silencing of genes similar or homologous in sequence to the injected dsRNA [13]. Since then RNAi has been extensively studied in different organisms and recently this area has been undergoing an explosion in information. The advent of functional genomics has led to identification and sequencing of many, if not all of the genes present in an organism. This has led to the identification of thousands of genes in a variety of organisms which encode proteins of unknown function. The specificity and simplicity in the mode of action of RNAi has provoked scientists to utilize this technique to silence or knock down target gene expression over the cumbersome classical genetic approach. By observing phenotypic effects of this treatment, one may be able to assign functions for several of these genes encoding proteins of unknown functions.

Mechanism of RNAi:

The inducers of RNAi are dsRNAs [14]. An RNaseIII-like enzyme called DICER cleaves dsRNA. DICER cleaves dsRNA into small RNAs of 21-25 nt in length. This 21-

25nt small RNA is called small interfering RNA (siRNA) [15, 16]. siRNA, then, acts as a guide for the remaining steps of RNAi [17]. siRNA interacts or binds to unidentified proteins to form a ribonucleoprotein complex termed as RNAi silencing complex [RISC]. This then binds to the target mRNA of perfect complementarity and leads to it's degradation. Recently, functionality of RNAi in tick salivary glands was demonstrated successfully which showed that RNAi could be effectively used to study functions of proteins in TSGs [18].

Micro RNAs (miRNAs) are also short interfering RNAs that have been shown to play a role in gene silencing [19]. siRNAs are produced from the target gene itself, for example, a double-stranded virus, transposons or transgene. Micro RNAs , on the other hand, are produced from self-complementary transcript regions within an organism that are non-protein coding. Both siRNAs and miRNAs are produced by the action of Dicer, probably by two different homologs, Dicer1 and Dicer2 [20]. Once the small RNAs are produced, they become associated with proteins to form the RISC as described above. siRISC binds to a target gene of absolute gene specificity (the same sequence as the dsRNA) whereas miRISC binds to target genes with partial sequence specificity and results in translational repression of that particular gene rather than mRNA degradation as in the case of siRISC (Figure 4).

We performed RNAi, both *in vitro* and *in vivo* by either soaking TSGs in a solution containing dsRNA of the target gene (Figure 5A) or by injecting or soaking unfed adult ticks with the dsRNA of the target gene (Figure 5B). The extent of silencing was then measured by monitoring for reduction in transcript and protein level of the target protein, in addition to aberrant feeding phenotypes *in vivo*.

References:

1) Sauer, J.R., McSwain, J.L., Bowman, A.S., Essenberg, R.C., 1995. Tick salivary gland physiology. Annual Review of Entomology 40, 245-267.
2) Bowman, A.S., Coons, L.B., Needham, G.R., Sauer, J.R., 1997. Tick Saliva: Recent advances and implications for vector competence. Medical and Veterinary Entomology 11, 277-285.
3) Ribeiro, J.M.C., Mather, T.N., 1998. *Ixodes scapularis*: salivary kininase activity is a metallo-dipeptidyl carboxypeptidase. Experimental Parasitology 89, 213-221.
4) Schmidt, S.P., Essenberg, R.C., Sauer, J.R., 1981. Evidence for a D1 dopamine receptor in the salivary glands of *Amblyomma americanum* (L.). Journal of Cyclic Nucleotide Research 7, 375-384.
5) Schmidt, S.P., Essenberg, R.C., Sauer, J.R., 1982. Dopamine sensitive adenylate cyclase in the salivary glands of the lone star tick. Comparative Biochemistry and Physiology 72, 9-14.
6) Bowman, A.S., Dillwith, J.W., Madden, R.D., Sauer, J.R., 1995. Regulation of free arachidonic acid levels in isolated salivary glands from the lone star tick: a role for dopamine. Archives of Insect Biochemistry and Physiology 29, 309-327.
7) Qian, Y., Essenberg, R.C., Dillwith, J.W., Bowman, A.S., Sauer, J.R., 1997. A specific prostaglandin E_2 receptor and its role in modulating salivary secretion in the female tick Amblyomma americanum (L.). Insect Biochemistry and Molecular Biology 27, 387-395.
8) Jahn, R., Sudhof, T.C., 1999. Membrane fusion and exocytosis. Annu Rev. Biochem 68, 863-911.

9) Karim, S., Essenberg, R.C, Dillwith, J.W., Tucker, J.S., Bowman, A.S., Sauer, J.R., 2002. Identification of SNARE and cell trafficking regulatory proteins in the salivary glands of the lone star tick, *Amblyomma americanum* (L.). Insect Biochem Mol Bio 32, 1711-21

10) Hannon, G.J., 2002. RNA interference. Nature 418, 244-251.

11) Bender, J., 2001. A vicious cycle: RNA silencing and DNA methylation in plants. Cell 106, 129- 132.

12) Bernstein, E., Candy, A.A., Hammond, S.M., Hannon, G.J., 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409, 363-366.

13) Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double stranded RNA in *C. elegans*. Nature 391, 806-811.

14) Timmons, L., Fire, A., 1998. Specific interference by ingested dsRNA. Nature 395, 854.

15) Hammond, S.M., Berstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates posttranscriptional gene silencing in Drosophila cells. Nature 404, 293-296.

16) Yang, D., Lu, H., Erickson, J.W., 2000. Evidence that processed small dsRNA may mediate sequence specific mRNA degradation during RNAi in Drosophila embryos. Curr. Biol. 10, 1191-1200.

17) Schwartz, D.S., Hutragner, G., Haley, B., Zamore, P.D., 2002. Evidence that siRNAs function as guides, not primers, in the Drosophila and human pathways. Mol. Cell 10, 537-548.

18) Aljamali, M.N., Bior, A.D., Sauer, J.R., Essenberg, R.C., 2003. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*. Insect Mol Biol 12, 299-305.

19) Bartel D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

20) Lee Y.S., Nakahara K., Pham J.W., Kim K., He Z., Sontheimer E.J., Carthew R.W., 2004. Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 117, 69–81.

21) Sauer J.R., Essenberg R.C., Bowman A.S., 2000. Salivary glands in ixodid ticks: control and mechanism of secretion. J Insect Physiol. 46, 1069-1078.

Figure Legends:

Table 1: Tick- borne diseases that are prevalent in the U.S.A and the pathogens that are the causative agents of the diseases.

Figure 1: The growth pattern of a lone star *A. americanum* female tick from its unfed adult stage to repletion stage. A. Unfed stage, B. 6 days of feeding, C. 12 days of feeding, and D. 13 days of feeding (repletion).

Figure 2: Photograph of the salivary glands within a female *A. americanum* tick. Salivary glands are located in the anterior end of the tick. The position of the gut and oxygen supplying trachea are also noted.

Figure 3: Overview of the process of protein and fluid secretion in TSGs. Adopted from Sauer et al [21].

Figure 4: Synthesis of siRNA and miRNA from dsRNA and the subsequent incorporation into siRISC and miRISC, respectively. Step 1: dsRNA is recognized and bound by DICER and is broken down into siRNA or miRNA. Step 2: siRNA or miRNA is acted upon by a helicase leading to strand separation. Step 3: One of the two strands is incorporated with a protein complex leading to the formation of siRISC or miRISC. siRISC binds to the mRNA of absolute sequence similarity and leads to its degradation. miRISC binds to an mRNA with partial sequence homology and leads to its translational repression.

Figure 5: A depiction of the RNAi experiments performed *in vitro* and *in vivo*.

A: Unfed female ticks were fed on sheep. Partially fed ticks (50-200 mg) were removed from the sheep and left glands were incubated with buffer and right glands with dsRNA.

The transcript levels, protein levels, and protein functions were analyzed and compared between the buffer incubated and dsRNA incubated glands.

B: Unfed female ticks were injected with either dsRNA or buffer. The buffer injected and dsRNA injected ticks were fed on the same sheep on adjacent cells. Partial fed ticks were removed and the transcript levels, protein levels, and protein functions were analyzed and compared between the salivary glands of the buffer injected and dsRNA injected ticks. Also, feeding abnormalities of ticks injected with dsRNA were monitored.

Disease	Microbe	Type	Range (U.S.A)
Lyme disease	*Borrelia burgdorferi*	bacteria (spirochete)	Northeast, WI, MN, CA
Tularemia	*Francisella tularensis*	bacteria	AR, MO, OK
Rocky Mountain spotted fever	*Rickettsia rickettsiae*	rickettsia	Southeast, West, Northeast, South Central
Ehrlichiosis	*Erhlichia chaffiensis*	rickettsia	South Central, Northeast, Middle Atlantic
Babesiosis	*Babesia species*	protozoa	Northeast, North Central, Northwest

Table 1

A-Unfed B- 6 days C-12 days D- 13 days

Figure 1

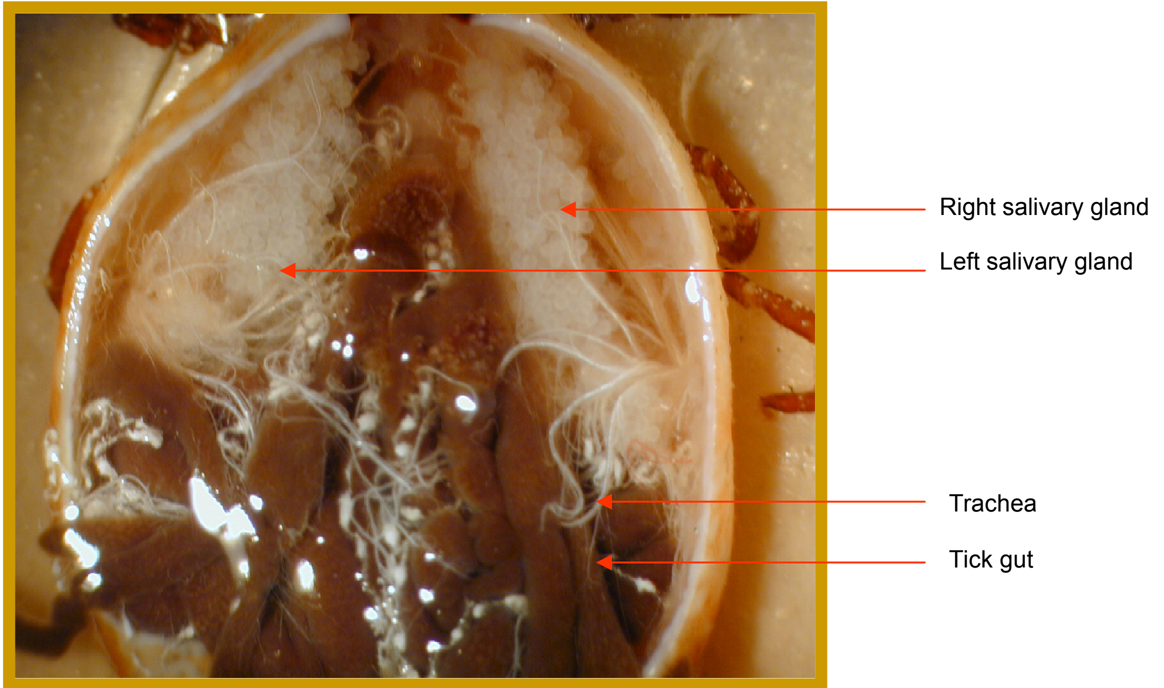


Figure 2

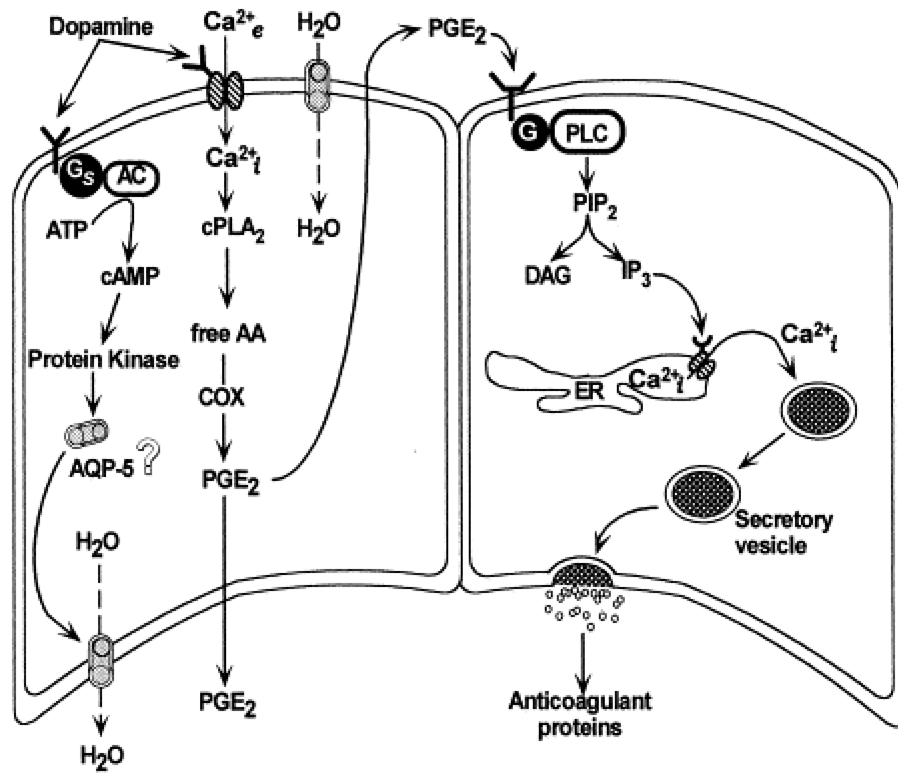


Figure 3

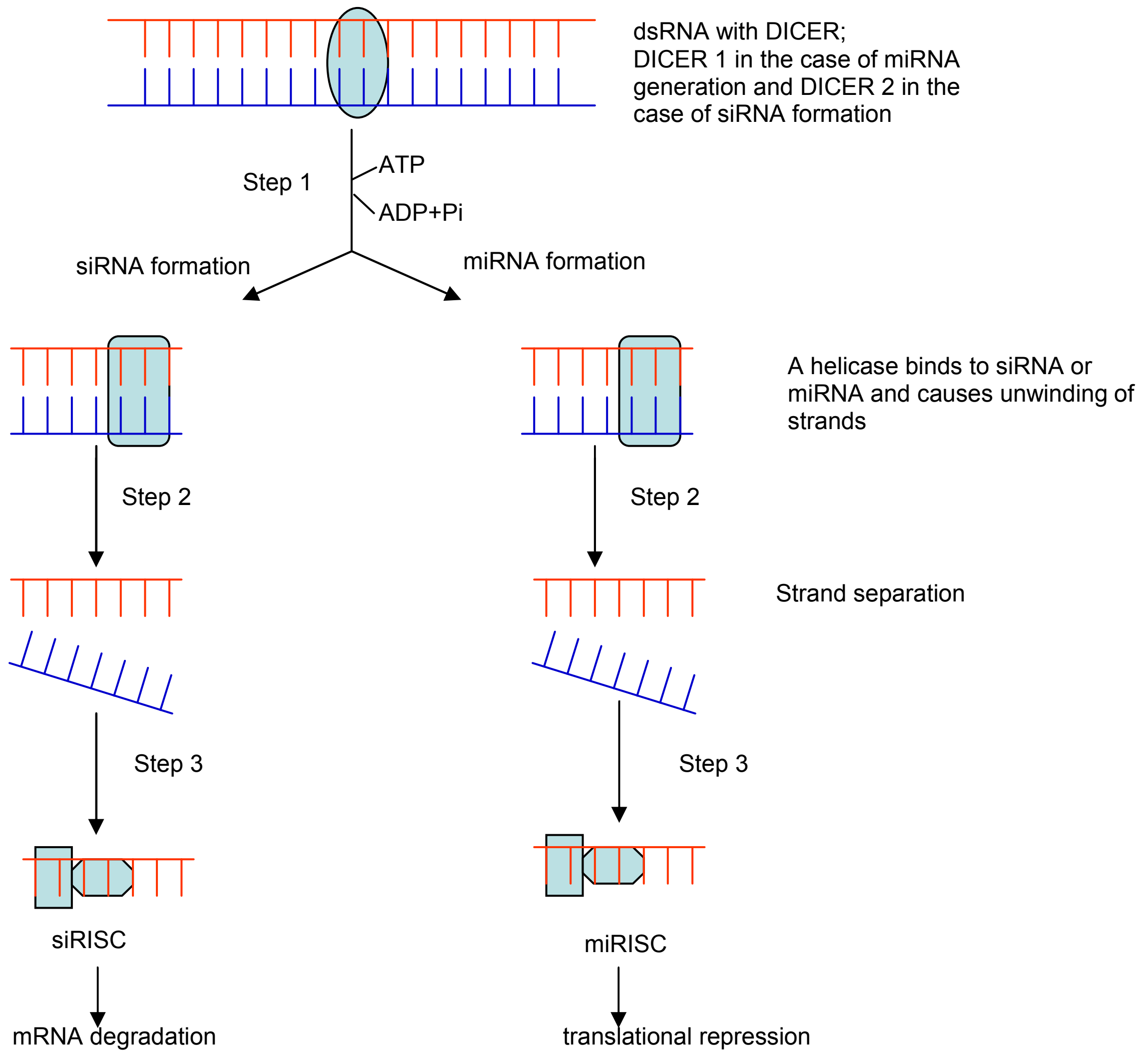


Figure 4

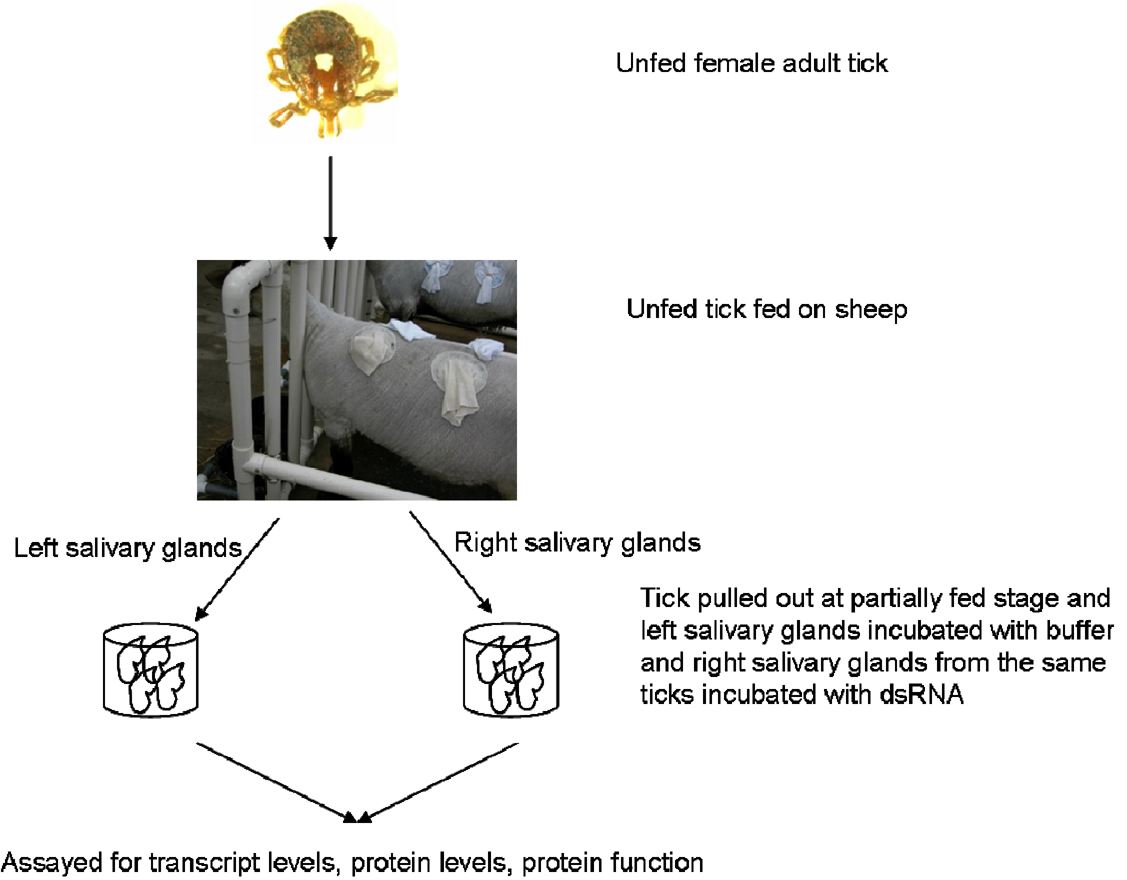
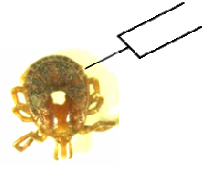


Figure 5A



Injected dsRNA or buffer into unfed female ticks

Fed the dsRNA injected ticks and buffer injected ticks in adjacent cells on a sheep

Pulled out the partially fed ticks and assayed for transcript levels, protein levels, protein function and phenotypic abnormalities by comparing with similar studies performed with buffer injected ticks

Figure 5B

Objectives:

1. Better understand the process of regulated exocytosis in the female lone star tick salivary glands using RNA interference (RNAi).
2. Dissect of the process of fluid secretion from the tick salivary glands thereby identifying key proteins in this process.

CHAPTER 2: OBJECTIVE 1

SNARE Proteins:

SNARE proteins represent a group of membrane proteins that are thought to play crucial roles in the process of membrane fusion and exocytosis [1, 2]. Depending on the membrane they are associated with, SNAREs are classified into target SNAREs (t-SNARE) and vesicle SNAREs (v-SNARE). Key known t-SNAREs are syntaxin and Synaptosome Associated Protein (SNAP25) in the neuronal cells and sec9p and ssop in yeast cells. The key known v-SNARE is synaptobrevin (vamp2) in neuronal cells and Snc2p in yeast cells. SNARE proteins have characteristic heptad repeats of about 60 amino acids, which are predicted to form coiled coils.

SNARE proteins, while regulating the process of membrane fusion and exocytosis, are thought to interact with many other SNARE and non-SNARE proteins. There was early debate among scientists whether SNARE proteins represent the minimal fusion machinery. In the recent past there has been an increase in evidence supporting the hypothesis that SNAREs are key but not sole components in the process of membrane fusion. The process appears to require the function of other proteins that interact with SNAREs [3, 4, 5, 6, 7].

Sequence analysis of SNARE proteins from different species has shown significant sequence variability among the members of the group from various species at the amino acid level. However, SNAREs identified to date all have a central ionic region characterized by one or more glutamine(s) in both syntaxin and SNAP25 and one or more

arginine(s) in synaptobrevin [8]. At some stage during vesicle exocytosis, SNAREs assemble into a tight complex called the SNARE complex. These SNARE complexes are SDS-resistant but heat sensitive. The ionic groups present in all SNAREs are thought to play a role in protein-protein interactions [9, 10]. In addition to SNARE-SNARE interaction there are several other proteins that interact with SNAREs [11]. A particular group of protein called the Sec1/munc18-like (SM) proteins, such as n-Sec1, binds to SNAREs, particularly syntaxins, to regulate their action [12, 13]. For example n-Sec1 binds to syntaxin and promotes the process of exocytosis. In addition to SM proteins, SNAREs have been found to interact with epithelial sodium channels and Ca^{2+} channels [14, 15]. It is now known that fusion of SNARE proteins and formation of one SNARE complex alone does not provide sufficient energy for the fusion reaction to take place. Rather, several SNARE complexes are formed which interact with Ca^{2+} channels, in forming pores and cause an increase in Ca^{2+} levels within the cell. Thus, it is becoming clear that though SNAREs play a prominent role, they require the assistance of several other proteins in the process of membrane fusion. The process of vesicle mediated exocytosis regulated by the functions of SNARE and SNARE associated proteins are summarized below (Figure 1) [16].

Of the key SNARE and SNARE associated proteins, we were able to clone two partial cDNA fragments from TSGs with one showing identity to the key v-SNARE synaptobrevin and the other to the SM protein n-Sec1 at the amino acid level. Once cloned, the next step in the project was to analyze the functions performed by both these proteins within the salivary glands. We took advantage of the simplicity, reliability and

quickness of RNA interference (RNAi) and exploited this technique to identify the functions of these proteins in TSGs.

References:

1) Edamatsu, M., 2001. The molecular mechanism of targeted vesicle transport in cytokinesis. Cell Struct. Funct. 26, 567-570

2) Gerber, S.H., Sudhof, T.C., 2002. Molecular determinants of regulated exocytosis. Diabetes 51, Suppl. 1, S3-S11

3) Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., Rothman, J.E., 1998. SNAREpins: minimal machinery for membrane fusion. Cell 92, 759-772.

4) McNew, J.A., Parlati, F., Fukuda, R., Johnston, R.J., Paz, K., Paumet, F., Sollner, T.H., Rothman, J.E., 2000. Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature 407, 153-159.

5) Ungermann, C., Sato, K., Wickner, W., 1998. Defining the functions of trans-SNARE pairs. Nature 396, 543-548.

6) Schoch, S., Deak, F., Konigstorfer, A., Mozhayeva, M., Sara, Y., Sudhof, T.C., Kavalali, E.T., 2001. SNARE function analyzed in synaptobrevin/vamp knockout mice. Science 294, 1117-1122.

7) Stevens, C.F., Williams, J.H., 2000."Kiss and run" exocytosis at hippocampal synapses. Proc. Natl. Acad. Sci. U.S.A. 97, 12828-12833.

8) Fasshauer, D., Sutton, R.B., Brunger, A.T., Jahn, R., 1998. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNARE's. Proc. Natl. Acad. Sci. U.S.A. 95, 15781-15786.

9) Ernst, J.A., Brunger, A.T., 2003. High-resolution structure, stability and synaptotagmin binding of a truncated neuronal SNARE complex. J. Biol. Chem. 278, 8630-8636.

10) Sutton, R.B., Fasshauer, D., Jahn, R., Brunger, A.T., 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 a resolution. Nature 395, 347-353.

11) Antonin, W., Holroyd, C., Fasshauer, D., Pabst, S., Von Mollard, G.F., Jahn, R., 2000. A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. EMBO J. 19, 6453-6464.

12) Barchlay, J.W., Craig, T.J., Fisher, R.J., Ciufo, L.F., Evans, G.J., Morgan, A., Burgoyne, R.D., 2003. Phosphorylation of munc18 by protein kinase C regulates the kinetics of exocytosis. J. Biol. Chem. 278, 10538-10545.

13) Ossig, R., Dascher, C., Trepte, H.H., Schmitt, H.D., Gallwitz, D., 1991. The yeast SLY gene products, suppressors of defects in the essential GTP-binding Ypt1 protein, may act in endoplasmic reticulum-to-Golgi transport. Mol. Cell Biol. 11, 2980-2993.

14) Bezprozvanny, I., Scheller, R.H., Tsien, R.W., 1995. Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature 378, 623-626.

15) Condliffe, S.B., Carratino, M.D., Frizzell, R.A., Zhang, H., 2003. Syntaxin 1A regulates ENaC function via domain-specific interactions. J. Biol. Chem. 278, 12796-12804.

16) Chen, Y.A., Scheller, R.H., 2001. SNARE- mediated membrane fusion. Nat. Rev. Mol. Cell Biol. 2, 98-106.

Figure legends:

Figure 1: SNARE–mediated exocytosis: a model [16]. The SM protein n-Sec1 is bound to the t-SNARE syntaxin. At appropriate conditions, n-Sec1 dissociates from syntaxin and this requires the GTP binding protein Rab. Once syntaxin is free, syntaxin, SNAP 25 and synaptobrevin/vamp bind to form a core complex. This complex tethers the vesicle to the plasma membrane and leads to subsequent fusion and release of vesicular contents. Post release, cytoplasmic proteins α-SNAP and NSF bind to the complex. NSF hydrolyzes ATP and this causes the release of independent SNARE proteins for subsequent cycles of membrane fusion.

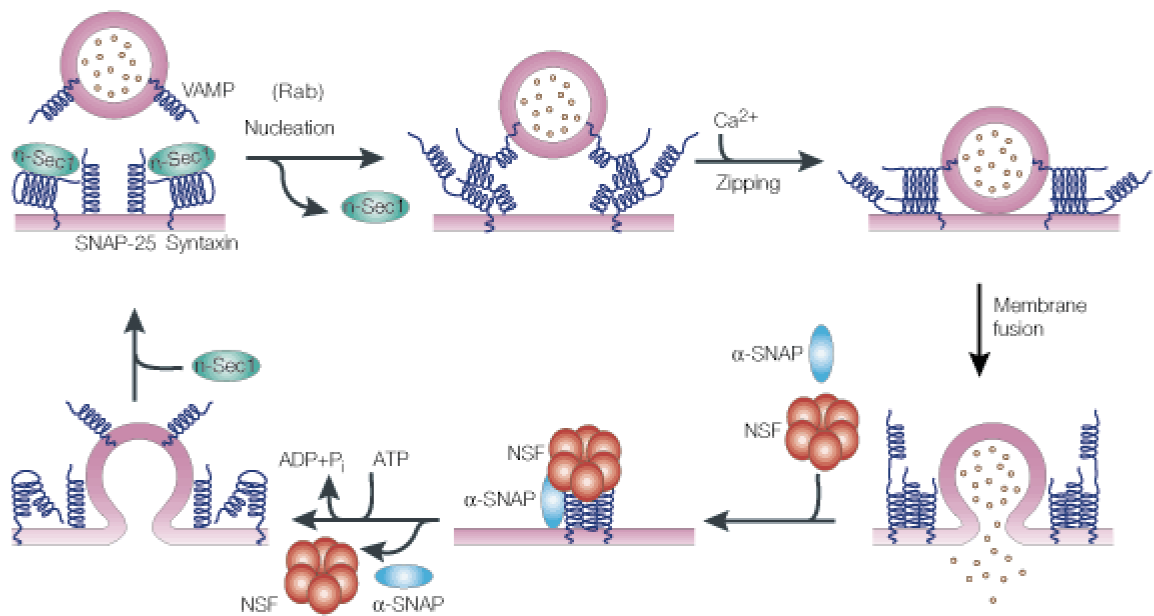


Figure 1

***Amblyomma americanum* salivary glands: Double-stranded RNA-mediated gene silencing of synaptobrevin homologue and inhibition of PGE_2 stimulated protein secretion (Published in the Journal of Insect Biochemistry and Molecular Biology)**

Abstract

Protein secretion into the saliva from the tick salivary glands is due to exocytosis of vesicular membrane bound granular material regulated by SNARE complex proteins after salivary gland stimulation by PGE_2 (Karim et al., 2002). Proteins associated with vesicles (v-SNAREs) are essential components of the exocytotic process. Synaptobrevin is a key v-SNARE in all secreting cells studied to date. A vesicle associated synaptobrevin cDNA fragment homologue from the salivary glands of partially fed lone star tick females was cloned and sequenced. Double-stranded (ds) RNA interference (RNAi) is an effective method to silence specific gene expression. The functional role of synaptobrevin in protein secretion in partially fed tick salivary glands was studied with an *in vitro* RNAi method. Incubation of isolated salivary glands with double-stranded RNA (dsRNA) transcribed from a tick salivary gland synaptobrevin cDNA fragment resulted in decreased expression at the transcript, a reduction in the level of synaptobrevin protein and inhibition of PGE_2 stimulated anticoagulant protein secretion by isolated salivary glands. We demonstrate the applicability of RNAi for studying individual steps in the mechanism of PGE_2 stimulated exocytosis in the salivary glands of ixodid ticks.

Introduction

Salivary glands are crucial to the ability of ixodid ticks to feed successfully. As feeding progresses the rate of salivary fluid secretion increases greatly, enabling the tick to concentrate the blood meal by returning excess water and ions to the host. In addition, bioactive proteins (e.g. anticoagulants, anti-inflammatory proteins and immunosuppressants) and prostaglandins are secreted as components of saliva to modulate interactions of the tick with the host (Sauer et al., 1995; Bowman et al., 1997). A local hormone role for prostaglandins is suggested by the presence of a specific PGE_2 receptor in the salivary glands of female lone star ticks (Qian et al., 1997). This PGE_2 receptor is linked with mobilization of Ca^{2+} via a phosphoinositide signaling pathway and is associated with stimulation of protein secretion (Qian et al., 1998, Yuan et al., 2000). Highly conserved intracellular SNAREs [Soluble N-ethylmaleimide-sensitive factor protein (NSF) attachment protein receptors] are associated with the mechanism of protein secretion in all secretary cells studied to date (Rizo and Sudhof, 2002). Members of this class of proteins are distributed in distinct sub cellular compartments. Intracellular SNAREs can be divided into two categories, the v-SNAREs located on carrier vesicles and the t-SNAREs present on target compartments. Assembly of cognate v- and t-SNARE proteins promotes the formation of extremely stable core complexes, and serve as functional receptors for cytosolic factors necessary for vesicle transport and vesicle fusion, NSF and soluble NSF attachment proteins (SNAPs) (Söllner et al., 1993). Besides SNAPs and NSF, various other molecules such as monomeric G-proteins are known to regulate SNARE protein interactions and activity (McMahon et al., 1995; Fujita et al., 1998; Beites et al., 1999; Lao et al., 2000). Significantly, SNARE complex

proteins have been identified in the salivary glands of the lone star tick. Antibodies for SNARE complex proteins inhibit PGE_2-stimulated secretion of anti-coagulant protein in permeabilized tick salivary glands (Karim et al., 2002).

RNA interference (RNAi), through the injection of a sequence specific double stranded RNA (dsRNA) into an organism, or incubation of tissues with the dsRNA offers a method of specifically inactivating expression (gene silencing) of the corresponding gene and thereby allows for an opportunity to investigate the corresponding proteins function (Carthew, 2001). RNAi was first observed in *Caenorhabditis elegans* by Fire et al., (1998) who demonstrated that the introduction of dsRNA into the cells inactivated the expression of the corresponding gene. Subsequently, it was found that gene silencing is physiological and occurs in many organisms from fungi to animals and that the process can be exploited to selectively "knock down" specific gene function (Sharp, 1999; Misquitta & Paterson, 1999; Catalanotto et al., 2000; Hammond et al., 2000). Our mechanistic understanding of RNAi derived mainly from biochemical work in cell extracts from different organisms (Hannon, 2002). The dsRNA-induced degradation of homologous RNAs can be divided simplistically into initiation and effector steps (Bernstein et al., 2001; Denli et al., 2003; Hannon, 2002; Cerutti, 2003). In the initiation step, a long dsRNA is processed into small interfering RNAs (siRNA) of about 21-23 nt (Zamore et al., 2000). This cleavage requires ATP and it is mediated by an RNase-III like dsRNA-specific ribonuclease, named Dicer in Drosophila (Zamore et al., 2000; Bernstein et al., 2001). In the effector step, the complex, known as the RNA-induced silencing complex (RISC) (Hammond et al., 2000) is proposed to undergo an ATP-dependent activation step that results in the unwinding of the double-stranded siRNA

(Nykanen et al., 2001). Activated RISC uses a single stranded siRNA as a guide to identify complementary RNAs (Nykanen et al., 2001; Martinez et al., 2002), and an endoribonuclease that has yet to be identified cleaves the target RNA across from the center of the guide siRNA (Hammond et al., 2000; Martinez et al., 2002; Elbashir et al., 2001). Finally, the cleaved RNA is thought to be degraded by exonucleases (Hammond et al., 2000). In this study, an exocytotic v-SNARE cDNA fragment of synaptobrevin was cloned and sequenced from partially fed ixodid tick salivary glands. We examined whether the corresponding dsRNA reduced expression of synaptobrevin mRNA and the synaptobrevin transcript in isolated salivary glands. We then investigated whether this treatment affected the ability of PGE_2 to stimulate anticoagulant protein secretion in isolated salivary glands.

Materials and methods

Materials

Restriction enzymes, Taq DNA polymerase, kits for plasmid DNA and polymerase chain reaction (PCR) products were purchased from Invitrogen (Carlsbad, CA, USA) and Qiagen (Valencia, CA, USA). Antibodies to rat synaptobrevin (rabbit: polyclonal), Rabbit IgG-HRP and rat brain tissue were purchased from StressGen (Victoria, BC, Canada). Mineral oil was obtained from Sigma (St. Louis, MO, USA).

2.2 Tick Rearing

Amblyomma americanum (L.) ticks were reared at Oklahoma State University's tick rearing facility, according to the methods of Patrick and Hair (1975). Immature ticks were fed on rabbits and adult ticks on sheep. All unfed ticks were maintained at 27-28°C and 90% relative humidity under 14h light/10h dark photoperiod before infestation of the hosts. Partially fed females were dissected within 4 h of being removed from the host.

2.3 Salivary glands

Tick tissues were dissected in ice-cold Medium-199 (Sigma) with Hanks' salts and 20 mM MOPS buffer (pH 7.0), 0.10 g/l streptomycin sulfate and 0.03 g/l penicillin. After removal, glands were washed gently in the same ice-cold buffer. The dissected tissues were immediately stored in RNAlater (Ambion, Austin, TX, USA) after dissection 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, 1X 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 synaptobrevin cDNA and RT-PCR

Using a tick specific EST575569 sequence (Accession No. **BM292709**) for synaptobrevin (Nene et al., 2002), gene specific primers were designed to amplify a cDNA fragment from salivary glands of the lone star tick. Total RNA was isolated from the salivary glands using an RNAqueous^{TM} total RNA isolation kit (Ambion). Concentration of total RNA was determined spectrophotometrically at 260 and 280 nm, aliquoted and stored at -80°C before use. Total RNA was reverse transcribed using M-MLV (Moloney Murine Leikemia virus) reverse transcriptase according to the Invitrogen protocol. For each set, cDNA was PCR amplified using gene specific primers (GSP) for both synaptobrevin (forward 5'-CCAACCCAACAGACAAGAGAA-3 and reverse 5'-GGATTAGCATCTCTCCACAC-3') and human β-actin primers (Stratagene, La Jolla, CA, USA) as the control by using a PCR program of 94°C for 4 mins, 35 cycles of 94°C for 30 secs, 60°C for 30 secs and 72°C for 30 secs.

2.5 Cloning and sequencing

After PCR amplification, gel-purified fragments were cloned into the pCR-II vector using a T/A cloning Kit (Invitrogen). Recombinant plasmids were then transformed into TOP 10F' One Shot Competent Cells and cultured on agar plates containing 50 μg/ml ampicillin, 0.2mM IPTG and 40 μg/ml X-gal for selection and amplification. White colonies (10) were picked from these plates and then grown in Luria Broth (LB) containing 50 μg/ml ampicillin. Plasmid DNA was purified with a Qiaprep spin Miniprep plasmid kit (Qiagen). Cloned recombinant plasmid DNA (250 ng) was tested for the presence of an insert by comparing its migration to the vector plasmid alone on 2% agarose gel electrophoresis; positive clones were then digested with

EcoRI and the product analyzed again by agarose gel electrophoresis to verify the insert. Recombinant plasmids were sequenced at the OSU Protein/Nucleic Acid Resource Facility from the T7 promoter using an ABI 373A automated DNA sequencer and Prism® Ready Reaction Dyedeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, CT, USA). The resulting sequences were used to search the DNA and protein databases.

dsRNA in vitro synthesis and incubation with tick salivary glands

The synaptobrevin cDNA-containing plasmids with inserts in opposite orientation were linearized by digesting with HindIII enzyme and precipitating with ethanol. Precipitated plasmid DNA was used as template for *in vitro* transcription using a MEGAscript kit (Ambion) following the manufacturer's instructions. The resulting dsRNA was analyzed by agarose gel electrophoresis to verify their size and if it was double stranded. Plasmids containing HBP (Histamine binding protein) inserts were digested with KpnI and XhoI. The digested plasmids were precipitated with ethanol and used as templates for *in vitro* transcription. The two single stranded RNA's were mixed together and incubated at 65°C for 5 minutes and later left to anneal at room temperature for 4 hours. All *in vitro* experiments were confirmed for specificity by using β-Actin and β subunit of NaK ATPase expression as negative controls for non-specific interference. In all experiments, right and left salivary glands from 20 partially fed female ticks were pre-incubated separately for 6 hrs at 37°C with 25 μg of dsRNA in TS/MOPS or TS/MOPS alone prior to analysis of synaptobrevin mRNA by RT-PCR or protein by Western blot. Integrated fluorescent intensity of bands was compared with a gel documentation system from Bio-Rad (Hercules, CA, USA).

Western blotting

Female tick salivary glands (20) were homogenized in ice-cold extraction buffer [(PBS, 1 mM-dithiothreitol, 2.5 mM EGTA, 1X Complete^{TM} Mini protease inhibitor cocktail) (Roche)]. Homogenized salivary glands were sonicated for 1 minute. Protein concentration in all samples was estimated by the Bradford method (1976). The protein standards used for SDS-PAGE were low molecular weight range from Bio-Rad. Tick salivary gland protein extracts (50 µg each) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and then transferred onto nitrocellulose membranes in a Transblot cell (Bio-Rad) following the manufacturer's instructions. The transfer buffer consisted of 25 mM Tris HCl, 192 mM glycine in 20% methanol. Nonspecific protein binding sites were blocked with 5% skim milk and the membranes were incubated with the polyclonal synaptobrevin antibody (StressGen) at dilutions of 1:2000. The antigen-antibody complexes were visualized with horseradish peroxidase-conjugated anti-rabbit IgG (StressGen) at a dilution of 1:10000 and detected with SuperSignal^{R} chemiluminescent substrate (Pierce, Rockford, IL, USA) on x-ray film.

Exocytosis of anticoagulant protein

The right and left salivary glands from 8 partially fed female ticks were divided into two tissue sets where one gland from each tick was used as the control and the other gland for the treatment set. Dissected salivary glands were pre-incubated with synaptobrevin dsRNA or dsRNA for the histamine binding protein for six hours at 37°C, other control glands were incubated in dissecting buffer alone. After incubation with dsRNA or buffer alone, glands were centrifuged for 1 min at 1000xg and then stimulated

with PGE_2 (0.1μM) for 5 minutes at room temperature. Glands were centrifuged at 1000xg for 1 minute and the supernatant used for determination of anticoagulant activity by the activated partial thromboplastin assay (APTT, intrinsic pathway) as described by Zhu et al., (1997). Time to initiation of coagulation (V_{max}) was determined by Softmax[R] PRO (Molecular Devices, Sunnyvale, CA, USA) software using a THERMO MAX plate reader (Molecular Devices). Each experiment was repeated three times.

Results

We previously demonstrated that vesicle-associated plasma membrane protein (VAMP or synaptobrevin) is present in salivary glands of the lone star tick (Karim et al., 2002). We amplified a cDNA for it using primers based upon the sequence of an EST from *Amblyomma variegatum* (Nene et al., 2002). The amplified 350 bp fragment (Figure 1A) was cloned in a TOPO TA cloning vector and sequenced. We also amplified 750 bp fragment of β-Actin (Figure 1B) and used it as control in all of our experiments. A search of the databases using BlastX (www.ncbi.nih.gov) demonstrated the sequence had 65% identity to human synaptobrevin at the amino acid level (Figure 1C).

The hallmarks of RNAi are its specificity, simplicity and the ease by which the method can be applied to an organism *in vitro* or *in vivo* (Fjose et al., 2001). Salivary glands incubated for 6 hrs with dsRNA, produced from the putative synaptobrevin gene clone from salivary glands of female lone star tick, demonstrated ~50% decrease in the synaptobrevin transcript (Figure 2A). The level of β-actin transcript as a negative control remained the same in the experimental and control groups (Figure 2B). Interestingly, when salivary glands were incubated with 10 μg of synaptobrevin dsRNA for 12 hrs, it reduced the synaptobrevin transcript level more than 90% at 27 PCR cycles (Fig. 3) but less at 35 cycles (data not shown). The level of β-subunit of NaK ATPase as well as β-Actin transcripts remained the same in experimental and control groups (Fig. 3). Further, salivary glands demonstrated less synaptobrevin protein as determined by Western blot analysis in dsRNA pre-incubated glands in a dsRNA dose-dependent manner (Fig. 4A & B). A dsRNA amount of 25 μg was able to maximally inhibit detectable synthesis of synaptobrevin protein.

We have previously shown that saliva of female lone star ticks contains anticoagulant protein (Zhu et al., 1997). Regulated exocytosis of anticoagulant protein in salivary glands is stimulated by PGE_2 (Yuan et al., 2000). To determine whether the RNAi of v-SNARE synaptobrevin expression affects anticoagulant secretion, glands were incubated with synaptobrevin dsRNA for 6 hrs at 37°C. Glands were washed with buffer and then stimulated with 0.1 μM PGE_2. PGE_2-stimulated secretion of anticoagulant protein in salivary glands was inhibited 47% after glands had been pre-incubated with synaptobrevin dsRNA as compared to controls (Figure 5). As a negative control and to confirm specificity of the dsRNA for synaptobrevin on anticoagulant protein secretion, glands were incubated with dsRNA for another secreted protein (HBP) for 6 hrs at 37°C (Aljamali et al.,2003). Glands were washed and stimulated with PGE_2 as described above. In this case, there was no difference in anticoagulant protein secretion between HBP dsRNA incubated glands and the glands incubated without dsRNA (data not shown).

Discussion

The above results demonstrate that incubation with dsRNA is an effective method for reducing gene expression in tick salivary glands. This method is specific, affecting only the gene targeted for disruption, and can be used to target genes in secretary tissue such as salivary glands. There is great interest in exploiting RNAi via the use of double-stranded RNA (dsRNA) as a means of assessing specific function(s) of individual genes (Hannon, 2002). Synaptobrevin is a v-SNARE protein thought to play a key role in the process of exocytotic protein secretion in secretary cells (Baumert et al., 1989). Synaptobrevin is present in the salivary glands of partially fed female lone star ticks and shown to be important in the process of exocytotic protein secretion in the salivary glands (Karim et al., 2002). In the present investigation the addition of dsRNA of the corresponding cDNA fragment of synaptobrevin to the tick salivary glands resulted in inhibition of PGE_2 stimulated protein secretion and considerable decrease in the synaptobrevin transcript and encoded synaptobrevin protein. Incubation of salivary glands with dsRNA for a secreted protein, HBP, had no affect on the secretary process. Although substantial inhibitory effects were observed with a high concentration (25 μg) of dsRNA to synaptobrevin, 100% reduction in all three parameters could not be achieved. The lack of complete inhibition of the secretary process could be due to several factors including absence of all features of the RNAi pathway found in other eukaryotic cells or the presence of other isoforms of synaptobrevin in the salivary glands. The mechanism of RNAi is still poorly understood but evidence indicates that cleavage of the target RNA occurs only in the region corresponding to the input dsRNA (Zamore et al., 2000). It is assumed that once cleaved, the transcript is susceptible to degradation

by nonspecific RNases. Little is known about the optimal sequence and conformation of the target mRNA that renders it susceptible to interference by a dsRNA molecule. We chose to use a dsRNA of 350 nucleotides. Since there is evidence that increasing the length of dsRNA increases the potency in mediating RNAi in *Drosophila* (Tuschl et al., 1999; Yang et al., 2000), it may be that a longer transcript of dsRNA would enhance the inhibition of protein secretion by tick salivary glands. This study supports other evidence that RNA interference can be triggered in tick salivary glands by dsRNA (Aljamali et al., 2003) and adds support to the hypothesis that a synaptobrevin homologue plays a key role in protein secretion in ixodid ticks. The results reported here also establish RNAi by incubation as method for inhibiting gene function in tick salivary glands. The ease and specificity by which synaptobrevin protein can be effectively "knocked down" in tick salivary glands makes this an ideal system for studying the mechanism of protein secretion in tick salivary glands.

Acknowledgments

The Director of the Oklahoma Agricultural Experiment Station approved this article for publication. We thank Drs. Haobo Jiang and Udaya DeSilva for reviewing the manuscript. This work was supported by National Science Foundation grant IBN 9974299.

References

Aljamali, M. N., Bior, A. A., Sauer, J. R. and Essenberg, R. C. 2003. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*. Insect Mol. Biol. 12(3), 299-305.

Baumert, M., Maycox, P. R., Navone, F., DeCamilli, P., Jahn, R. 1989. Synaptobrevin: an integral membrane protein of 18,000 Daltons present in small synaptic vesicles of rat brain. EMBO J. 8, 379-384.

Beites, C. L., Xie, H., Bowser, R. and Trimble, W. S. 1999. The septin CDCre1-1 binds syntaxin and inhibits exocytosis. Nature Neuroscience 2, 434-439.

Bernstein, E., Denli, A. M. and Hannon, G. J. 2001. The rest is silence. RNA 7(11), 1509-1521.

Bowman, A. S., Coons, L. B., Needham, G. R., Sauer, J. R. 1997. Tick saliva: recent advances and implications for vector competence. Med. Vet. Entomol. 11, 277-285.

Bradford, M. 1976. A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-256.

Carthew, R. W. 2001. Gene silencing by double-stranded RNA. Curr. Opin. Cell Biol. 13, 244-248.

Catalanotto, C., Azzalin, G., Macino, G. and Cogoni, C. 2000. Gene silencing in worms and fungi. Nature 404, 245.

Cerutti, H. 2003. RNA interference: traveling in the cell and gaining functions? Trends in Genetics 19(1), 39-46.

Denli, A. M. and Hannon, G. J. 2003. RNAi: an ever-growing puzzle. Trends in Biochemical Sciences 28(4), 196-201.

Elbashir, S. M., Lendeckel, W. and Tuschl, T. 2001. RNA interference is mediated by 21- and 22 nucleotide RNAs. Genes Dev. 15(2), 188-200.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811.

Fjose, A., Ellingsen, S., Wargelius, A. and Seo, H. C. 2001. RNA interference. Mechanisms and applications. Ann. Rev. Biotechnol. 7, 31-57.

Fujita, Y., Shirataki, H., Sakisaka, T., Asakura, T., Ohyo, T., Kotani, H., Yokoyama, S., Nishioka, H., Matsuura, Y., Mizoguchi, A., Scheller, R. H. and Takai, Y. 1998. Tomosyn: a syntaxin-1-binding protein that forms a novel complex in the neurotransmitter release process. Neuron 20, 905-915.

Hammond, S. M., Bernstein, E., Beach, D. and Hannon, G. J. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404, 293-296.

Hannon, G. J. 2002. RNA interference. Nature 418, 244-251.

Karim, S., Essenberg, R. C., Dillwith, J. W., Tucker, J. S., Bowman, A. S. and Sauer, J. R. 2002. Identification of SNARE and cell trafficking regulatory proteins in the salivary glands of the lone star tick, *Amblyomma americanum* (L.). Insect Biochem. Mol. Biol. 32, 1711-1721.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lao, G., Scheuss, V., Gerwin, C. M., Su, Q., Mochida, S., Retting, J., and Sheng, Z. H. 2000. Syntaphilin: a syntaxin-1 clamp that controls SNARE assembly. Neuron 25, 191-201.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., Tuschl, T. 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110(5): 563-574.

McMahon, H. T., Missler, M., Li, C., and Sudhof, T. C. 1995. Complexins: cystolic proteins that regulate SNAP receptor function. Cell 83, 111-119.

Misquitta, L. and Paterson, B. M. 1999. Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. Proc. Nat. Acad. Sci. USA 96, 1451-1456.

Nene, V., Lee, D., Quackenbush, J., Skilton, R., Mwaura, s., Gardner, M. J. and Bishop, R. 2002. AvGI, an index of genes transcribed in the salivary glands of the ixodid tick *Amblyomma variegatum*. Int. J. Parasitol. 32, 1447-1456.

Nykanen, A., Haley, B. and Zamore, P. D. 2001. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107(3), 309-321.

Patrick, C. D. and Hair, J. A. 1975. Laboratory rearing procedures and equipment for multi-host ticks. (Acarina: Ixodidae). J. Med. Entomol. 12, 389-390.

Qian, Y., Yuan, J., Essenberg, R. C., Bowman, A. S., Shook, A. L., Dillwith, J. W. and Sauer, J. R. 1998. Prostaglandin E_2 in the salivary glands of the female tick, *Amblyomma americanum* (L.): calcium mobilization and exocytosis. Insect Biochem. Mol. Biol. 28, 221-228.

Qian, Y., Essenberg, R. C., Dillwith, J. W., Bowman, A. S., Sauer, J. R. 1997. A specific prostaglandin E_2 receptor and its role in modulating salivary secretion in the female tick, *Amblyomma americanum* (L.). Insect Biochem. Mol. Biol. 27, 387-395.

Rizo, J. and Sudhof, T. C. 2002. SNAREs and Munc18 in synaptic vesicle fusion. Nat. Rev. Neurosci. 3, 641-653.

Sauer, J. R., McSwain, J. L., Bowman, A. S., Essenberg, R. C. 1995. Tick salivary gland physiology. Ann. Rev. Entomol. 40, 245-267.

Sharp, P. A. 1999. RNAi and double-stranded RNA. Genes Dev. 13, 139-141.

Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. 1993. SNAP receptors implicated in vesicle targeting and fusion. Nature 362, 318-324.

Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D. P. and Sharp, P. A. 1999. Targeted mRNA degradation by double-stranded RNA *in vitro*. Genes Dev. 13, 3191-3197.

Yang, D., Lu, H. and Erickson, J. W. 2000. Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. Curr. Biol. 10, 1191-1200.

Yuan, J., Bowman, A. S. Aljamali, M., Payne, M. R., Tucker, J. S., Dillwith, J. W., Essenberg, R. C., Sauer, J. R. 2000. Prostaglandin E_2-stimulated secretion of protein in the salivary glands of the lone star tick via a phosphoinositide signaling pathway. Insect Biochem. Mol. Biol. 30, 1099-1106.

Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101, 25-33.

Zhu, K., Sauer, J. R., Bowman, A. S., Dillwith, J. W. 1997. Identification and characterization of anticoagulant activities in the saliva of the lone star tick, *Amblyomma americanum* (L.). J. Parasitol. 83, 38-43.

Figure legends

Figure 1: RT-PCR of cDNA from partially fed tick salivary glands using synaptobrevin and β-actin gene specific primers. (A) 1. Low DNA Mass^{TM} ladder 2. tick salivary gland synaptobrevin, (B) 1. Low DNA Mass^{TM} ladder, 2. tick salivary gland β-actin. (C): Amino acid blast alignment between the cloned female *Amblyomma americanum* (A.a.) tick salivary gland synaptobrevin and corresponding part of *Homo sapiens* (H.s.)

(Score = 108 bits (270). E value = 2 x 10^{23}

Identities = 46/70 (65%), Positives = 58/70 (82%))

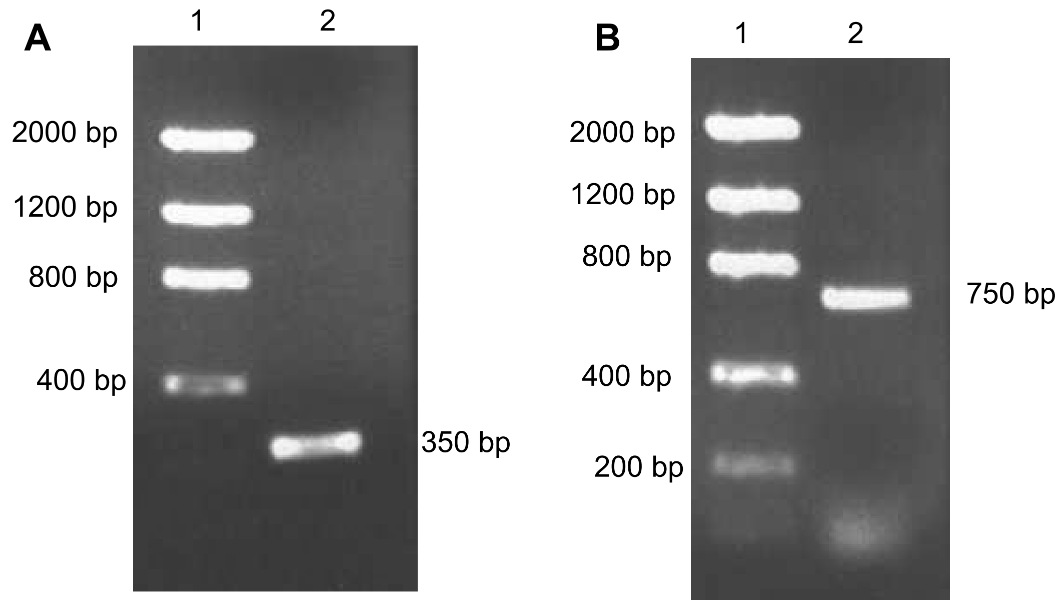
Figure 2: *In vitro* RNAi in the salivary glands. RT-PCR of total RNA prepared from salivary glands using synaptobrevin (A), Lane 1, Low DNA Mass^{TM} Ladder, 2. Control salivary glands, synaptobrevin transcript, 3. dsRNA treated glands, synaptobrevin transcript and β-actin GSPs. (B) Lane 1, Low DNA Mass^{TM} Ladder, 2. Control salivary glands, β-actin transcript, 3. dsRNA treated salivary glands, β-actin transcript, C) Integrated fluorescence intensity of the synaptobrevin bands in control and dsRNA treated salivary glands. Results are mean ± S. E., n=3.

Figure 3: Genetic specificity of RNAi in tick salivary glands to corresponding cDNA of synaptobrevin. A) RT-PCR of total RNA prepared from salivary glands, treated with synaptobrevin dsRNA , Lane 1, Low DNA Mass^{TM} Ladder, 2. Control salivary glands, synaptobrevin transcript, 3. dsRNA treated glands, synaptobrevin transcript, 4. Control salivary glands, NaK ATPase transcript, 5. synptobrevin dsRNA treated glands, NaK ATPase transcript. B). RT-PCR of total RNA prepared from salivary glands treated with NaK ATPase dsRNA, 1. Low DNA Mass^{TM} ladder, 2. Control salivary glands, NaK ATPase transcript, 3. NaK ATPase dsRNA treated glands, NaK ATPase transcript, 4.

control salivary glands, synaptobrevin transcript, 5. NaK ATPase dsRNA treated glands, synaptobrevin transcript.

Figure 4: Western blot analysis of the tick salivary glands incubated with two concentrations of synaptobrevin dsRNA *in vitro* A) 15 μg synaptobrevin dsRNA , 1. Control, rat brain, 2. control salivary glands, 3. synaptobrevin dsRNA incubated salivary glands B) 25 μg synaptobrevin dsRNA, 1. Control salivary glands and 2, synaptobrevin dsRNA incubated salivary glands.

Figure 5: Inhibition of the 0.1 μM PGE_2 stimulated anticoagulant protein secretion (compared to uninhibited control) in isolated whole salivary glands after 6 hours pre-incubation of salivary glands with dsRNA for corresponding cDNA fragment of synaptobrevin. Results are mean ± S.E., n = 3.



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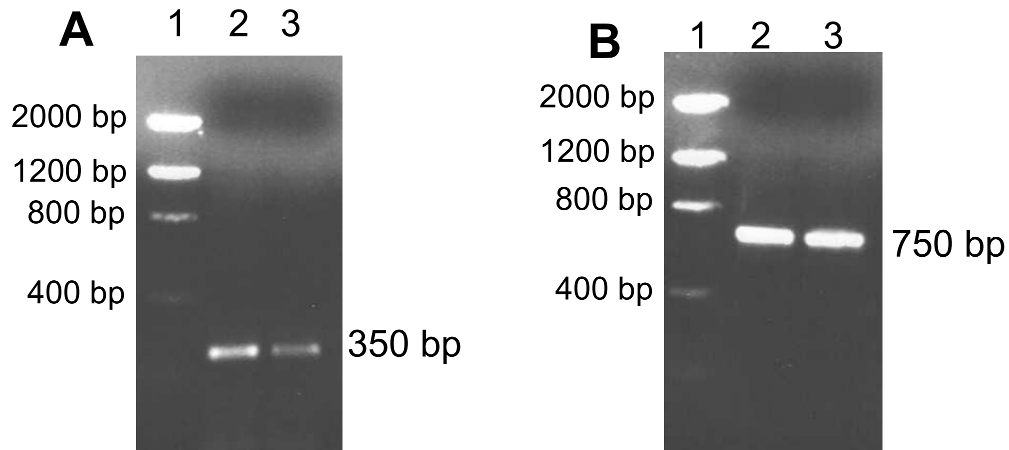
+CFKVKTTAP+RYCVRPNSGI++P V V+VMLQPF+YDP EK+KHKFMVQT+ AP
H. s:40 VCFKVKTTAPRRYCVRPNSGIIDPGSTVTVSVMLQPFDYDPNEKSKHKFMVQTIFAPPNT 99

A. a: 183 VNQDTVWRDA 212
+ + VW++A
H. s.: 100 SDMEAVWKEA 109

FIGURE 1



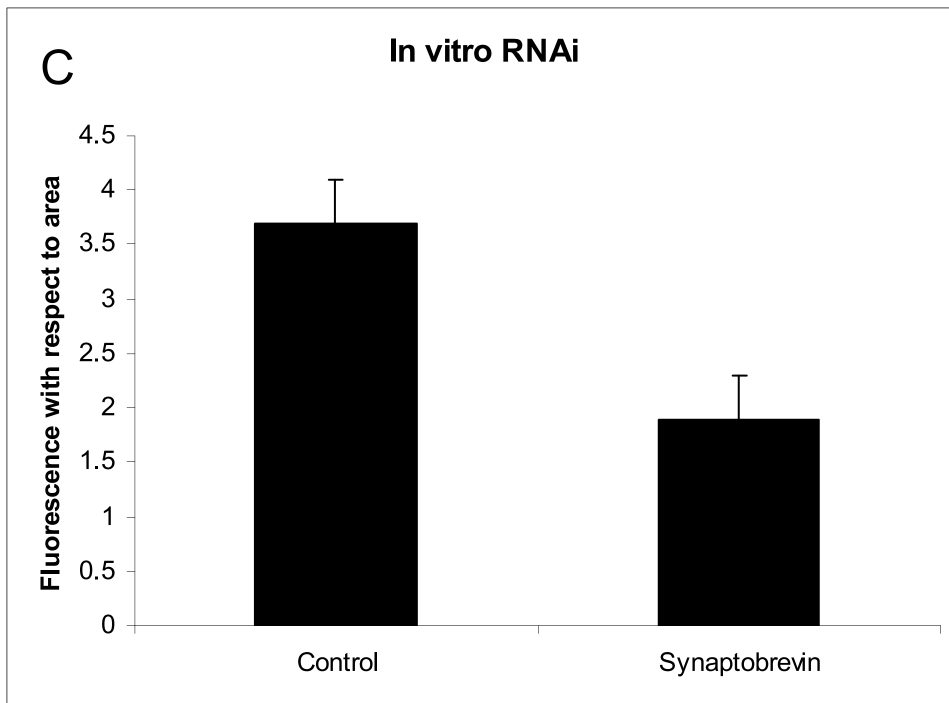


FIGURE 2

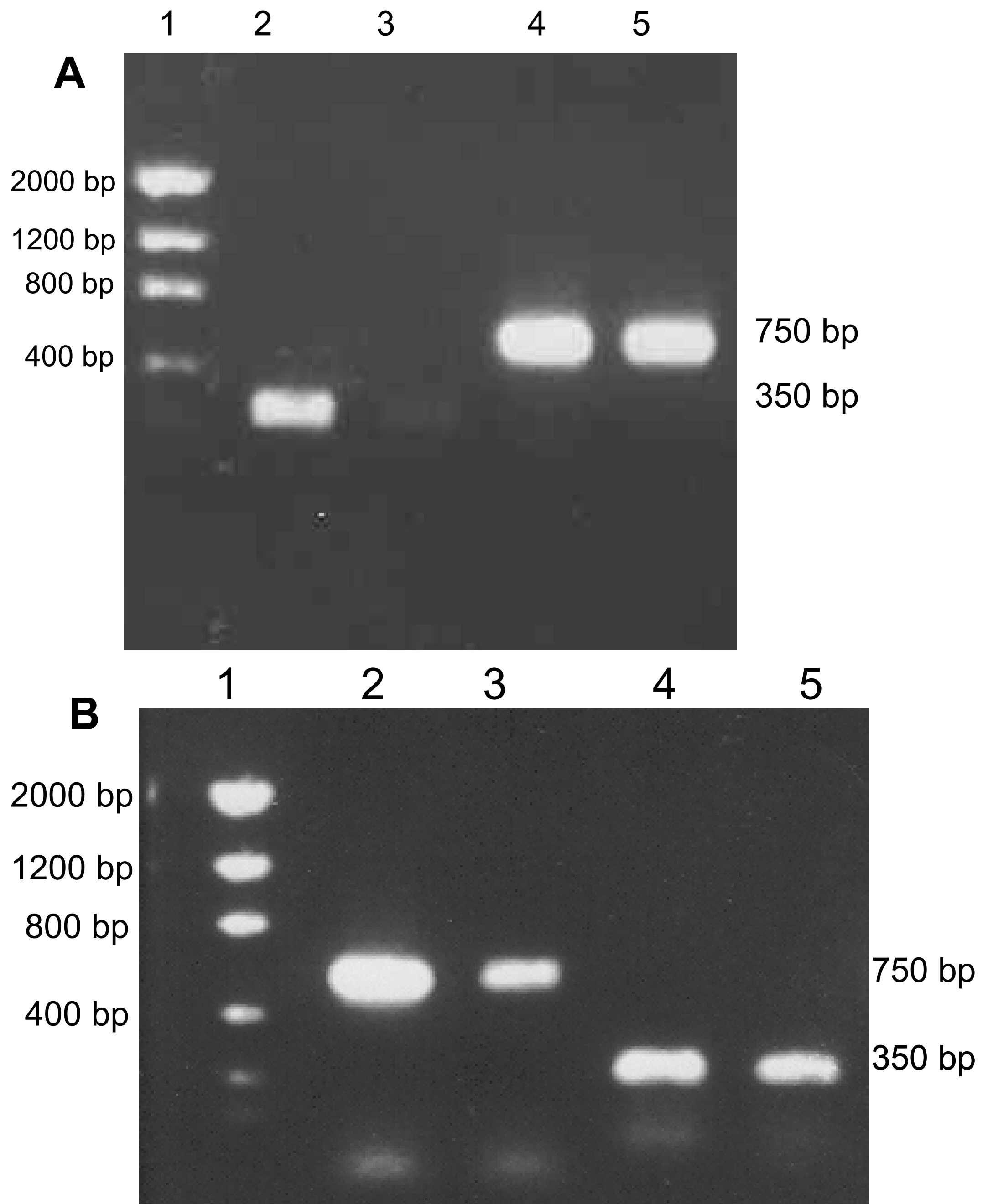


FIGURE 3

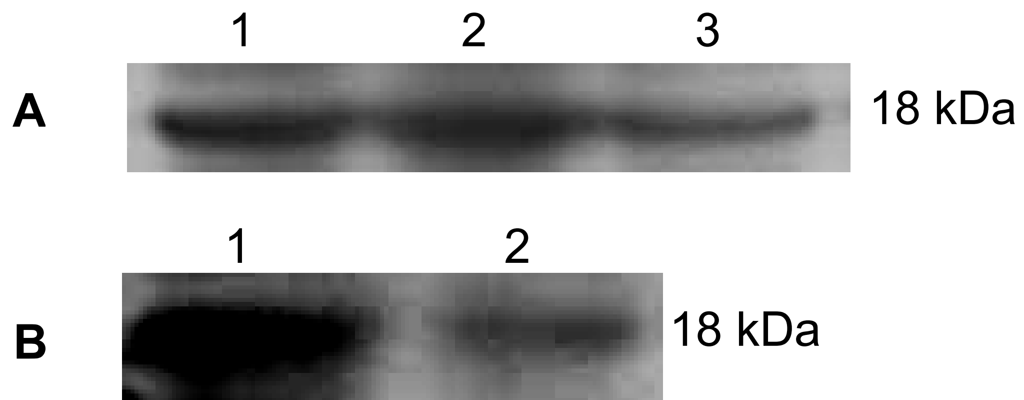


FIGURE 4

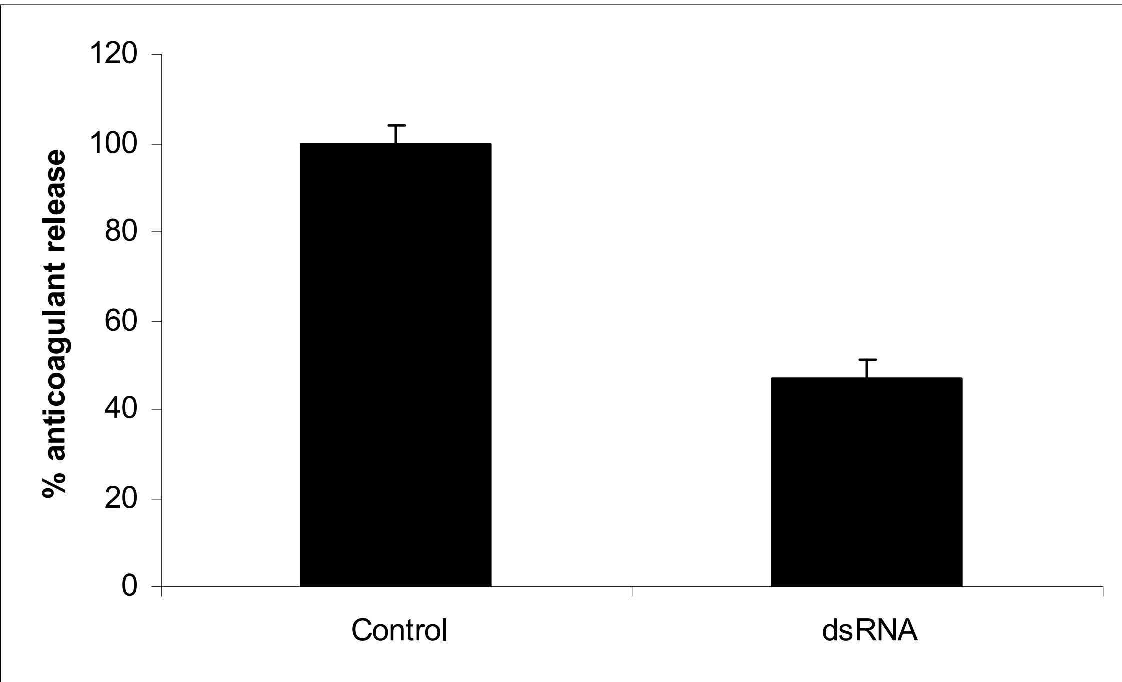


FIGURE 5

Amblyomma americanum **salivary gland homolog of nSec1 is essential for saliva protein secretion (Published in Biochemical and Biophysical Research Communications)**

Abstract

SNARE (Soluble N-ethylmaleimide sensitive factor Attachment protein Receptor) proteins assemble in tight core complexes which promote fusion of carrier vesicles with target compartments. Members of this class of proteins are expressed in all eukaryotic cells and distributed in distinct sub cellular compartments. All vesicle transport mechanisms known to date have an essential requirement for a member of the Sec1 protein family, including the nSec1 in regulated exocytosis. A homolog of nSec1 was cloned and sequenced from the salivary glands of partially fed female ticks. Double-stranded RNA was used to specifically reduce the amount of nSec1 mRNA and protein in female adult tick salivary glands. This reduction was accompanied by a decrease in anticoagulant protein release by the glands and by abnormalities in feeding by dsRNA treated ticks. We report the efficacy of double-stranded RNA-mediated interference (RNAi) in "knocking down" nSec1 both *in vivo* and *in vitro* in tick salivary glands and the applicability of this technique for studying the mechanism of exocytosis in tick salivary glands.

Introduction

Tick salivary glands are crucial in the ability of ixodid ticks to feed successfully on a host. As feeding progresses, the rate of salivary fluid secretion increases greatly, enabling the tick to concentrate the blood meal by returning excess water and ions to the host. Numerous bioactive proteins (e.g. anticoagulants, anti-inflammatory proteins and immunosuppressants) and prostaglandins are secreted as components of saliva to modulate interactions of the tick with the host (1, 2). Ticks feed exclusively on host blood in each of their developmental stages: larvae, nymph and adult.

Exocytosis is a highly orchestrated process involving the docking and fusion of secretary vesicles with the plasma membrane (3, 4). According to the Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor (SNARE) hypothesis of vesicle fusion, vesicles dock at a target membrane through the 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 promote the 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) (5). Besides SNAPs and NSF, various other molecules are known to regulate SNARE protein interactions and activities (6-9). Among these molecules, the Sec-1 related proteins play a crucial role in modulating SNARE complex formation (10). As demonstrated for the neuronal isoform munc18-1 (also known as nSec1 or rbSec1), these proteins bind with high affinity to syntaxins and regulate their association with cognate SNAREs (11-17).

nSec1 is critical for vesicle trafficking in yeast and is proposed to play an important role in the exocytosis process. Carr et al., (18) proposed that Sec1p functions to promote exocytosis after SNARE complexes have been assembled. Loss of Sec1 function results in an accumulation of vesicles at specific steps in secretion (19-21). Yang et al., (16) helped support the hypothesis that Sec1 proteins function as syntaxin chaperons during vesicle docking, priming and membrane fusion. Over expression of Sec1 in *Drosophila melanogaster* results in a reduction of synaptic transmission (22, 23).

Posttranscriptional gene suppression by RNA interference (RNAi) provides an effective technique to "knock down" specific gene expression in tick salivary glands' (24, 25). To gain insight into the complexity of *A. americanum* salivary glands exocytotic pathway, a cDNA fragment of an nSec1 homologue was cloned and sequenced from partially fed tick salivary glands. The use of double-stranded RNA based upon this sequence confirmed an important role of nSec1 in the mechanism of protein secretion in tick salivary glands.

Results

We previously demonstrated that a tick salivary gland homolog of nSec1 protein is present in salivary glands of the lone star tick (26). We amplified a cDNA fragment for it using primers based upon the sequence of an EST from *Amblyomma americanum* (27). The amplified 410 bp fragment (Figure 1A) was cloned in a pCRII vector and sequenced. We also amplified a 750 bp fragment of β-actin (Figure 1B) and used it as a control in all our experiments. A search of the database using BlastX (www.ncbi.nlm.nih.gov/BLAST/) demonstrated that the nSec1 sequence had 74% identity to *Mus musculus* nSec1 at the amino acid level (Figure 1C).

Salivary glands incubated for 6 hrs with dsRNA, synthesized from the putative nSec1 gene demonstrated ~60% decrease in nSec1 transcript (Figure 2A). Unfed live ticks injected *in vivo* with nSec1 dsRNA showed ~30% decrease in transcript level after 12 days of feeding as compared to the buffer injected controls (Figure 2B). The level of β-actin transcript level as a negative control remained the same in experimental and control groups both in *vivo* and *in vitro* (Figure 2A & 2B). Alternatively, salivary glands from 8 ticks were incubated for 6 hrs with Na-K ATPase dsRNA and buffer. Left glands were incubated with buffer and right with NaK ATPase dsRNA. The levels of NaK ATPase and nSec1 transcripts were compared between the buffer incubated glands and dsRNA incubated glands. There was a 70% reduction of NaK ATPase transcript levels in the NaK ATPase treated glands. But nSec1 mRNA levels remained the same confirming the specificity of RNAi (Figure 2D & 2E). The nSec1 protein level was also found to be reduced in both *in vivo* (~75%) and *in vitro* (~60%) dsRNA treated salivary glands as compared to control glands (Figure 3A-3D).

nSec1 dsRNA injected ticks fed on the host attained an average repletion weight of 628 mg as compared to control ticks repletion weight of 719 mg. nSec1 dsRNA injected ticks attained repletion in 14.5±2 days as compared to control ticks average repletion time of 11± 1 days (Figure 4A).

We have previously shown that saliva from partially fed female lone star ticks contains anticoagulant proteins (28). Regulated exocytosis of anticoagulant protein in isolated salivary glands is stimulated by PGE_2 (29). To determine whether RNAi of nSec1 affects anticoagulant secretion, isolated glands were incubated with nSec1 dsRNA. Glands were washed with buffer and then stimulated with 0.1 μM PGE_2. PGE_2-stimulated secretion of anticoagulant protein in salivary glands was inhibited 20% as compared to controls (Figure 6). Other salivary glands were dissected from ticks in which dsRNA for nSec1 was injected into unfed ticks prior to feeding. These salivary glands also demonstrated a 20% reduction in secretion of anticoagulant protein as compared to controls (Figure 6). To confirm the specificity, we used histamine binding protein (HBP) dsRNA in a separate experiment. Here, glands were incubated with or without HBP dsRNA and the experiment was performed in a similar fashion as done with nSec1 dsRNA. In this case there was no difference in PGE_2 stimulated secretion of anticoagulant protein between glands incubated with buffer and those incubated with HBP dsRNA (data not shown).

Discussion

There is great interest in exploiting RNAi via the use of double stranded RNA as a means of assessing specific function(s) of individual genes in cells of vertebrates and invertebrates (30). In the SNARE model for protein secretion, a critical component of the fusion process is the Sec1p protein, known in mammals as munc18, nSec1 or rbSec1 (11-13). This soluble protein is thought to be critical for vesicle trafficking. A tick homolog of nSec1 is present in salivary glands of partially fed female lone star ticks and has previously been shown to be important in the process of exocytotic protein secretion in the salivary glands (26). Apart from the presence of nSec1 in neuronal cells, nSec1 mRNA has also been detected in pancreatic endocrine islet and in insulin-producing cells (31, 32).

We have cloned a partial sequence of the tick nSec1 gene and used it to synthesize a complimentary dsRNA. The dsRNA used either in isolated salivary glands or in whole ticks decreased the amount of nSec1 mRNA and protein, and diminished the ability of the salivary glands to secrete anticoagulant protein. As a negative control, use of a dsRNA for a partial sequence of the β-subunit of NaK ATPase reduced the expression of NaK ATPase but had no affect on the expression of nSec1. These results add evidence that nSec1 is important in the process of protein secretion in tick salivary glands. The results also further demonstrate that dsRNA is an effective and simple method for reducing gene expression in tick salivary glands. This method is specific, affecting only the gene targeted for disruption, and useful in targeting genes required for salivary gland secretion.

This study adds data to the increasing evidence suggesting that exocytotic proteins, previously shown to be of vital importance for neuronal exocytosis, are also essential components of the non-neuronal exocytotic pathway. Application of dsRNA from the corresponding cDNA fragment of nSec1 to the salivary glands both *in vivo* and *in vitro* resulted in inhibition of PGE_2 stimulated protein secretion and considerable decrease in the nSec1 transcript, encoded nSec1 protein and prolonged feeding of treated ticks on the host.

The success of prolonged tick feeding on a host depends on several factors in tick saliva, primarily proteins and prostaglandins (1), by which they modulate the host response at the tick-host interface. Deleting or reducing one of the SNARE complex related proteins might cause an aberrant phenotypic response during tick feeding. The groups of dsRNA injected ticks remaining attached for longer periods and feeding aberrantly represents a clear example of aberrant phenotypic expression possibly resulting from disruption of the tick's ability to secrete proteins necessary for feeding.
The unique feeding pattern and significant decrease in nSec1 transcript, encoded protein and anticoagulant secretions indicate the ability of dsRNA to enter the salivary glands from the hemocoel similar to *in vivo* RNAi injection experiments in *C. elegans* that produced mutant phenotypes in the whole worm and even its progeny (33, 34). Interestingly, a decrease in transcript of 60% was observed after application of RNAi *in vitro* as compared to 30% in vivo. This difference may be due to dilution of dsRNA in the feeding stage of the female tick which increases approximately 100X in size during feeding.

It is uncertain if an nSec1 is present and is targeted in other tissues that may affect *in vivo* results. However, it is well established that exocytosis is mediated by SNARE proteins in tick salivary glands (25, 26). The present challenge is to identify the potential SNARE regulators present in salivary glands and to assign functions to specific secretion events. In the present study, we demonstrated further that an nSec1 homolog is required for exocytosis. More work is required to determine more specifically how nSec1 interacts with SNARE complex proteins and the physiology of protein secretion in the salivary glands.

Materials and methods

Materials

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). Antibodies to rat neural Sec1 (rabbit: polyclonal), Rabbit IgG-HRP and rat brain tissue were purchased from StressGen (Victoria, BC, Canada). Mineral oil was obtained from Sigma (St. Louis, MO, USA).

Tick rearing

Amblyomma americanum (L.) ticks were reared at Oklahoma State University's tick rearing facility according to the methods of Patrick and Hair (35). Immature ticks were fed on rabbits and adult ticks on sheep. All unfed ticks were maintained at 27-28°C and 90% relative humidity under 14h light/10h dark photoperiod before infestation of the hosts. Ticks weighing 50-200 mg after 11-15 days of attachment were removed from sheep in the control and experimental groups. Salivary glands were dissected from ticks within 4 h of being removed from the host.

Salivary glands

Tick salivary glands 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 -80°C in 0.5 M piperazine N, N-bis-2-ethane sulfonic acid, pH 6.8, containing 20 mM EGTA, 1X 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 homolog of nSec1 cDNA and RT-PCR

Using a tick specific EST sequence for nSec1 (27), gene specific primers were designed to amplify a cDNA fragment from salivary glands of the lone star tick. Total RNA was isolated from the salivary glands using an RNAqueous™ total RNA isolation kit (Ambion). Concentration of total RNA was determined spectrophotometrically at 260 and 280 nm and it was aliquoted and stored at -80°C before use. Total RNA was reverse transcribed using M-MLV (Moloney Murine Leukemia virus) reverse transcriptase according to the Invitrogen protocol. For each gene, cDNA was PCR amplified using gene specific primers (GSP) for nSec1 (forward 5'-TGACGAAAAGGACGACCTATGG-3' and reverse 5'-CTTGGACAGAATGTAGAGCAGGATG-3'), tick salivary gland NaK ATPase β-subunit (forward 5'-CGGCAAGGAGGGAGAATACTACAAG-3' and reverse 5'-CAAACTGCTGGGCAATGGAG-3') and human β-actin primers (Stratagene, La Jolla, CA, USA) as the control by using a PCR program of 94°C for 4 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Annealing temperature used for NaK ATPase primers was 65° C for 30 secs.

Cloning and sequencing

After PCR amplification, gel-purified fragments were cloned into the pCR-II vector using a TOPO T/A cloning Kit (Invitrogen). Recombinant plasmids were then transformed into TOP 10F' One Shot Competent Cells and cultured on agar plates containing 50 μg/ml ampicillin, 0.2 mM IPTG and 40 μg/ml X-gal for selection and

amplification. White colonies (10 colonies) were picked from these plates and then grown in Luria Broth (LB) containing 50 μg/ml ampicillin. Plasmid DNA was purified with Qiaprep spin Miniprep plasmid kit (Qiagen). Cloned recombinant plasmid DNA (250 ng) was tested for the presence of an insert by comparing its migration to the vector plasmid alone on 2% agarose gel electrophoresis; positive clones were then digested with *Eco*RI and the product analyzed again by agarose gel electrophoresis to verify the insert. Recombinant plasmids were sequenced at the OSU Protein/Nucleic Acid Resource Facility with M13 reverse primer using an ABI 3700 automated DNA sequencer and Big Dye dye terminators. The resulting sequences were used to search the DNA and protein databases.

dsRNA *in vitro* synthesis and incubation with tick salivary glands

The nSec1 cDNA-containing plasmids with inserts in opposite orientation were linearized by digesting with *HindIII* enzyme and precipitated with ethanol. Plasmids containing HBP (Histamine binding protein) inserts were digested with *KpnI* and *XhoI* and the plasmids containing Na K ATPase were digested with *EcoRV* and *SacI*. The digested plasmids were ethanol precipitated and used as templates for *in vitro* transcription using a MEGAscript kit (Ambion) following the manufacturer's instructions. The two ssRNAs were mixed together and incubated at 65^0C for 5 minutes and later left to anneal at room temperature for 4 hours. All *in vitro* experiments used β-Actin and β subunit of Na K ATPase expression as negative controls for non-specific interference. In all *in vitro* experiments, right and left salivary glands from 20 partially fed female ticks were pre-incubated separately for 6 hrs at 37°C with 15 μg of dsRNA in M199/MOPS or M199/MOPS alone prior to analysis of nSec1 mRNA by RT-PCR or

protein expression by Western blot. Integrated fluorescent intensity of bands was compared with a gel documentation system from Bio-Rad (Hercules, CA, USA). A total number of 50 unfed female ticks were injected with 450 ng of dsRNA into each tick. After injection, ticks were kept overnight in a 37°C water bath to monitor tick survival after injection. Buffer and dsRNA injected ticks were infested in separate cells on host sheep for feeding.

Western blotting

Twenty partially fed female tick salivary glands treated with dsRNA or not were homogenized in ice-cold extraction buffer [(PBS, 1 mM-dithiothreitol, 2.5 mM EGTA, 1X Complete™ Mini protease inhibitor cocktail) (Roche)]. Homogenized salivary glands were sonicated for 1 minute. Protein concentration in all samples was estimated by the Bradford method (36). The protein standards used for SDS-PAGE were low molecular weight range from Bio-Rad. Tick salivary gland protein extracts (50 μg each) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (37) and then transferred onto nitrocellulose membranes in a Transblot cell (Bio-Rad) following the manufacturer's instructions. The transfer buffer consisted of 25 mM Tris HCl, 192 mM glycine in 20% methanol. Nonspecific protein binding sites were blocked with 5% skim milk and the membranes were incubated with the polyclonal nSec1 antibody (StressGen) at a dilution of 1:2000. The antigen-antibody complexes were visualized with horseradish peroxidase-conjugated anti-rabbit IgG (StressGen) at a dilution of 1:1000 and detected with SuperSignal® chemiluminescent substrate (Pierce, Rockford, IL, USA) on x-ray film.

Exocytosis of anticoagulant protein

The right and left salivary glands from 8 partially fed female ticks were divided into two tissue sets where one gland from each tick was used as the control and the other gland as the treatment set. Dissected salivary glands were pre-incubated with nSec1 dsRNA for six hours at 37°C while control glands were incubated in buffer alone or irrelevant dsRNA such as HBP. After incubation with dsRNA or buffer alone, glands were centrifuged for 1 min at 1000xg and then stimulated with PGE_2 (0.1μM) for 5 minutes at room temperature. Glands were centrifuged at 1000xg for 1 minute and the supernatant used for determination of anticoagulant activity by the activated partial thromboplastin assay (APTT, intrinsic pathway) as described by Zhu et al., (28). Time to initiation of coagulation (V_{max}) was determined by Softmax^R PRO (Molecular Devices, Sunnyvale, CA, USA) software using a THERMO MAX plate reader (Molecular Devices). Each experiment was repeated three times. Alternatively, 900ng of nSec 1 dsRNA was injected into unfed ticks. Control ticks were injected with buffer. The injected ticks were kept at 37^0C above a water bath overnight. Each tick group was infested in a separate cell on the host sheep for feeding. Ticks that weighed 50-200 mg (11-15 days) were removed and the anticoagulant protein secretion studies were performed as mentioned above.

Statistics

All data are expressed as mean ± SEM. Statistical significance was determined by Student's t test.

Acknowledgments

We thank Drs. Haobo Jiang and Udaya DeSilva for reviewing the manuscript. This work was supported by the National Science Foundation grant IBN 9974299 and project 1446 funds from the Oklahoma Agricultural Experiment Station. The Director of the Oklahoma Agricultural Experiment Station approved this article for publication.

References

1. Sauer, J R, McSwain, J L, Bowman, A S and Essenberg, R C. Tick salivary gland physiology. Ann. Rev. Entomol. 1995; 40: 245-267.
2. Bowman, A S, Coons, L B, Needham, G R and Sauer, J R. Tick saliva: recent advances and implications for vector competence. Med. Vet. Entomol. 1997; 11: 277-285.
3. Bennet, M K and Scheller, R H. The molecular machinery for secretion is conserved from yeast to neurons. Proc. Natl. Acad. Sci. USA. 1993; 90: 2559-2563.
4. Sudhof T C. The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 1995; 83: 111-119.
5. Sollner, T, Whiteheart, S W, Brunner, M, Erdjument-Bromage, H, Geromanos, S, Tempst, P and Rothman, J E. SNAP receptors implicated in vesicle targeting and fusion. Nature 1993; 362: 318-324.
6. McMahon, H T, Missler, M, Li, C and Sudhof, T C. Complexins: cystolic proteins that regulate SNAP receptor function. Cell 1995; 83: 111-119.
7. Fujita, Y, Shirataki, H, Sakisaka, T, Asakura, T, Ohyo, T, Kotani, H, Yokoyama, S, Nishioka, H, Matsuura, Y, Mizoguchi, A, Scheller, R H and Takai, Y. Tomosyn: a syntaxin-1-binding protein that forms a novel complex in the neurotransmitter release process. Neuron 1998; 20: 905-915.
8. Beites, C L, Xie, H, Bowser, R and Trimble, W S. The septin CDCre1-1 binds syntaxin and inhibits exocytosis. Nature Neuroscience 1999; 2: 434-439.

9. Lao, G, Scheuss, V, Gerwin, C M, Su, Q, Mochida, S, Retting, J and Sheng, Z H. Syntaphilin: a syntaxin-1 clamp that controls SNARE assembly. Neuron 2000; 25: 191-201.
10. Halachmi, N and Lev, Z. The Sec1 family: a novel family of proteins involved in synaptic transmission and general secretion. J. Neurochem. 1996; 66: 889-897.
11. Hata, Y, Slaughter, C A and Sudhof T C. Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. Nature 1993; 366: 347-351.
12. Pevsner, J, Hsu, S C and Scheller, R H. nSec1: a neural-specific syntaxin-binding protein. Proc. Natl. Acad. Sci. USA, 1994; 89: 8414-8418.
13. Garcia, E P, Gatti, E, Butler, M, Burton, J and De Camilli, P. A rat brain Sec1 homologue related to Rop and UNC18 interacts with syntaxin. Proc. Natl. Acad. Sci. USA 1994; 91: 2003-2007.
14. Garcia, E P, McPherson, P S, Chilcote, T J, Takei, K and De Camilli, P. rbSec1 and B colocalize with syntaxin 1 and SNAP-25 throughout the axon, but not in a stable complex with syntaxin. J. Cell Biol. 1995; 129: 105-120.
15. Dulubova, I, Sugita, S, Hill, S, Hosaka, M, Fernandez, L, Sudhof, T C and Rizo, J. A conformational switch in syntaxin during exocytosis: role of munc18. EMBO J. 1999; 18: 4372-4382.
16. Yang, B, Steegmaier, M, Gonzales, L C and Scheller, R H. nSec1 binds a closed conformation of Syntaxin1A. J. Cell Biol. 2000; 148: 247-252.
17. Misura, K M S, Scheller, R H and Weis, W I. Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature 2000; 404: 355-362.

18. Carr, C M, Grote, E, Munson, M, Hughson, F M and Novick, P J. Sec1p binds to SNARE complexes and concentrates at sites of secretion. J. Cell Biol. 1999; 146: 333-344.

19. Novick, P, and Scheckman, R. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. USA. 1979; 76: 1858-1862.

20. Ossig, R, Dascher, C, Trepte, H H, Schmitt, H D and Gallwitz, D. The yeast SLY gene products, suppressors of defects in essential GTP-binding Ypt1 protein, may act in endoplasmic reticulum-to-Golgi transport. Mol. Cell. Biol. 1991; 11: 2980-2993.

21. Cowles, C R, Emr, S D and Horazdovsky, B F. Mutations in the VPS45 gene, a SEC1 homolog, result in vacuolar protein sorting defects and accumulation of membrane vesicles.J. Cell Sci. 1994; 107: 3449-3459.

22. Schultze, K L, Littleton, J T, Salzberg, A, Halachmi, N, Stern, M, Lev, Z and Bellen, H J. rop, a *Drosophila* homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release in vivo. Neuron 1994; 13: 1099-1108.

23. Wu, M N, Littleton, J T, Bhat, M A, Prokop, A and Bellen, H J. ROP, the *Drosophila* Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage dependent manner. EMBO J. 1998; 17: 127-139.

24. Aljamali, M N, Bior, A A, Sauer, J R and Essenberg, R C. 2003. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*. Insect Mol. Biol. 2003; 12: 299-305.

25. Karim, S, Ramakrishnan, V G, Tucker, J S, Essenberg, R C and Sauer, J R. *Amblyomma americanum* salivary glands: Double stranded RNA-mediated gene silencing of synaptobrevin homologue and inhibition of PGE_2 stimulated protein secretion. Insect Biochem. Mol. Biol. 2004; 34: 407-413.

26. Karim, S, Essenberg, R C, Dillwith, J W, Tucker, J S, Bowman, A S and Sauer, J R. Identification of SNARE and cell trafficking regulatory proteins in the salivary glands of the lone star tick, *Amblyomma americanum* (L.). Insect Biochem. Mol. Biol. 2002; 32: 1711-1721.

27. Hill, C A and Gutierrez, J A. Analysis of the expressed genome of the lone star tick, *Amblyomma americanum* (Acari: Ixodidae) using an expressed sequence tag approach. Microb. Comp. Genomics 2000; 5: 89-101.

28. Zhu, K, Sauer, J R, Bowman, A S and Dillwith, J W. Identification and characterization of anticoagulant activities in the saliva of the lone star tick, *Amblyomma americanum* (L.). J. Parasitol. 1997; 83: 38-43.

29. Yuan, J, Bowman, A S, Aljamali, M, Payne, M R, Tucker, J S, Dillwith, J W, Essenberg, R C and Sauer, J R. Prostaglandin E_2-stimulated secretion of protein in the salivary glands of the lone star tick via a phosphoinositide signaling pathway. Insect Biochem. Mol. Biol. 2000; 30: 1099-1106.

30. Hannon, G J. RNA interference. Nature 2002; 418: 244-251.

31. Katagiri, H, Terasaki, J, Murata, T, Ishihara, H, Ogihara, T, Inukai, K, Fukushima, Y, Anai, M, Kikuchi, M, Miyazaki, J, Yazaki, Y and Oka, Y A. Novel isoforms of syntaxin-binding protein homologues to yeast Sec1 expressed ubiquitously in mammalian cells. J. Biol. Chem. 1995; 42: 4963-4966.

32. Jacobsson, G, Bean, A J, Scheller, R H, Juntti-Berggren, L, Deeney, J T, Berggren, P O and Meister, B. 1994. Identification of synaptic proteins and their isoforms mRNAs in compartments of pancreatic endocrine cells. Proc. Natl. Acad. Sci. USA 1994; 91: 12487-12491.

33. Fire, A, Xu, S, Montgomery, M K, Kostas, S A, Driver, S E and Mello, C C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabdities elegans*. Nature 1998; 391: 806-811.

34. Grishok, A, Pasquinelli, A E, Conte, D, Di, N, Parrish, S, Ha, I, Baille, D L, Fire, A, Ruvkun, G and Mello, C C. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell. 2001; 106: 23-34.

35. Patrick, C D and Hair, J A. Laboratory rearing procedures and equipment for multi-host ticks. (Acarina: Ixodidae). J. Med. Entomol. 1975; 12: 389-390.

36. Bradford, M. A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 1976;72: 248-256.

37. Laemmli, U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.

Figure legends

FIGURE 1:

RT-PCR of cDNA from partially fed female tick salivary glands using nSec1 and β-actin gene specific primers (A) 1. Low DNA Mass^{TM} ladder, 2. partially fed female tick adult salivary gland nSec1, (B) 1. Low DNA Mass^{TM} ladder, 2. partially fed female tick salivary gland β-actin. (C) Amino acid blast alignment between the cloned female *Amblyomma americanum* (A.a.) tick salivary gland nSec1 and corresponding part of the gene sequence from *Mus musculus* (M. m.).

[(Score = 204 bits (520), E value = 2 x 10^{52}, Identities = 102/137 (74%), Positives = 117/137 (84%)].

FIGURE 2A, B and C:

In vivo and *in vitro* effect of RNAi on nSec1 in the salivary glands of partially fed female ticks (A) RT-PCR of total RNA prepared from salivary glands using nSec1 and β-actin gene specific primers (A-Lanes 1-2, *in vitro* Control, nSec1 transcript amplified, Lanes 3-4, *in vitro* nSec1 dsRNA, nSec1 transcript amplified, Lanes 5-6, *In vitro* control, β-actin amplified transcript, Lanes 7-8, *in vitro* nSec1 dsRNA treated glands, β-actin amplified transcript). (B) Lane 1, *in vivo* control glands, nSec1 amplified transcript, Lane 2, *in vivo* nSec1 dsRNA injected glands, nSec1 amplified transcript, Lane 3, *in vivo* control, β-actin amplified transcript and Lane 4, *in vivo* nSec1 dsRNA injected glands, β-actin amplified transcript). (C) Integrated fluorescence intensity of the nSec1 RT- PCR bands in control and dsRNA treated salivary glands after *in vitro* incubation or injection *in vivo*.

Figure 2D and E:

In vitro effect of RNAi on Na K ATPase in the salivary glands of partially fed female ticks, (D) RT-PCR of total RNA prepared from salivary glands using nSec1 and NaK ATPase gene specific primers (Lane 1, Control Na K ATPase, lane 2, dsRNA incubated NaK ATPase, Lane 3, Control nSec1 and Lane 4, NaK ATPase dsRNA incubated salivary glands amplified with nSec1 primers) and (E) Integrated fluorescence intensity of the samples analyzed in D.

FIGURE 3:

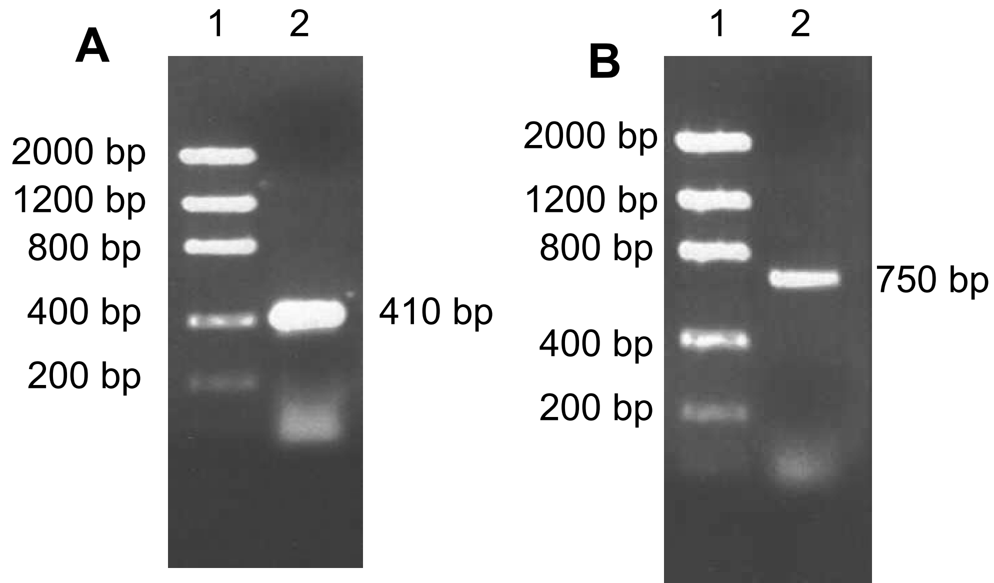
Western blot analysis of nSec1 protein in the partially fed female tick salivary glands (A) incubated in vitro with 15 μg dsRNA nSec1 (Lane 1, Control, lane 2, nSec1 dsRNA treated glands), (B) integrated optical density of the samples in A, (C) *in vivo* with 0.9 μg dsRNA nSec1 (Lane 1, Control, lane 2, nSec1 dsRNA treated glands) and (D) integrated optical density of the samples analyzed in C.

FIGURE 4:

Tick feeding after 0.45μg nSec1 dsRNA injection into unfed ticks prior to attachment to the host (A) Tick size and attachment data were recorded from Day 6 to the final day of tick repletion. Data were tabulated as tick width, repletion weight and average days of repletion. (B) Photo of the effect of nSec1 dsRNA injection on tick feeding as compared to controls.

Figure 5:

Secretions from salivary glands: Inhibition of the 0.1 μM PGE2 stimulated anticoagulant protein secretion (compared to treated control) in isolated whole salivary glands treated with dsRNA for the corresponding cDNA fragment of nSec1 *in vivo*(0.45μg)and *in vitro* (15μg).



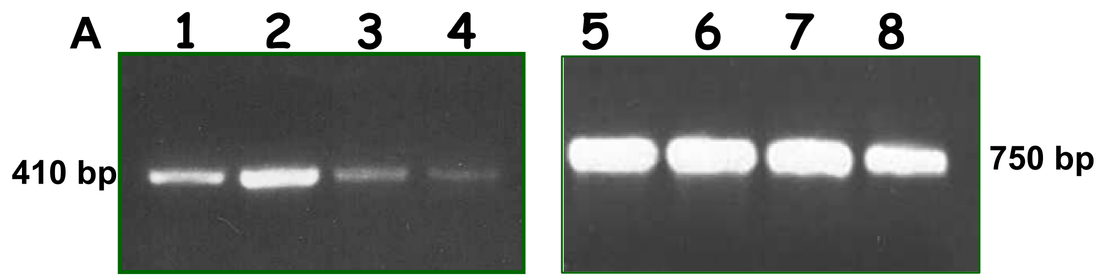
C

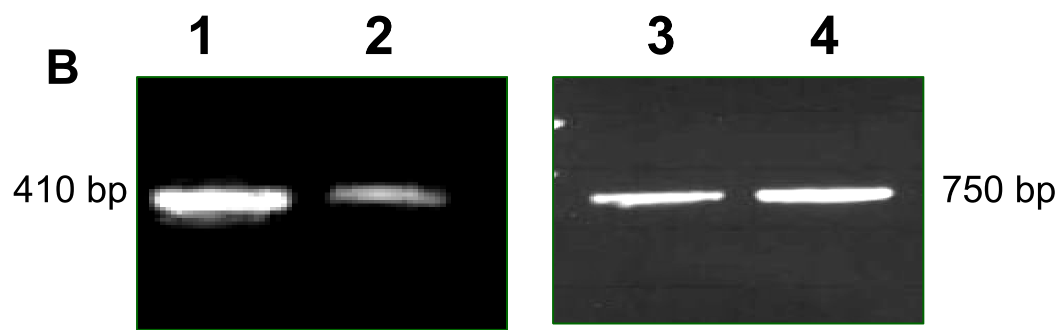
```
A.a.:  14 DEKDDLWIELRHQHIAVVSQAVTKQLKKFIESKRMTSSGDKSSLKDLTTMIKKMPQYQKE 193
          DE DDLWI LRH+HIA VSQ VT+ LK F  SKRM ++G+K++++DL +M+KKMPQYQKE
M.m.: 282 DEDDDLWIALRHKHIAEVSQEVTRSLKDFSSSKRM-NTGEKTTMRDLSQMLKKMPQYQKE 340

A.a.: 194 LNKYSTQLHLAEDCMKCYQGYVDRLCKVEQDLAMGTDAEGEKIKDPMRNIVPILLDTSVS 373
          L+KYST LHLAEDCMK YQG VD+LC+VEQDLAMGTDAEGEKIKDPMR IVPILLD +VS
M.m.: 341 LSKYSTHLHLAEDCMKHYQGTVDKLCRVEQDLAMGTDAEGEKIKDPMRAIVPILLDANVS 400

A.a.: 374 NFDKIRIILLYILSKKG 424
            DKIRIILLYI  K G
M.m.: 401 TYDKIRIILLYIFLKNG 417
```

FIGURE 1





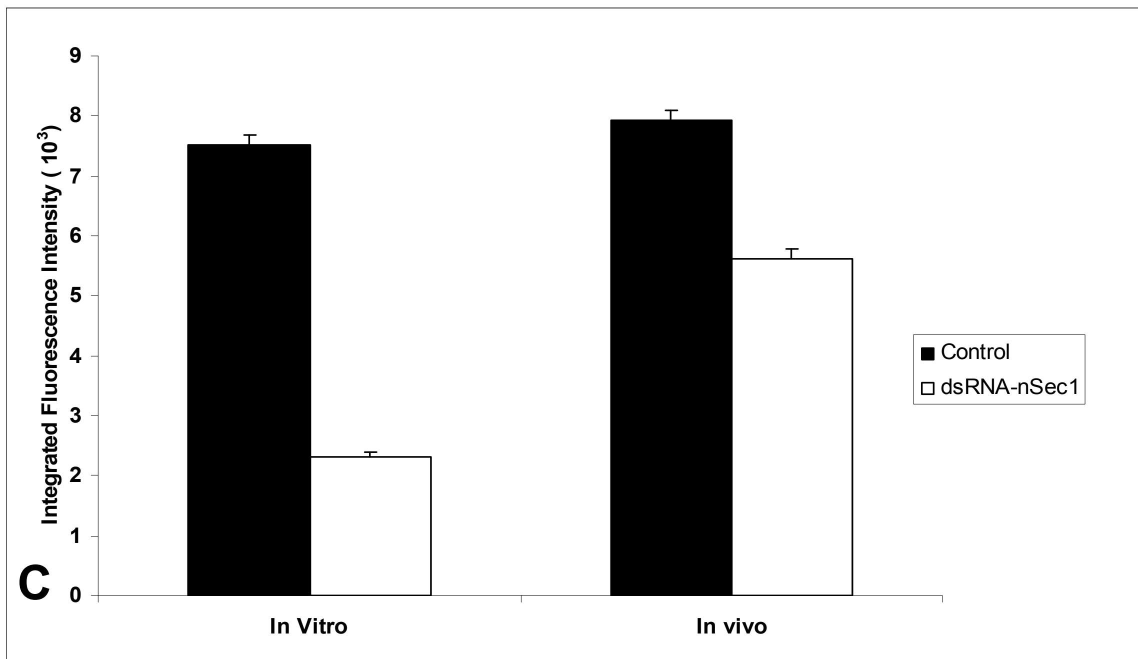


Figure 2

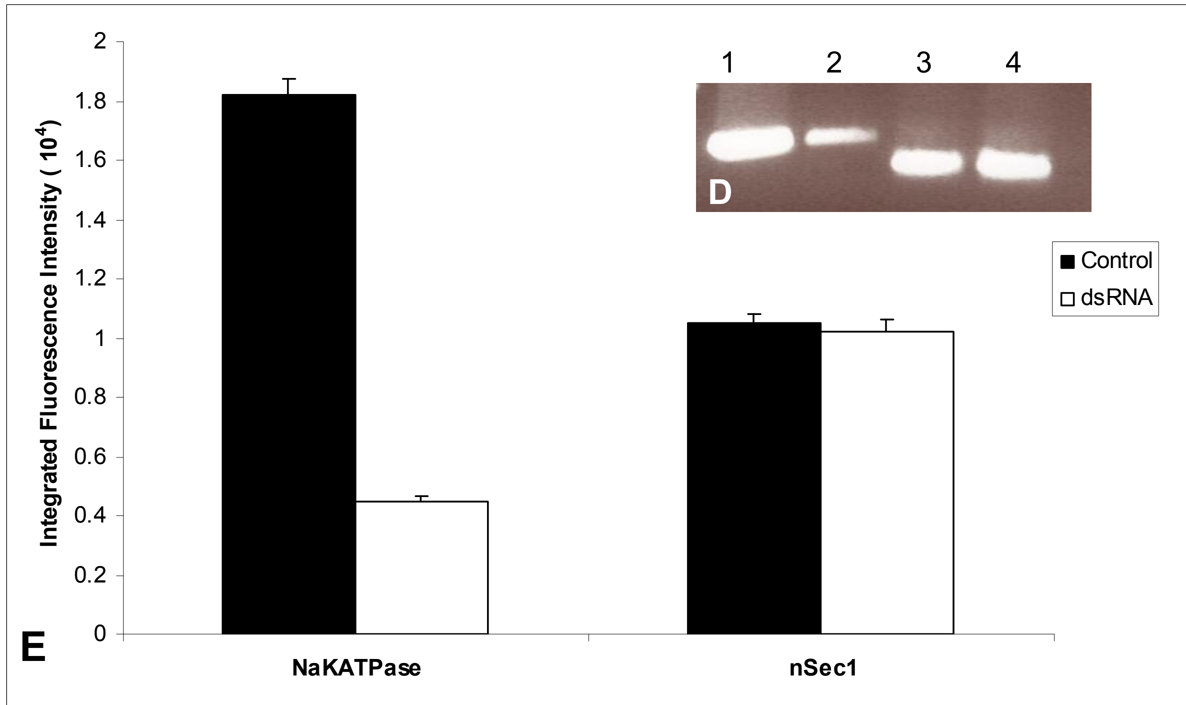
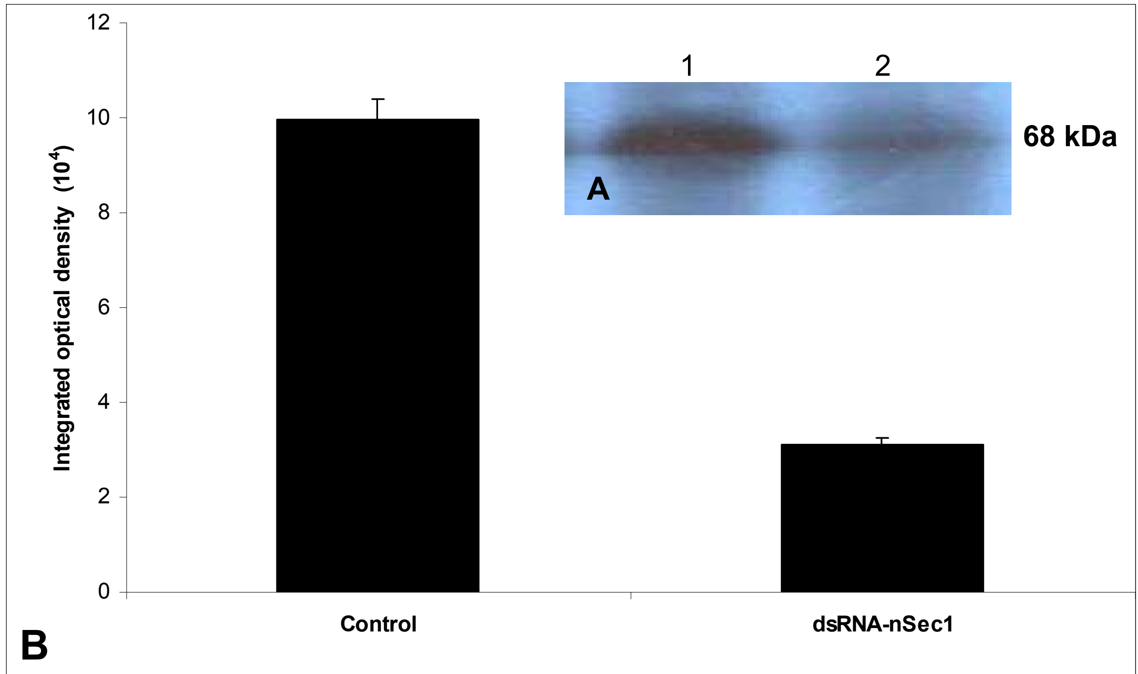


Figure 2



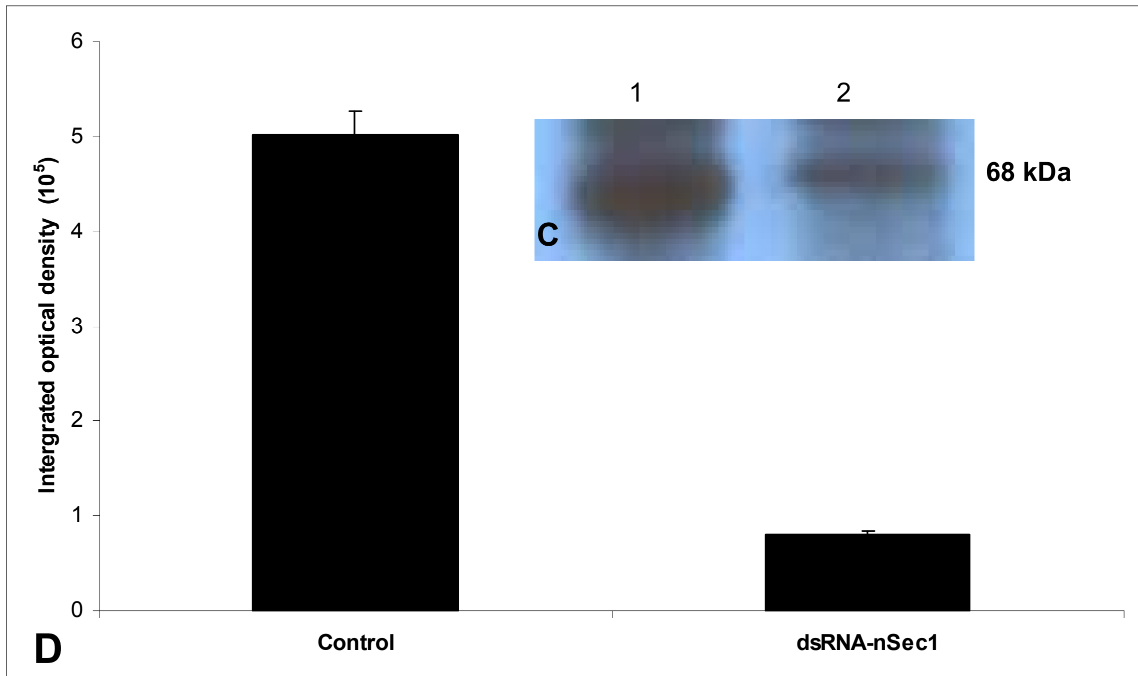


FIGURE 3

A

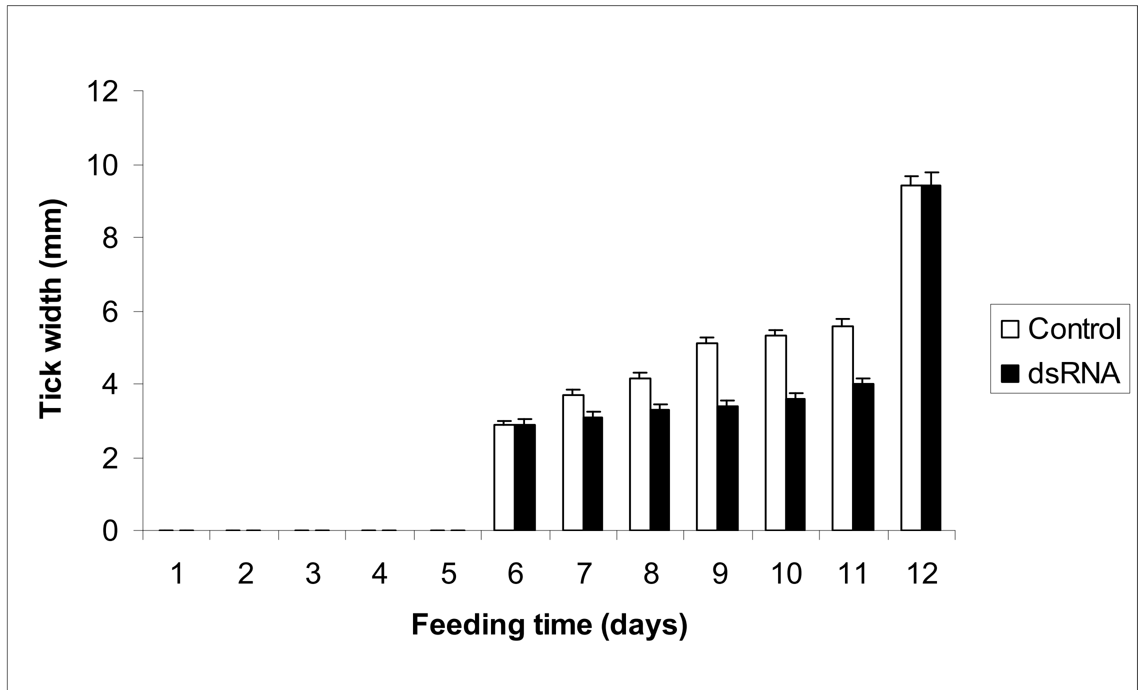


FIGURE 4

B

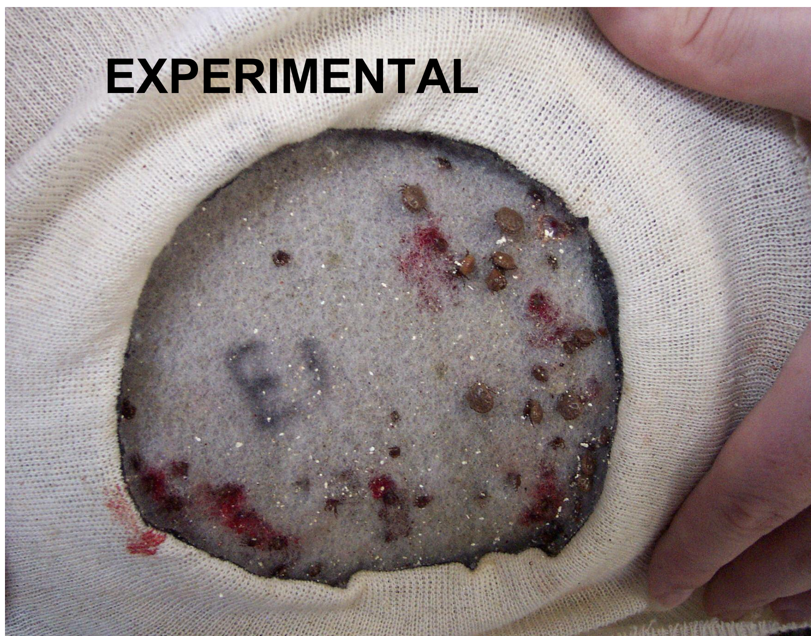


FIGURE 4

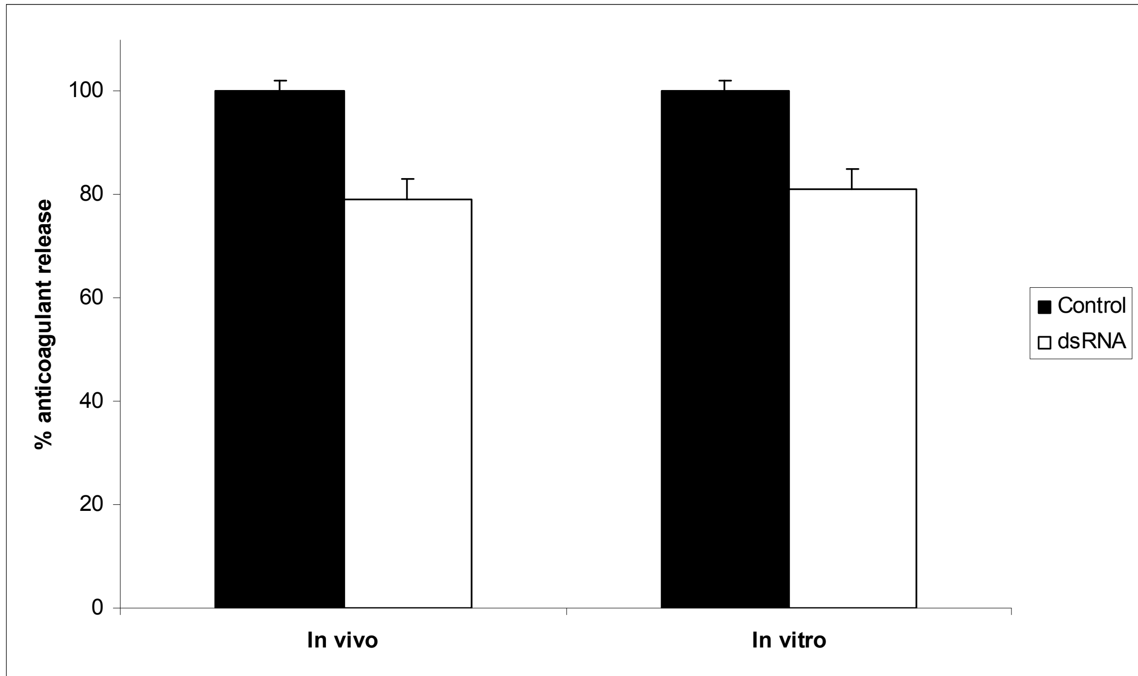


FIGURE 5

CHAPTER 3: OBJECTIVE 2

Ixodid tick saliva is hypertonic for Na^+ and Cl^- relative to hemolymph and midgut but is isotonic for K^+ [1]. Ticks consume large volumes of blood and non-blood tissues as well. Ticks excrete excess salt and fluid back into the host. About one third to one half of consumed blood is secreted back into the host through the salivary glands thereby concentrating the blood meal. The concentrations of Na^+ and Cl^- in the saliva do not balance each other. The concentration of Na^+ is slightly higher when compared with Cl^-. This means that there should be some other anion that balances Na^+ [1]. Moreover, tick saliva is very alkaline with a pH of 9.5. Due to this it is hypothesized that the anion that balances Na^+ might be a bicarbonate ion. Much is known about control of secretion from tick salivary glands. However, the proteins that are functionally involved in the process of secretion are yet to be identified. In an effort to better understand the process, we wanted to identify important channel proteins that are being expressed in tick salivary glands and their functional significance in terms of tick feeding and reproduction.

Sodium Potassium ATPase (NaK ATPase):

ATPases are active transporters that catalyze the transport of ions across membranes against a concentration gradient through the expense of an ATP molecule. ATPases that transport cations have been classified into three main classes F-ATPase, V-ATPase and P-ATPase [2].

NaK ATPases belong to the P-ATPase family and catalyze the active export of Na^+ ions and import of K^+ ions. The key feature of P type ATPases is the formation of a

covalent acyl phosphate-enzyme intermediate and the inhibition by submicromolar concentrations of vanadate [3]. Several ATPases including Ca^{2+} ATPase, H^{+}ATPase and K^{+}ATPase belong to the family of P type ATPase.

NaK ATPases generally exists as a heterodimer with the subunits designated as α and β. The α subunit is the catalytically active subunit containing the site for ATP binding, phosphorylation and inhibitor binding. The molecular weight of the α subunit is ≈ 100kDa. Extensive research has been carried out and significant information has been obtained about the structure and function of the α subunit of the enzyme from various species.

The β subunit has been known to be essential for the normal functioning of the enzyme. But the exact function of this subunit is yet to be elucidated. Prior experiments had shown the importance of NaK ATPase in the process of fluid secretion in tick salivary glands [4]. The importance of NaK ATPase in the process of fluid secretion in TSGs and the known significance of the α subunit encouraged us to work on the β subunit and monitor for aberrant phenotypes in ticks not expressing this protein which would be a test of the importance of the β subunit in the normal functioning of the holoenzyme.

The β subunit has several amino acid sequence isoforms namely β_1, β_2 and β_3 [5]. The β subunit, on SDS-PAGE, generally appears as a glycosylated broad 60-80kDa band. Deglycosylation results in a band of ≈34kDa. The domain structures of all known β subunits have a short N-terminal cytoplasmic domain, a single transmembrane domain and an extracellular C terminal domain. Another significant feature of all known β subunits is the presence of six cysteine residues in the extracellular domain. These

cysteine residues are 100% conserved in β subunits of all NaK ATPases identified so far. These six cysteine residues have been found to form three disulfide bridges [6, 7].

Although the α subunit has the sites for phosphorylation, ATP and inhibitor binding, it is well known that the minimal functional complex of NaK ATPase is the αβ heterodimer. The β subunit has been recently found to be important for the stabilization, maturation and normal enzymatic activity of NaK ATPase. The glycosylation on the β subunit has been found to render stability for the enzyme to trypsinolysis. The disulfide bonds increase the strength of the holoenzyme and prevent the enzyme from being converted to the unstable or inactive form [8]. The results obtained from experiments performed by several research groups show that the β subunit also plays a crucial role in the occlusion of K^+ [9, 10] and that both the α and β subunits should be expressed and should interact for the enzyme to be fully functional.

Sodium potassium chloride co-transporter (NKCC):

NKCC is a key channel protein that plays an important role in mediating the movement of ions across membranes in cells of various organisms. This protein is helpful in maintaining the cell ion homeostasis and volume control [11] by functioning as a secondary active transporter that utilizes the sodium ion gradient generated by the NaK ATPase to facilitate the inward migration of 1 Na^+, 1 K^+ and 2 Cl^- ions in several secretory epithelia.

NKCC belongs to the family of Cation Chloride Co-transporters (CCC) [12]. This ion transporter generally catalyzes the movement of 1 Na^+, 1 K^+ and 2 Cl^- ions across membranes in an electro neutral fashion. Certain co-transporters have been found to transport $2Na^+$, $1K^+$ and $3Cl^-$ [13].

Two main isoforms of NKCC have been found to exist in animal cells. NKCC isoform1 [NKCC1] is present in most cells and hence referred to as the housekeeping isoform or secretory isoform. NKCC isoform 2 [NKCC2] commonly referred to as the re absorptive isoform is found in mammalian kidney cells. Reports indicate the presence of a few variants of both these isoforms. NKCC1 and NKCC2 encode proteins of ≈ 132kDa and 121kDa respectively. On a SDS-PAGE both NKCC1 and NKCC2 run as a smear with a molecular weight of 160-170kDa and 150-160kDa respectively. All NKCCs are highly sensitive to inhibition by bumetamide and other loop diuretics. NKCC2 is more sensitive to bumetamide than NKCC1 [14].

NKCC1 is present in the basolateral membrane and mediates the movement of Na^+, K^+ and Cl^- into the cells. On the other hand NKCC2 is present in the apical membrane of kidney cells and aids in the re-absorption of these ions from urine. NKCC's activities are regulated by a variety of features including the concentrations of the ions transported, hormones such as noradrenalin and vasopressin, reduced oxygen tension, increased intracellular Mg^{2+} concentration and reduced intracellular Cl^- concentration [15, 16, 17, 18]. These regulatory factors modulate the activities of the transporter through two routes, namely co-transporter phosphorylation and protein-protein interactions [19, 20].

NKCC1 has been found to have 12 membrane spanning domains and large N and C terminal regions. There is an extracellular domain between the seventh and eighth transmembrane domains that is glycosylated [21]. It has been found that regions in the transmembrane domains 2, 4 and 7 are crucial for the binding of ions and thus crucial for the enzyme's normal function.

Bicarbonate transporters:

Bicarbonate transport proteins [BT] have been identified in plasma membranes of all mammalian cell types. BTs play a key role in maintaining and regulating the intracellular pH. They do this by regulating the movement of bicarbonate ions across membranes. Tick saliva, as mentioned earlier, has an alkaline pH of 9.5. This led to the hypothesis that there might be a BT expressed in the TSG membrane which functions in regulating the pH of the saliva by facilitating the movement of bicarbonate ions into the lumen.

BT proteins can be divided into two major groups based on sequence similarities. The first group includes the Anion Exchanger (AE) family of Cl^-/HCO_3^- exchangers, NBC group of Na^+/HCO_3^- co-transporters and Na^+ dependent Cl^-/ HCO_3^- co-transporters. The second group is the SLC26a family of sulfate transporting proteins [22].

The BT group of proteins has been reported to be very important in pH_i recovery following ischemia of ferret hearts [23]. In that study BT proteins were found to restore pH_i by 47% indicating the importance of this group of proteins, among the other transport proteins, in the pH recovery process. Thus, it is more likely for one or more proteins of these physiologically important BT proteins to be expressed in the salivary glands of ticks and also other tissues of ticks. Identifying them will improve our understanding of the mechanism of secretion and might also lead us to the identification of a few tick protective antigens.

Aquaporins:

Aquaporins represent a group of integral membrane proteins that facilitate the movement of water molecules. The first aquaporin was discovered in the year 1992 [24].

Since then, there have been 13 different types of aquaporins that have been identified in mammals (AQP0 to AQP12) [25]. Aquaporins are small proteins of about 28kDa in molecular weight. They have been found to be comprised of six transmembrane helices, three extracellular and two intracellular loops. The first intracellular and the third extracellular loops have been found to have a characteristic motif, comprising two repeating Asn- Pro- Ala triplets (NPA) [26]. The NPA motifs render the flexibility for the loops to fold and connect to form the hydrophilic pore for the water molecule to permeate them. The Asn residue is extremely crucial for rendering the ability of aquaporins to allow water to permeate without the flow of protons, though certain aquaporins have been found to be able to transport certain molecules in addition to water. Recently, there are reports indicating possible aquaporin-like proteins that seem to have unusual NPA motifs [27]. Aquaporins have been found to exist as tetramers in plasma membranes, with each monomer having the ability to transmit water. Aquaporins can be classified into two categories, namely, aquaporins and aquaglyceroporins, based on their ability to transport just water or glycerol or urea in addition to water. The aquaporins are AQPs 0, 1, 2, 4, 5, 6 and 8 while AQPs 3, 7, 9 and 10 are the aquaglyceroporins. In salivary glands of higher eukaryotes, AQPs 1, 5 and 8 are expressed [26]. AQP5 has been identified to be the most physiologically important aquaporin, with its loss of function being implicated in some diseases.

Chloride Channels:

Chloride channels are a group of membrane proteins that is ubiquitously expressed from bacteria to mammals [28]. They are found to be associated with the plasma membrane and also with intracellular vesicle membranes. The first chloride

channel identified was denoted as ClC-0 [29]. Following that discovery, eight other chloride channels, denoted as ClC-1 to 7 and ClC-Ka and ClC-Kb, have been identified in mammals [30]. The family of ClC proteins in mammals is classified into three groups based on their sequence identity to the ClC-0 cloned from fish. ClC-1, 2 and ClC-K comprise the first group and are localized to the plasma membrane. ClC-3 to 5 form the second group and are found to be associated with the endosomes and synaptic vesicles and seem to play an important role in intracellular acidification. ClC-6 and ClC-7 form the third group of ClC proteins and are also associated with the endosomes [30]. Surprisingly, the bacterial ClC protein, denoted as ClC-e1 has recently been shown to function as an electrogenic exchanger that exchanges $2Cl^-$ ions for $1H^+$ ion [31]. All the ClC channel proteins are found to exist as homo-dimers with one pore in each monomer.

We were successful in cloning partial cDNA fragments coding for a few of the above mentioned proteins from tick salivary glands. The cDNA fragments cloned had significant amino acid sequence identity to the members of the following classes: Sodium Potassium Chloride Co-transporter (NKCC), Sodium Bicarbonate Co-transporter (NBC), Aquaporin-7 (AQP-7) and Chloride channel-2 (ClC-2) from other species. RNAi was performed on each of the 4 genes and the results are presented below. In the process of analyzing the functions performed by these proteins in TSGs by RNAi, we also have identified that RNAi can be successfully performed by soaking ticks in a solution containing dsRNA, a method that is novel for tick RNAi studies. The advantages and the shortcomings of this methodology are also discussed.

References:

1) Guenther, P.E., Barker, D.M., Sauer, J.R., 1980. Sodium, chloride and water balance in feeding gulf coast tick, *Amblyomma maculatum* (KOCH) Ann. Entomol. Soc. Am 73, 485-488.

2) Pedersen, P.L., Carafoli, E., 1987 Ion motive ATPase II. Trends Biochem Sci. 12, 186-189.

3) Cantley, L.C., Jr. Josephson, L., Warner, R., Yanagisawa, M., Lechene, C., Guidutti, G., 1977. Vanadate is a potent NaK ATPase inhibitor found in ATP derived from muscle. J Biol. Chem. 252, 7421-7423.

4) McSwain, J.L., Luo, C., DeSilva, G.A., Palmer, M.J., Tucker, J.S., Sauer, J.R., Essenberg, R.C., 1997. Cloning and sequence of a gene for a homologue of the C subunit of the V-ATPase from the salivary gland of the tick Amblyomma americanum (L.). Insect Mol.Biol. 6, 67-76.

5) Horrisberger, J.D., Lemas, V., Kraehenbuhl, J.P., Rossier, B.C., 1991. Structure function relationship of NaK ATPase. A.Rev.Physiol. 53, 565-584.

6) Kirley, T.L., 1989. Determination of three disulfide bonds and one free sufhydryl in the β subunit of NaK ATPase. J Biol. Chem. 264, 7185-7192.

7) Miller, R.P., Farley, R.A., 1990. β subunit of NaK ATPase contains three disulfide bonds. Biochemistry, N.Y. 29, 1524-1532.

8) Kawamura, M., Nagamo, K., 1984. Evidence for essential disulfide bonds in the β subunit of NaK ATPase. Biochem. Biophys. Acta. 774, 188-192.

9) Capasso, J.M., Hoving, S., Tal, D.M., Goldshleger, R., Karlish, S.J., 1992. Extensive digestion of NaK ATPase by specific and nonspecific proteases with preservation of cation occlusion sites. J. Biol. Chem. 267, 1150-1158.

10) Lutsenko, S., Kaplan, J.H., 1993. An essential role for the extracellular domain of the NaK ATPase β subunit in cation occlusion. Biochemistry N.Y. 32, 6737-6743.

11) Ussing, H.H., 1982. Volume regulation of frog skin epithelium, Acta Physiol. Scand. 114, 363-369.

12) Mount, D.B., Delpire, E., Gamba, G., Hall, A.E., Poch, E., Jr. Hoover, R.S., and Hebert, S.C., 1998. The electroneutral cation-chloride cotransporters. J. Exp. Biol. 201, 2091-2102.

13) Russell, J.M., 2000. Sodium potassium chloride cotransport, Physiol. Rev. 80, 211-276.

14) Isenvins, P., Jacoby, S.C., Payne, J.A., Forbush III, B., 1998. Comparison of NaKCl cotransporters. J. Biol. Chem. 273, 11295-11301.

15) Matthews, J.B., Smith, J.A., Mun, E.C., Sicklick, J.K., 1998. Osmotic regulation of intestinal epithelial NaKCl cotransport: role of Cl^- and F-actin. Am. J. Physiol. 274, C697-C706.

16) Jiang, G., Klein, J.D., O'Neill, W.C., 2001. Growth factors stimulate the NaK2Cl cotransporter NKCC1 through a novel Cl^- dependent mechanism, Am. J. Physiol, Cell Physiol 281, C1948-C1953.

17) Muzyamba, M.C., Cossins, A.R., Gibson, J.S., 1999. Regulation of NaK2Cl cotransport in Turkey red cells: the role of oxygen tension and protein phosphorylation, J. Physiol. (Lond) 517, 421-429.

18) Flatman, P.W., 2001. Deoxygenation stimulates NaK2Cl in ferret erythrocytes. J. Physiol. (Lond) 397, 471-487.

19) Lytle, C., 1997. Activation of the avian erythrocyte NaKCl cotransport protein by cell shrinkage, cAMP, fluoride and calcyculin A involves phosphorylation at common sites. J. Biol.Chem. 272, 15069-15077.

20) Darman, R.B., Flemmer, A., Forbush, B., 2001. Modulation of ion transport by direct targeting of protein phosphatase type I to the NaKCl cotransporter. J. Biol. Chem. 276, 34359-34362.

21) Moore-Hoon, M.L., Turner, R.J., 1998. Molecular and topological characterization of the rat parotid NaK2Cl cotransporter. Biochem Biophys Acta 1373, 261-269.

22) Sterling, D., and Casey, J.R., 2002. Bicarbonate transport proteins. Biochem. Cell Biol.80, 483-497.

23) Vandenberg, J.I., Metcalfe, J.C., and Grace, A.A., 1993. Intracellular pH recovery during respiratory acidosis in perfused hearts. Am. J. Physiol. Cell Physiol.266, C489-C497.

24) Preston, G.M., Carroll, T.P., Guggino, W.B., and Agre P., 1992. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science. 256, 385-387.

25) Agre, P., Sasaki, S., and Chrispeels, M.J., 1993. Aquaporins: a family of water channel proteins. Am. J. Physiol. 265, 461.

26) Agre, P., 2004. Nobel Lecture. Aquaporin water channels. Biosci. Rep. 24, 127-163.

27) Ishibashi, K., 2006. Aquaporin subfamily with unusual NPA boxes. Biochem. Biophys. Acta. 1758, 989-993.

28) Jentsch, T. J. , Steinmeyer, K., and Schwarz, G. 1990. Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes. Nature 348, 510–514

29) Chen, T.-Y., 2005. Structure and function of CLC channels. Annu. Rev. Physiol. 67, 809–839.

30) Jentsch, T.J., Neagoe, I., and Scheel, O., 2005. CLC chloride channels and transporters. Curr. Opin. Neurobiol. 15, 319-325.

31) Accardi, A., and Miller, C., 2004. Secondary active transport mediated by a prokaryotic homologue of ClC Cl^- channels. Nature 427, 803–807.

Functional Requirement of Sodium Potassium Chloride Co-transporter (NKCC) in the Success of Female *Amblyomma americanum* Tick Feeding. (Prepared for: Biochemical and Biophysical Research Communications).

Abstract:

The discovery of RNA interference (RNAi) has enabled the identification of several tick protective antigens that might be useful in future studies to design vaccines against ticks. Tick salivary glands are the major route of secretion of fluid, proteins and pathogens into the host. We report here the cloning of a partial cDNA fragment from the salivary glands of the lone star tick, *Amblyomma americanum*, that is homologous to sodium potassium chloride co-transporter isoforms 1 and 2 (NKCC1, 2). Confocal microscopy studies with a polyclonal antibody against the co-transporter localized the protein primarily to the basolateral membrane and intracellular regions and to a lesser extent to the apical membrane of cells of the salivary glands. We then performed RNAi studies on the co-transporter both *in vitro* and *in vivo* and the results obtained demonstrated reduction in the transcript levels of the co-transporter *in vitro* and reduced and slower feeding when ticks were injected with the double stranded RNA (dsRNA) to a tick salivary gland cDNA fragment of NKCC.

Key Words: Tick, salivary gland, RNAi, Co-transporter.

Introduction:

Ticks are known worldwide as arthropods that vector human and livestock disease-causing pathogens resulting in millions of dollars of loss to the livestock industry and many human health problems. Ticks achieve feeding success by overcoming host immune, inflammatory and pain producing responses. Their salivary gland secretions play a central role in the process of tick feeding [1], [2].

RNA interference (RNAi) [3], [4] is a simple and powerful reverse genetic tool that has simplified the tedious process of identifying tick protective antigens [5]. Ticks are relatively less studied as vectors of disease-causing pathogens when compared to their arthropod counterparts, mosquitoes. Ticks transmit the causative agents of several diseases including the Lyme disease pathogen, causing severe loss to livestock and humans. The tick genome has yet to be sequenced and hence the advent of RNAi has proven to be extremely useful for identifying and analyzing genes whose functions are unknown [6-13]. The ability to inject whole ticks with double stranded RNA (dsRNA) and monitoring for aberrant feeding, has simplified the identification of functionally required proteins for successful tick feeding.

An *Amblyomma americanum* female tick feeds for up to 14 days on the host with slow feeding for up to 11 days and a rapid feeding stage for two days before repleting and dropping off the host. In the rapid phase of feeding, ticks secrete large volumes of excess fluid to concentrate the blood meal [14]. Several fluid and ion permeabilizing channels are hypothesized to perform vital functions within the tick salivary glands and gut for this to be accomplished.

Sodium potassium chloride co-transporters (NKCC) are electroneutral symporters that have been identified in various organisms [15], [16]. They are integral membrane proteins with 12 transmembrane domains identified to function as pores that enable the outward migration of sodium, potassium and chloride ions in an electro-neutral fashion, though the stoichiometry has been found to vary between species [16], [17]. In secretory epithelia, however, the channel has been found to permit the movement of ions in the reverse direction with basolateral localization [18]. The co-transporters are an integral member of several transporting proteins that function co-operatively for a cell to maintain the membrane potential and volume. Here, we demonstrate cloning and sequencing of a partial cDNA fragment from female *A.americanum* salivary glands with sequence homology to NKCC1 and NKCC2. We tested the functional requirement of this protein within the ticks by employing RNAi. We demonstrate gene specific down regulation of NKCC at the mRNA level *in vitro*. Injecting dsRNAs specific to NKCC reduced the tick's ability to feed successfully when compared to the ticks that were injected with buffer alone. Confocal fluorescence microscopy studies using mouse polyclonal anti- co transporter antibody localized the protein to the basolateral membrane and intracellular regions. Apical localization was also observed, though to a lesser extent. The results obtained indicate the functional requirement of NKCC protein in ticks, and could be studied further to explore the possibility of using this protein, possibly in combination with other functionally important tick proteins, as a potential combinatorial anti-tick vaccine

Materials and methods:

Ticks:

Female *A.americanum* ticks were reared at the Oklahoma State University tick rearing facility according to the methods described by Patrick and Hair [19]. Ticks weighing 50-200mgs in weight were forcibly removed from sheep and salivary glands dissected within 3 hours.

Tick salivary glands:

Salivary glands 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. Dissected glands were used immediately for RNAi experiments.

Cloning and sequencing:

Total RNA was isolated from 6 female *A.americanum* tick salivary glands using RNAqueous RNA isolation kit (Ambion, Austin, TX, USA). The quality and concentration of the RNA was measured spectrophotometrically by reading the absorbance at 260nm and ratios of A260/230 and A260/280. cDNA was synthesized from the RNA by using SuperScript II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was subsequently used as a template in PCR using gene specific primers for NKCC and nSec-1 (control). The PCR fragment of about 200 bp was gel purified and cloned into TOPO TA PCR 2.0 vector (Invitrogen). Subsequent to transformation, the plasmid DNA was isolated using MiniPrep kit (Qiagen, Valencia, CA, USA). Database search (BLASTX) confirmed the sequence to be a partial cDNA fragment encoding NKCC.

Synthesis of dsRNA:

Plasmid DNA was linearized with restriction enzymes EcoRV and Hind III, both the restriction sites being downstream to the promoter used for transcription. The linearized plasmids were used as templates for *in vitro* transcription using MEGAscript SP6 and T7 kits (Ambion). The transcribed RNAs were pooled and incubated at 75^0C for 5 minutes followed by incubation at room temperature to let the complementary RNAs anneal to form dsRNA.

In vitro RNAi:

Salivary glands were dissected as described above. Left and right glands from 6 ticks were incubated with M199/MOPS buffer alone or 10µg dsRNA in M199/MOPS buffer solution. The incubation was carried out at 37^0C for 6 hours. Post incubation, RNA was isolated using an RNAqueous kit (Ambion). The RNA was reverse transcribed and used for quantitative real-time PCR analysis.

In vivo RNAi:

Groups of 20 ticks were used for *in vivo* studies. Unfed female *A.americanum* ticks were injected posteriorly on the dorsal surface with 1µg dsRNA in 1µl injection buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA) . Injections were done using a 31gauge needle. Control ticks were injected with injection buffer alone. Mortality and tick behaviour post injection were monitored by incubating the injected ticks over a 37^0C water bath for 18 hours. Ticks were then allowed to feed on sheep.

Confocal Microscopy:

Salivary glands from partially fed female ticks were dissected and fixed in a 4% solution of formaldehyde in phosphate buffered saline (PBS). Thin paraffin sections (4 micron

thickness) were made from these glands and mounted on glass slides (Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK, USA). The sections were immersed in xylene solution three times and washed each time using 80%, 95% and 100% ethanol respectively. After washing, the sections on slides were incubated with blocking solution for 1 hour (3% BSA in PBS). The slides were treated with primary mouse NKCC monoclonal antibody (U Iowa Hybridoma Core Facility, Iowa City, IA, USA) in 1:100 dilutions in PBS and incubated overnight. Following this, slides were washed with PBS and treated with 1: 1000 dilutions of secondary antibody (Alexa Fluor 633 goat anti mouse IgG) (Invitrogen) in PBS. After subsequent washes in PBS, localization patterns of NKCC within the glands were analyzed under a Leica TCSSP2 laser scanning confocal microscope (Leica, Bannockburn, IL, USA).

Quantitative Real-Time PCR:

Quantitative Real-Time PCR was performed using SYBR-GREEN chemistry in an AB7500 Real- Time PCR machine (Applied Biosystems, Foster City, CA, USA). Real time PCR data was analyzed using the $\Delta\Delta C_t$ method.

Statistics:

The bar graph presented represents the mean values with the error bars denoting the range of values. Student's t-test was used to confirm statistical significance of the Q-RT PCR results.

Results:

An EST project in *A.americanum* larvae [20] identified a contig with sequence identity to NKCC. We designed PCR primer sequences based on this sequence to amplify a partial cDNA fragment from female adult *A.americanum* salivary glands. The cDNA fragment

of 252 bp (GenBank Accession number: 851692) in length was cloned and was found to be 79% identical to *A.aegypti* NKCC isoform A and 73% identical to *D.melanogaster* NKCC isoforms A and B at the amino acid level (Figure 1A and 1B). To test the functional importance of NKCC in successful tick feeding, we used the cDNA fragment as a template for the synthesis of dsRNA. The synthesized dsRNA was either used for *in vitro* or for *in vivo* studies.

In vitro RNAi studies:

Salivary glands from 6 partially fed female *A.americanum* ticks were incubated with buffer alone or dsRNA of NKCC for 6 hrs. The amounts of the transcript levels of NKCC in the buffer incubated and dsRNA incubated glands were compared by using quantitative RT PCR after incubation. The level of NKCC transcript was reduced approximately 60% in the dsRNA incubated glands (Figure 2).

In vivo RNAi studies:

Unfed female *A.americanum* ticks were injected with dsRNA and allowed to feed on sheep for up to 14 days or forcibly removed from host animal when they were in the weight range of 50-200 mg. Three buffer injected and three dsRNA injected ticks were removed when they weighed 50-200 mg and their salivary glands dissected. The level of NKCC transcript in the salivary glands was compared between buffer injected and dsRNA injected ticks and the levels were not found to be significantly different as observed *in vitro* (data not shown). The results differ from prior *in vivo* applications of RNAi in ticks where successful silencing of the target gene in the salivary glands was achieved both *in vitro* and *in vivo* [6], [7]. This result may indicate that achieving RNAi mediated silencing within salivary glands *in vivo* might be target dependent i.e the

amount of dsRNA needed to silence a target gene might vary from gene to gene. Additionally, getting successful silencing within salivary glands might require increased amounts of dsRNA when compared to the amounts that might be required to silence the same genes in other tissues. This is consistent with results observed in mosquito salivary glands where higher amounts of dsRNA were required for effective silencing within the salivary glands than in other tissues [21].

Despite the inability to demonstrate *in vivo* silencing within salivary glands, we still observed aberrant feeding patterns in ticks injected with dsRNA. We observed that 60% of the ticks did not grow to repletion after 15 days and were loosely attached to the host (Figure 3A, 3B). We measured the width of 5 control and 5 dsRNA injected ticks as a measure of their growth rate on days 11, 12, 13 and 14 of their feeding, the days of maximum growth before repletion and dropping from the host. The dsRNA injected ticks did not increase in width while control buffer injected ticks increased in width and began repleting after 14 days post attachment to the host (Figure 3C)

Confocal microscopy:

Localization with an antibody to NKCC demonstrated reactivity primarily in basolateral membranes and intracellular regions resembling that seen in other secretary epithelia (Figure 4A and 4B). In rat thick ascending limbs, membrane expression of NKCC was minimal and the majority of localization was found in intracellular vesicles [22]. The same report also demonstrated involvement of vesicle associated membrane protein (VAMP), a vesicle associated Soluble N-ethylmaleimide sensitive fusion protein Attachment Receptor (SNARE) protein to be involved in the localization of NKCC to the apical membrane in response to cAMP stimulus. Prior studies of silencing VAMP protein

[8] within tick salivary glands decreased the levels of protein secretion from the dsRNA treated glands when compared to the buffer treated glands. Silencing both the VAMP and the NKCC might further disable the tick's ability to be a successful feeder. This approach of silencing more than one tick protein might be extremely valuable in the design of effective anti tick vaccines.

Discussion:

Ixodid ticks are arthropod vectors of human and livestock pathogens that cause severe illness and economic losses to the livestock industry. The relatively long feeding of a female ixodid tick on a host requires the tick to overcome numerous defense challenges from the host. It is largely understood that tick salivary glands and the secretion from them are very important for ticks to stay ahead of the host. The amount of blood ixodid ticks typically consume during the feeding process is an under estimate if the gain in weight alone is taken into consideration. This is because ticks excrete about one third to half of the total ingested fluid back into the host through their salivary glands thereby concentrating the blood meal [23]. Ticks almost certainly require the functions of numerous ion and water transporting channel proteins in their gut and salivary gland membranes for the process of secretion and digestion. Na K ATPase inhibitor ouabain has been found to significantly inhibit fluid secretion from tick salivary glands. A cDNA encoding for V-ATPase has been cloned from tick salivary glands and bafilomycin, its inhibitor, has also been found to inhibit fluid secretion though to a lesser extent [24]. In an effort to identify other proteins that might be functionally important for this process in ticks, we performed RNAi studies on NKCC protein.

Co-transporters, in general, are a functionally important group of transporting proteins that aid in the movement of ions and substances like glucose that are required by a cell.. The process of fluid secretion and maintaining membrane potential requires the participation of numerous membrane proteins. The roles performed by each member of this large group and the mechanism of regulation of this process are complex. It is widely accepted that Na K ATPase is the primary protein in the process of secretion that acts by

the direct hydrolysis of the energy reserve ATP, subsequently creating an inward directed Na^+ gradient. This gradient is required for the action of secondary active transporters like the NKCC protein which creates an outward directed Cl^- gradient. This then activates the opening of apically localized chloride channels that mediate the outward flow of Cl^- ions. Sodium enters the lumen either through apical channels or paracellularly and water migrates into the lumen by the mediation of aquaporins or through the paracellular route. We were able to show silencing of NKCC in salivary glands *in vitro* at the transcript level. However, *in vivo*, we were only able to see phenotypic differences in the ticks injected with the NKCC dsRNA and not able to demonstrate differences in the transcript level. We hypothesize that this observation could be due to 1) A typical female *A.americanum* tick feeds for up to 14 days and in this process gains over 100 fold increase in weight. An unfed tick weighs approximately 4 mg and when replete about 600 mg. This might dilute the dsRNA and the effective concentration within the tick would decrease as feeding progresses. 2) We tested the silencing of NKCC within the tick salivary glands and the dsRNA might not have permeated into the salivary glands. This is in contrast to similar studies performed earlier using histamine binding protein (HBP) and n-Sec1 dsRNAs [6], [9]. The phenotype observed might indicate the functional requirement of NKCC in other tissues apart from the salivary glands, most likely, the gut. Also, ticks that had grown to 50-200 mg were selected for measuring and comparing RNA levels between control and dsRNA injected ticks. Ticks that were able to grow to this weight range might have been the ones that were not susceptible to dsRNA and this might also explain the inability to demonstrate reduction in transcript levels. Large numbers of ticks are required if one needs to compare RNA levels in ticks that are in the

early stages of feeding or ones that weigh in the range of early feeding ticks. 3) Gene to gene variability and stability differences among the different mRNAs could also explain this observation. In a separate study in which we had soaked ticks in a dsRNA solution (unpublished results), we observed silencing of three different genes in whole ticks but were unable to show silencing within the salivary glands. The reasons for this lack of silencing within the salivary glands require further study. We also speculate that negative regulators of RNAi, as observed in *C.elegans* neurons, might be expressed within tick salivary glands which might negate the silencing [25] and [26]. To address this, we tried cloning the genes homologous to the genes in *C.elegans* without success. Also, any efforts of trying to demonstrate a direct involvement of NKCC in fluid secretion from tick salivary glands were unsuccessful. *In vitro* incubation of glands for several hours with dsRNA made them non functional to measure fluid secretion whereas glands dissected from ticks that were injected with dsRNA did not demonstrate any differences in fluid secretion levels. This might again be due to the factors discussed above. Localization studies performed using polyclonal antibodies against NKCC identified the protein to be present primarily in basolateral membrane and intracellular regions. This indicates that within tick salivary glands, NKCC might function as secondary active transporters allowing the migration of Na^+, Cl^- and K^+ ions into the cell which is comparable to the results obtained from work done on other secretary epithelia. NKCC appears to be concentrated in neck cells surrounding the salivary duct. Prior localization studies of other proteins performed within tick salivary glands (unpublished results) also showed the proteins to be localized more to the neck cells. Also, tick salivary glands exhibit strong auto fluorescence, especially in the neck cells at wavelengths in the range

of 400-550nm. The functions of neck cells and the reason for increased fluorescence intensities in those cells are unknown and present a challenging and interesting area to explore in future studies. Though RNAi is effective in ticks, there are still difficulties in getting successful silencing of target genes in all the tissues. Future work should be directed at understanding these differences. The results presented demonstrate the functional requirement of NKCC for ticks to feed normally, though silencing of the gene within the salivary glands was not observed *in vivo*. Further studies on NKCC may indicate silencing of the same gene in other tissues such as the gut, a tissue which is also necessary for the process of fluid elimination and concentration of the blood meal in feeding ixodid ticks.

References:

1) J.R. Sauer, J.L. McSwain, A.S. Bowman and R.C. Essenberg, Tick salivary gland physiology, *Annu. Rev. Entomol.* **40** (1995), pp. 245–267.

2) J.R. Sauer, R.C. Essenberg and A.S. Bowman, Salivary glands in ixodid ticks: control and mechanism of secretion, *J. Insect Physiol.* **46** (2000), pp. 1069-1078.

3) A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver and C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature* **391** (1998), pp. 806–811.

4) G.L. Sen, H.M. Blau, A brief history of RNAi: the silence of the genes, *FASEB J.* **20** (2006), pp. 1293-1299.

5) J. de la Fuente, C. Almazán, E.F. Blouin, V. Naranjo and K.M. Kocan, RNA interference screening in ticks for identification of protective antigens, *Parasitol. Res.* **96** (2005), pp. 137–141.

6) M.N. Aljamali, A.A. Bior, J.R. Sauer and R.C. Essenberg, RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*, *Insect Mol. Biol.* **12** (2003), pp. 299–305.

7) S. Narasimhan, R. R. Montgomery, K. DePonte, C. Tschudi, N. Marcantonio, J. F. Anderson, J. R. Sauer, M. Cappello, F. S. Kantor, and E. Fikrig, Disruption of *Ixodes scapularis* anticoagulation by using RNA interference, *Proc. Natl. Acad. Sci U.S.A*. **101** (2004), pp. 1141-1146.

8) S. Karim, V.G. Ramakrishnan, J.S. Tucker, R.C. Essenberg and J.R. Sauer, *Amblyomma americanum* salivary glands: double stranded RNA-mediated gene silencing

of synaptobrevin homologue and inhibition of PGE_2 stimulated protein secretion, *Insect Biochem. Mol. Biol.* **34** (2004), pp. 407–413.

9) S. Karim, V.G. Ramakrishnan, J.S. Tucker, R.C. Essenberg and J.R. Sauer, *Amblyomma americanum* salivary gland homolog of nSec1 is essential for saliva protein secretion, *Biochem Biophys Res Commun*. **324** (2004), pp.1256–1263.

10) V.G. Ramakrishnan, M.N. Aljamali, J.R. Sauer and R.C. Essenberg, Application of RNA interference in tick salivary gland research, *J. Biomol. Tech.* **16** (2005), pp. 297-305.

11) S. Karim, N.J. Miller, J. Valenzuela, J.R. Sauer and T.N. Mather, RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding, *Biochem. Biophys. Res. Commun.* **334** (2005), pp.1336-1342.

12) J. de la Fuente, C. Almazán, V. Naranjo, E.F. Blouin, J.M. Meyer and K.M. Kocan, Autocidal control of ticks by silencing of a single gene by RNA interference, *Biochem. Biophys. Res. Commun.* **344** (2006), pp. 332-338.

13) J. de la Fuente, C. Almazán, E. F. Blouin, V. Naranjo and K. M. Kocan, Reduction of tick infections with *Anaplasma marginale* and *A. phagocytophilum* by targeting the tick protective antigen subolesin, *Parasitol. Res.* (2006) (Epub. ahead of print).

14) J.R.Sauer and R.C. Essenberg, Role of cyclic nucleotides and calcium in controlling tick salivary gland function, *Am. Zool*. **24** (1984), pp. 217-227.

15) M. Haas and B. Forbush III, The Na-K-Cl cotransporter of secretary epithelia, *Annu. Rev. Physiol.* **62** (2000), pp. 515-534.

16) M. Haas, B. Forbush III, The Na-KCl cotransporters, *J. Bioenerg. Biomembr.* **30** (1998), pp. 161– 172.

17) J.M. Russell, Cation-coupled chloride influx in squid axon. Role of potassium and stoichiometry of the transport process, *J. Gen. Physiol.* **81** (1983), pp. 909– 925.

18) C. Lytle, J-C Xu, D. Biemesderfer, B. Forbush III, Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies, *Am. J. Physiol.* **269** (1995), pp. C1496– 1505.

19) C.D. Patrick and J.A. Hair, Laboratory rearing procedures and equipment for multi-host ticks (Acarina: Ixodidae), *J. Med. Entomol.* **12** (1975), pp. 389–390.

20) C.A. Hill, J.A. Gutierrez, Analysis of the expressed genome of the lone star tick, *Amblyomma americanum* (Acari: Ixodidae) using an expressed sequence tag approach, *Microb. Comp. Genomics* **5** (2000), pp. 89-101.

21) B. Boisson, J. Jacques, V. Choumet, E. Martin, J. Xu, K. Vernick, C. Bourgouin, Gene silencing in mosquito salivary glands by RNAi, *FEBS Letters* **580** (2005), pp. 1988-1992.

22) P.A. Ortiz, cAMP increases surface expression of NKCC2 in rat thick ascending limbs: role of VAMP, *Am J Physiol Renal Physiol*. **290** (2006), pp. F608-616.

23) J.R. Sauer and J.A. Hair, The quantity of blood ingested by the lone star tick (Acarina: Ixodidae), *Annals of the Entomological Society of America* **65** (1972), pp. 1065–1068.

24) J.L. McSwain. C. Luo, G.A. DeSilva, M.J. Palmer, J.S. Tucker, J.R. Sauer and R.C. Essenberg, Cloning and sequence of a gene for a homologue of the C subunit of the V-ATPase from the salivary gland of the tick *Amblyomma americanum* (L), *Insect Mol Biol.* **6** (1997), pp. 67-76.

25) S. Kennedy, D. Wang and G. Ruvkun, A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans, Nature* **427** (2004), pp. 645-649.

26) L. Timmons, Endogenous inhibitors of RNA interference in *Caenorhabditis elegans*, *Bioessays* **26** (2004), pp. 715-718.

Figure legends:

Figure 1:

A) Amino acid alignment of the cDNA fragment amplified using gene specific primers for NKCC from tick salivary glands to the NKCC from the mosquito *Aedes aegypti.* B) RT-PCR using tick salivary glands cDNA as template. Lane1: Low DNA Mass Ladder, lane 2: PCR amplified cDNA fragment (NKCC) and lane 3: PCR amplified control cDNA fragment (nSec1).

Figure 2:

In vitro RNAi within tick salivary glands: Transcript levels of NKCC compared between control and treated glands by performing qRTPCR.

Figure 3:

A) Picture of buffer injected ticks feeding on sheep after 14 days of infestation on the animal. B) Picture of NKCC dsRNA injected ticks feeding on sheep after 14 days of infestation on the animal. C) Average widths (mm) of 5 buffer injected and 5 NKCC dsRNA injected ticks measured as they feed on sheep from days 11 to days 14 post infestation.

Figure 4:

A) Immuno fluorescence observed using a confocal microscope with no secondary antibody. B) Immuno fluorescence observed using a confocal microscope after incubations with primary mouse NKCC monoclonal antibody and secondary Alexa Fluor 633 goat anti mouse IgG.

Figure 1A

```
A.a     249    LLRQYSTGASLIVMTLPMPRKGTCTAPMYMAWLEMLTKDMPPFLLVRGNQTSVLTFY
               LL+QYS  ASLIV+++P+PRKG  +AP+YM+WLEMLTKDMPPFLLVRGNQTSVLTFY
Ae.ae   970    LLQQYSKNASLIVLSMPIPRKGIVSAPLYMSWLEMLTKDMPPFLLVRGNQTSVLTFY
```

Score = 100 bits (248), Expect = 3e-20

Identities = 46/58 (79%), Positives = 54/58 (93%), Gaps = 0/58 (0%)

Frame = -1

A.a- Amblyomma americanum, Ae.ae- Aedes aegypti.

Figure 1B

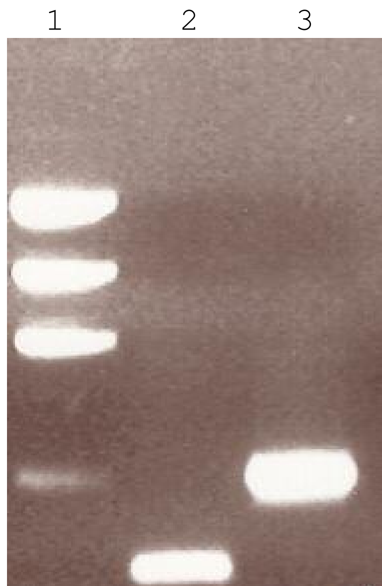


Figure 2

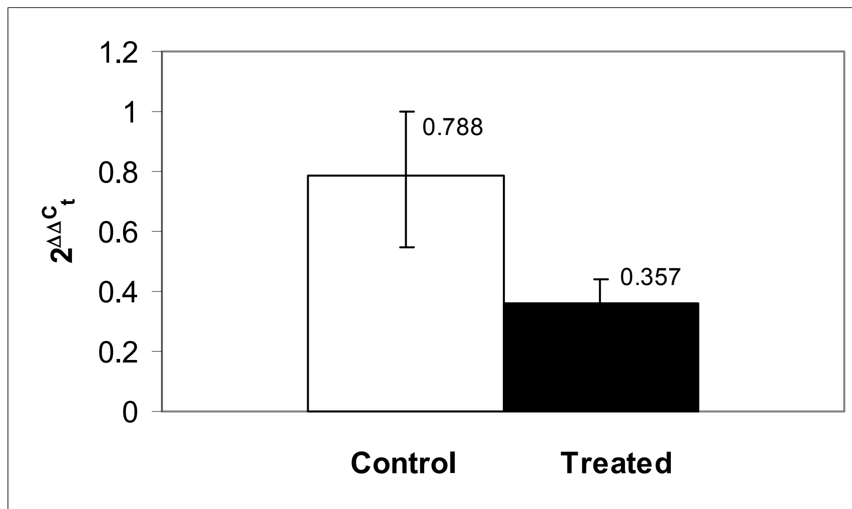


Figure 3A

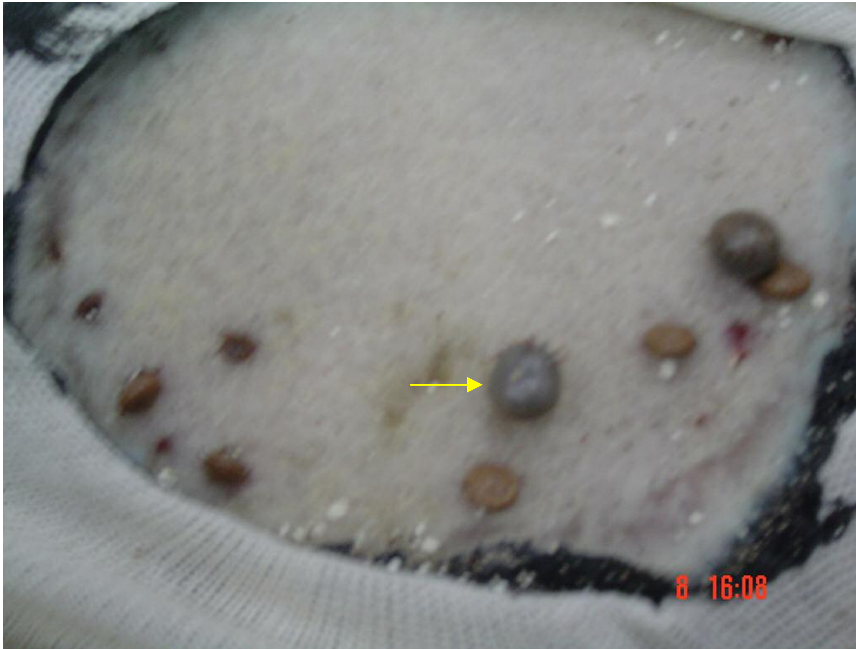


Figure 3B

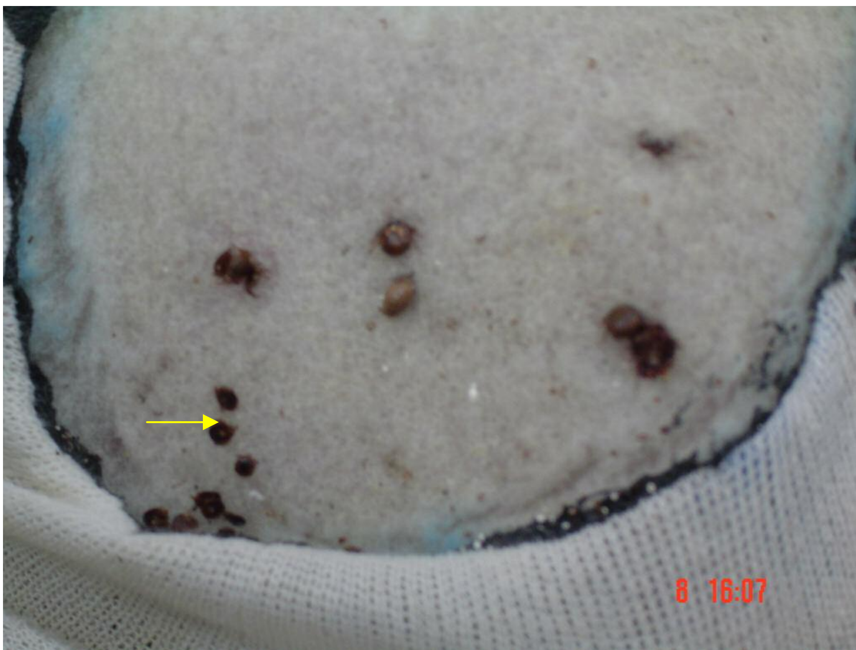


Figure 3C

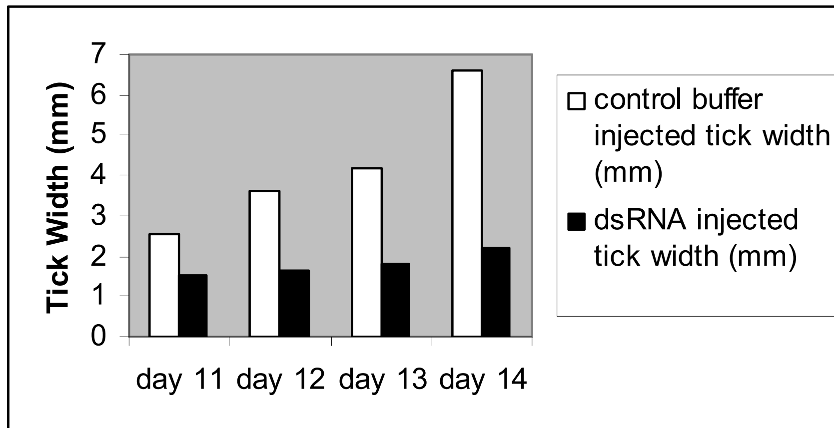


Figure 4A

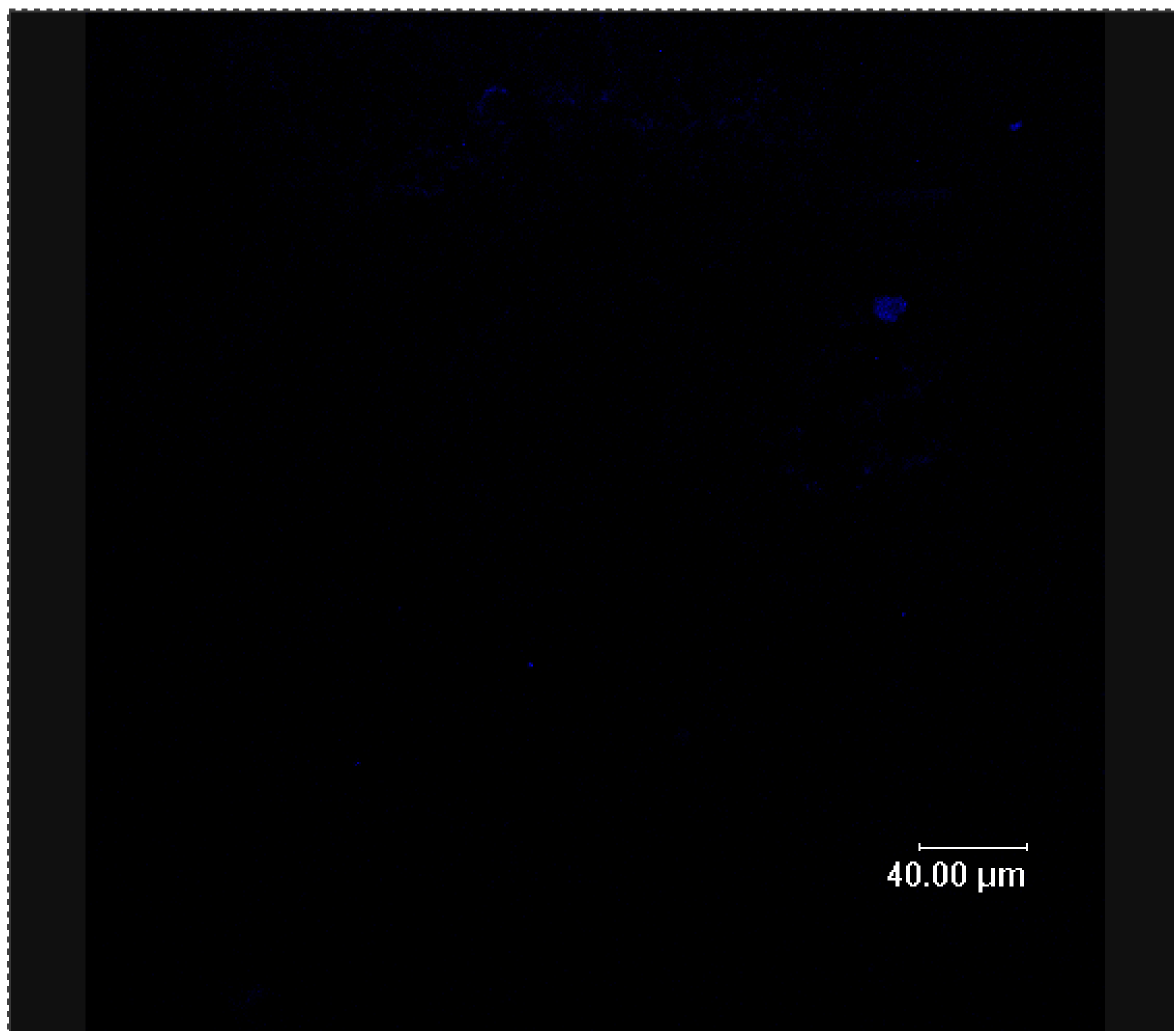
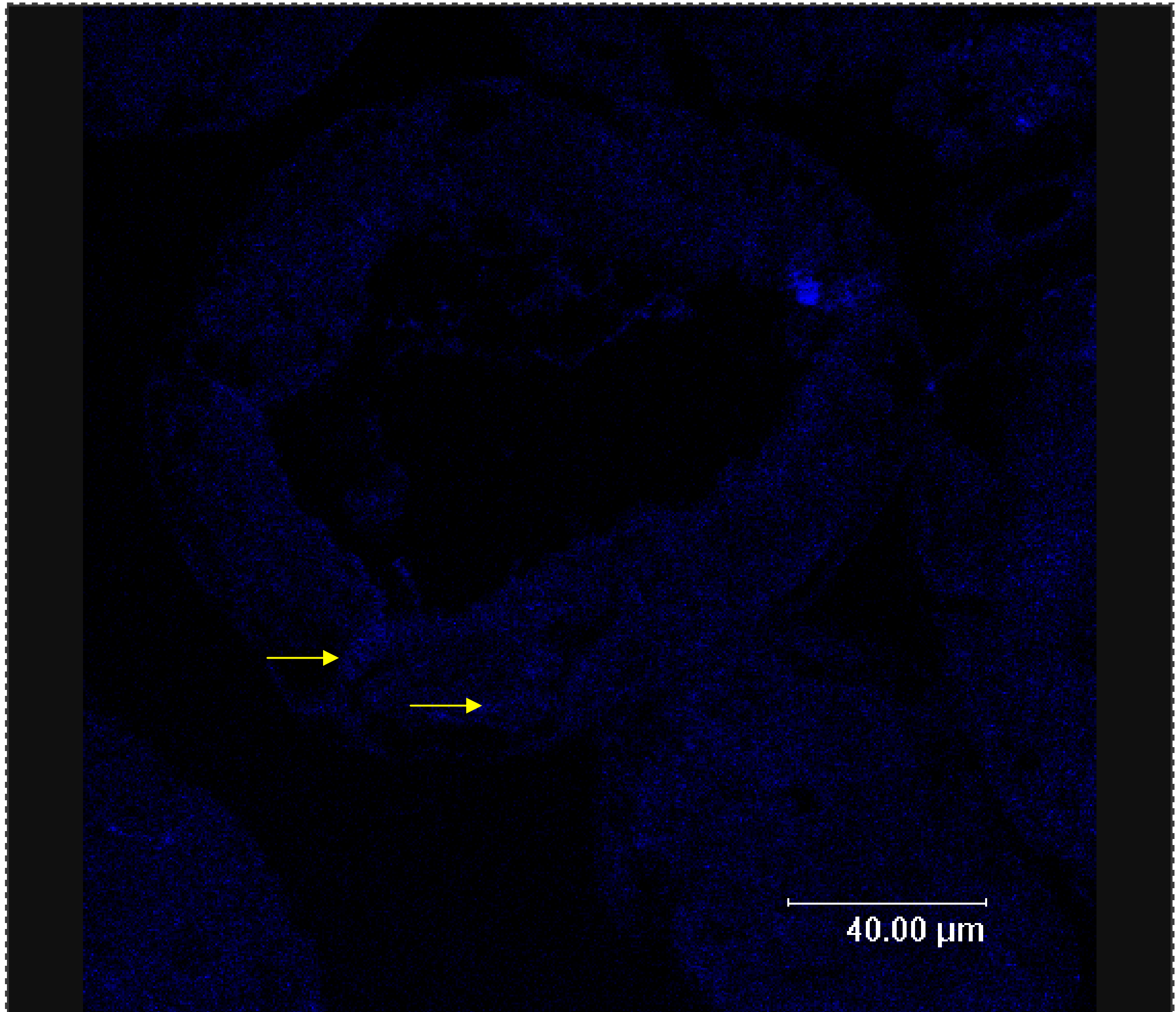


Figure 4B



RNA interference (RNAi) by soaking ticks in dsRNA solution- Application in understanding tick salivary secretions. (Prepared for: Biochemical and Biophysical Research Communications).

Abstract

Ticks are obligate blood-sucking arthropods and important vectors of pathogens that cause numerous human and livestock diseases that cause millions of dollars of loss to the livestock industry worldwide. Understanding the molecular mechanisms involved in ticks' ability to vector disease causing pathogens is therefore of great health and economic importance. Tick salivary glands are the major route of secretion of proteins, fluid and other factors that overcome host immune responses. Tick salivary glands are also the route for secretion of pathogens to the host. We have cloned and sequenced partial cDNAs of three salivary gland genes, aquaporin 7 (AQP-7), chloride channel 2 (ClC-2) and sodium bicarbonate co-transporter (NBC) that were hypothesized to be functionally important for salivary gland secretion and normal feeding in the lone star tick *Amblyomma americanum*. We then performed RNA interference (RNAi) on these genes by soaking unfed adult female *Amblyomma americanum* ticks in solutions containing dsRNA and then allowing the ticks to feed for up to 17 days on host animals. We demonstrate reduced and prolonged feeding behaviors and reduced levels of transcripts of the genes of interest in whole ticks but surprisingly not in the salivary glands as compared to untreated controls. This is the first report of successfully down-regulating target genes and causing aberrant tick feeding behavior after using the simplified technique of soaking ticks in solutions containing double stranded RNA (dsRNA) of physiologically relevant genes. Keywords: RNAi, ticks, AQP, NBC, ClC.

Introduction*:*

Ixodid ticks are unique in their feeding habits in comparison to other blood feeding arthropods and insects. They feed on hosts for relatively long periods by overcoming host immune, inflammatory and pain producing responses [1]. Additionally, ticks transmit numerous disease- causing agents to livestock and humans. Significant human diseases caused by ticks in the USA are Lyme disease and Rocky Mountain spotted fever, the former resulting in untold illness and suffering annually. Understanding the process of secretion from the ticks, or from the salivary glands, the major route of secretion and excretion of excess fluid, might provide substantial information about how ticks feed successfully and more importantly identify targets that could serve as potential anti-tick vaccines.

One aspect of tick feeding that is largely under-emphasized is the copious fluid a tick secretes back into the host via the salivary glands thereby concentrating the blood meal. An *Amblyomma americanum* female tick secretes approximately one third to one half of the fluid ingested back into the host to concentrate the blood meal [2]. Identification of the transport proteins that function in this process is therefore of great importance in better understanding the mechanism and regulation of fluid secretion from tick salivary glands. Aquaporin-7 (AQP-7) is a relatively newly discovered member of the growing list of aquaporins and has been identified to be specifically expressed in the adipose tissue and testes in mammals [3], [4]. AQP-7 is classified into the class of aquaglyceroporins [5] due to its permeability to glycerol and urea in addition to water. Chloride channels are considered to be important in the movement of chloride ions and in maintaining the membrane potential, pH regulation and cell volume control [6], [7]. Loss

of chloride channel-2 (ClC-2) function in mice leads to degeneration of testes and retina [8]. In addition, ClC-2 has been identified to be the channel responsible for the hyper polarization – activated chloride current in the salivary glands of mice [9]. Sodium bicarbonate co-transporter (NBC) is a member of the SLC4 family of bicarbonate transporters and has been identified in a variety of species [10], [11]. NBC functions to maintain and regulate pH. These proteins are more likely to be functionally required for ticks to secrete and feed as the process of secretion holds a central and crucial place in their ability to be voracious long term feeders.

RNAi [12], [13], a powerful reverse genetics tool has been successfully applied over a wide range of research areas including tick research [14- 20]. In this report, we utilized RNAi to silence genes encoding AQP7, ClC-2 and NBC by soaking unfed female *A. americanum* ticks in a solution containing dsRNA. The soaked ticks were then allowed to feed and we monitored for differences in the feeding patterns. We were able to demonstrate reduction in the transcript levels of the target genes, in addition to feeding abnormalities in ticks that were soaked in each of the three dsRNAs. Silencing was observed in whole ticks but surprisingly not within the salivary glands, the tissue from which the cDNA fragments were isolated. Despite this, this is the first report of successfully using RNAi in tick research by the simplified method of soaking ticks in a dsRNA solution. The method of soaking ticks in a dsRNA solution is much simple than injecting or capillary feeding which involve considerable time and labor.

Materials and methods:

Tick rearing:

A. americanum female ticks were reared at the Oklahoma State University tick rearing facility according to methods described by Patrick and Hair [21]. Adult females were fed on sheep along with half the number of males to enable mating and normal growth. Female ticks were either allowed to feed till repletion (up to 17 days) or removed from sheep when they weighed 50-200mg for PCR, cloning and comparing transcript levels post- RNAi. Repletion represents the end point of feeding of a female tick after which the tick voluntarily detaches from the host skin and starts the preparatory stages for laying eggs in case of adults, or molts into the next stage of feeding in case of larvae and nymphs.

Tick salivary glands:

In order to measure transcript levels of target genes within the salivary glands, female ticks were dissected within 4 hours after removal from the host. Tick salivary glands were dissected in ice-cold 100 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer containing 20 mM ethylene glycol bis-(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (pH 6.8) and used immediately for experiments.

Cloning of AQP-7, ClC-2 and NBC:

Total RNA was isolated from 8 salivary glands of partially fed female ticks using RNAqueous total RNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. cDNA was synthesized from total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using their standard protocol. The cDNA was then used as a template for PCR amplifying partial cDNA fragments of the

three target genes using gene specific primers. The PCR products were gel purified (Qiagen, Valencia, CA, USA) and cloned into a TOPO TA PCRII vector (Invitrogen). The cloned insert was transformed and plasmid DNA was isolated using Miniprep kit (Qiagen). Recombinant plasmids were sequenced at the OSU Protein/Nucleic Acid Resource Facility using an ABI 373A automated DNA sequencer and Prism® Ready Reaction Dyedeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, CT, USA). The sequences were then fed onto a database (BLASTX) to check for sequence identity at the amino acid level (www.ncbi.nih.gov/BLAST/).

Synthesis of dsRNA:

Recombinant plasmids were linearized using appropriate restriction enzymes with recognition sites downstream to the insert sequence. The linearized plasmids were the templates for *in vitro* transcription reactions, using the MEGAscript RNAi and SP6 kits (Ambion) following the manufacturer's protocol. The complementary single stranded RNAs were pooled together and incubated at 75^0C for 5 minutes and allowed to anneal at room temperature.

Soaking ticks in dsRNA containing solution:

Groups of 15 female unfed *A.americanum* ticks were used for each target gene and control experiments. Before soaking the ticks in a dsRNA solution, a few drops of acetone were applied to the posterior end of the dorsal surface of the ticks. Following this they were incubated in a solution of M199/Mops containing 0.1% DMSO alone or with 0.1% DMSO and 10 µg of the dsRNA for the target gene. The incubations were performed in a 0.5 ml microfuge tube, with 5 ticks in each tube, over a 37^0C water bath

for 90 minutes. After incubation, ticks were removed from the solution and infested on sheep along with untreated male ticks.

RNA isolation from whole ticks:

Two ticks from each group were removed from host sheep after approximately 12 days of feeding (50-200mg) and used for RNA isolation. Whole ticks were ground in liquid nitrogen using a mortar and pestle. The tissue samples were then homogenized and used for RNA isolation using RNAqueous (Ambion) following the manufacturer's guidelines. Two more female ticks from each group were removed from the host sheep after 12 days and used for dissection of salivary glands. Total RNA was isolated from the salivary glands as detailed earlier. cDNA synthesis was also performed as mentioned earlier.

Real Time PCR:

cDNA synthesized from each of the groups of ticks were used for real time PCR assays using the SYBR Green chemistry in an AB 7500 real time PCR machine (Applied Biosystems, Foster City, CA, USA) and gene specific primers.

Other methods:

Replete ticks were weighed and placed in individual vials and allowed to lay eggs. The vials were placed in a humid chamber at room temperature. After the replete ticks completed the process of laying eggs (up to 2 weeks), the ticks were removed from the vial and the weights of the egg masses were measured. The ratio of egg mass: replete weight was noted [22].

Statistics:

The bar graph presented represents the mean values with the error bars denoting the range of values. Student's t-test was used to confirm statistical significance of the Q-RT PCR results.

Results:

Cloning of AQP-7, ClC-2 and NBC:

Partial cDNA fragments of the three genes were cloned from tick salivary glands. The cloned fragment of AQP-7 (GenBank Accession number) was 50% identical to the AQP-7 from the African grass rat *Arvicanthis niloticus*. The ClC-2 fragment was 68% identical to the mosquito *Aedes aegypti* ClC-2 (GenBank Accession number) while the NBC (GenBank Accession number) fragment showed 37% identity to the NBC from the purple sea urchin *Strongylocentrotus purpuratus.* (Figure 1A, 1B and 1C).

RNAi Experiments:

Transcript levels:

The RNA levels of the target genes were compared between the untreated and dsRNA soaked (treated) ticks, both within whole ticks and within tick salivary glands. We observed RNAi mediated knock down of expression of the target gene for each of the three genes under investigation in whole ticks, though the degree of knock down was different for each gene. The transcript levels of AQP-7 decreased by 80%, ClC-2 decreased by 50% and the NBC decreased by 70% in response to their respective dsRNAs. All values were calibrated with the expression levels of an endogenous control (Figure 2).

Aberrant feeding patterns:

Untreated and dsRNA treated ticks were fed on the same sheep in adjacent cells. Each of the three groups of treated ticks showed no mortality and attached firmly onto the host animal. Ticks were allowed to grow to repletion and the time required for repletion was noted (Figure 2). All of the control ticks repleted by 14 days while some dsRNA

treated ticks took as long as 18 days to replete. The weights of ticks that grew to repletion were measured (Figures 3). The differences between the average weights of control and any of the three dsRNA treated ticks were not significantly different, though each of the dsRNA treated group of ticks had average weights lower than the control ticks. However, the minimum weight of a replete tick in the treated group was 32% lower than the minimum weight of a replete tick in the control group for the ClC-2 and NBC dsRNA treated tick groups while this difference was even more significant (54%) in the AQP-7 dsRNA treated ticks. This might be due to tick to tick variability. We then tested the effect of silencing each one of these genes on tick reproduction. Replete ticks were allowed to lay eggs, and the ratios of egg mass/replete weights were calculated (Figure 4). RNAi mediated knock down of each of the three targeted genes reduced the egg mass/ repletion weights ratio by about 9% (NBC and ClC-2 dsRNA) and 14% (AQP-7 dsRNA). RNAi mediated silencing of all the three genes in one experiment might result in a more adverse phenotype and hence hamper tick feeding.

Discussion:

Ticks as vectors of pathogens have drawn considerable interest in understanding how the pathogens survive within the tick and identifying proteins that are expressed within the ticks that might play an important role in the pathogen's ability to survive, grow and be transmitted to a vertebrate host [23- 26]. While this yields valuable information about one of the several pathogens a tick could transmit, studying the process of secretion from the ticks might provide information that could indicate a direction for stopping or reducing tick feeding and possibly pathogen transmission. With this rationale in mind, we targeted genes encoding proteins that are hypothesized to be important in the process of fluid secretion from tick salivary glands.

Ticks secrete copious amounts of fluid back into the host thereby concentrating the blood meal which facilitates tick feeding for relatively long periods on the host. Several transport proteins must function in tandem for secretion to proceed normally. Past research has identified considerable information about control of tick salivary gland secretion but, most of the ion and water transport proteins have yet to be identified [27]. In addition to secretion, ion and water channeling proteins function in cell volume control, regulating pH and maintaining membrane potential and are expected to be necessary in other tissues of the tick.

In an effort to determine the identity of potential ion and water transport proteins in the salivary glands, we cloned cDNA fragments for AQP-7, ClC-2 and NBC. AQP-7 is the first known aquaporin to be cloned from the ticks. Tick saliva is alkaline (pH 9.4) and the Na^+ and Cl^- stoichiometry do not balance with the Na^+ ion concentration slightly higher than the Cl^- ion concentration [28]. This observation suggests that, the anion that

balances excess Na^+ may be HCO_3^- and hence our interest in NBC. ClC-2 is a ubiquitously expressed chloride channel protein that helps maintain optimal intra cellular and extra cellular chloride concentrations in various secretary epithelia. We therefore picked these genes for RNAi studies.

RNAi has been successfully employed in ticks for both *in vitro* and *in vivo* research. *In vivo* studies were performed by injecting long dsRNAs into unfed ticks and then infesting them on the host to feed. In this study, we have performed the RNAi experiments by soaking the ticks in a dsRNA solution containing DMSO. Before soaking them in this solution, we applied a few drops of acetone to remove the waxy coating on the cuticle. Soaking experiments have been performed successfully in the nematode *C. elegans*, an organism that does not have a thick cuticle [29]. Ixodid ticks have a hard tough cuticle and so to facilitate entry of dsRNA into the ticks, we used the membrane permeabilizer DMSO. Tick survival in a solution containing 0.1% DMSO was tested at different time points beginning at 30 minutes (data not shown). Ticks survived for two hours in the solution with no marked differences in behavior when compared to non treated ticks. Once infested on the host, experimental ticks were compared with groups of ticks that were incubated with the solution containing the same constituents except for dsRNA. All the genes studied, when silenced by RNAi, caused aberrant tick feeding patterns including slower feeding and a slightly lower repletion weight as compared to the non-dsRNA treated ticks. However, all the ticks fed to repletion and there was no tick mortality observed. The data indicate the feasibility of soaking ticks in solutions containing dsRNA and demonstrate the functional importance of the three proteins targeted in this study. Although, the dsRNA treated ticks demonstrated aberrant feeding

as compared to controls, the changes were small. This could indicate that additional proteins are expressed in ticks that may complement the functions of the targeted proteins. Also, since these proteins are hypothesized to be functionally important, there might be isoforms that are expressed in tick cells, as observed in other secretary epithelia, which might replace the loss of the targeted proteins and hence the reason for the small effect on tick feeding. The reduction in the transcript levels were observed only in whole ticks and not in tick salivary glands although the targeted cDNA fragments were isolated from salivary glands. Hence, the difference in feeding patterns might be significantly different if we were able to achieve successful silencing of the genes in the salivary glands. As already observed in prior RNAi studies in mosquitoes [30], achieving effective silencing within tick salivary glands might require higher amounts of dsRNA. In one earlier study of RNAi in ticks, silencing of the target gene was not observed within salivary glands *in vivo* indicating a similar possibility as observed in mosquitoes, though in several others, salivary gland expression could be reduced. Performing RNAi studies in ticks by injecting and soaking the same dsRNAs will indicate the effectiveness of one method over the other. Immuno fluorescence studies of these proteins within the tick salivary glands might be an useful experiment to better understand their functions based on their patterns of localization within the salivary gland acini.

The results presented here do not offer direct evidence of the functions performed by these proteins in ticks, the reasons for this is discussed in detail (manuscript submitted). However, the ability of gene silencing through RNAi by soaking ticks presents a convenient way of performing such studies, especially for large scale studies. Such large scale studies, certainly, will quicken the process of understanding tick biology

and direct us towards identifying one or a combination of proteins that might function in biologically controlling ticks and their pathogenic effects.

References:

1) J.R. Sauer, J.L. McSwain, A.S. Bowman and R.C. Essenberg, Tick salivary gland physiology, *Annu. Rev. Entomol.* **40** (1995), pp. 245–267.

2) J.R. Sauer and R.C. Essenberg, Role of cyclic nucleotides and calcium in controlling tick salivary gland function, *Am. Zool*. **24** (1984), pp. 217-227.

3) H. Kuriyama, S. Kawamoto, N. Ishida, I. Ohno, S. Mita, Y. Matsuzawa, K. Matsubara and K. Okubo, Molecular Cloning and Expression of a Novel Human Aquaporin from Adipose Tissue with Glycerol Permeability, *Biochem. Biophys. Res. Commun*. **241** (1997), pp. 53-58

4) K. Ishibashi, M. Kuwahara, Y. Gu, Y. Kageyama, A. Tohsaka, F. Suzuki, F. Marumo and S. Sasaki, Cloning and Functional Expression of a New Water Channel Abundantly Expressed in the Testis Permeable to Water, Glycerol, and Urea, *J. Biol. Chem*. **272** (1997), pp. 20782-20786.

5) P. Agre, Nobel Lecture. Aquaporin water channels, *Biosci. Rep*. **24** (2004), pp. 127-163.

6) T.J. Jentsch, V. Stein, F. Weinreich and A.A. Zdebik, Molecular structure and physiological function of chloride channels, *Physiol. Rev.* **8**2 (2002), pp. 503-568.

7) A. Thiemann, S. Gründer, M. Pusch and T.J. Jentsch, A chloride channel widely expressed in epithelial and non-epithelial cells, *Nature* **356** (1992), pp. 57–60.

8) M.R. Bösl, V. Stein, C. Hübner, A.A. Zdebik, S.E. Jordt, A.K. Mukhophadhyay, M.S. Davidoff, A.F. Holstein, and T.J. Jentsch, Male germ cells and photoreceptors, both depending on close cell-cell interactions, degenerate upon ClC-2 Cl^--channel disruption, *EMBO J* **20** (2001), pp. 1289-1299.

9) A. Dinudom, J.A.Young and D.I. Cook, Na^+ and Cl^- conductances are controlled by cytosolic Cl^- concentration in the intralobular duct cells of mouse mandibular glands, *J Membr Biol* **135** (1993), pp. 289-295.

10) W.F. Boron and E.L. Boulpaep, The electrogenic Na/HCO3 cotransporter, *Kidney Int.* **36** (1989), pp. 392-402.

11) D. Sterling and J.R. Casey, Bicarbonate transport proteins, *Biochem Cell Biol.* **80** (2002), pp. 483-497.

12) A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver and C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature* **391** (1998), pp. 806–811.

13) G.L. Sen, H.M. Blau, A brief history of RNAi: the silence of the genes, *FASEB J.* **20** (2006), pp. 1293-1299.

14) M.N. Aljamali, A.A. Bior, J.R. Sauer and R.C. Essenberg, RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*, *Insect Mol. Biol.* **12** (2003), pp. 299–305.

15) S. Narasimhan, R. R. Montgomery, K. DePonte, C. Tschudi, N. Marcantonio, J. F. Anderson, J. R. Sauer, M. Cappello, F. S. Kantor, and E. Fikrig, Disruption of *Ixodes scapularis* anticoagulation by using RNA interference, *Proc. Natl. Acad. Sci U.S.A*. **101** (2004), pp. 1141-1146.

16) S. Karim, V.G. Ramakrishnan, J.S. Tucker, R.C. Essenberg and J.R. Sauer, *Amblyomma americanum* salivary glands: double stranded RNA-mediated gene silencing of synaptobrevin homologue and inhibition of PGE_2 stimulated protein secretion, *Insect Biochem. Mol. Biol.* **34** (2004), pp. 407–413.

18) S. Karim, V.G. Ramakrishnan, J.S. Tucker, R.C. Essenberg and J.R. Sauer, *Amblyomma americanum* salivary gland homolog of nSec1 is essential for saliva protein secretion, *Biochem Biophys Res Commun*. **324** (2004), pp.1256–1263.

19) V.G. Ramakrishnan, M.N. Aljamali, J.R. Sauer and R.C. Essenberg, Application of RNA interference in tick salivary gland research, *J. Biomol. Tech.* **16** (2005), pp. 297-305.

20) S. Karim, N.J. Miller, J. Valenzuela, J.R. Sauer and T.N. Mather, RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding, *Biochem. Biophys. Res. Commun.* **334** (2005), pp.1336-1342.

21) C.D. Patrick and J.A. Hair, Laboratory rearing procedures and equipment for multi-host ticks (Acarina: Ixodidae), *J. Med. Entomol.* **12** (1975), pp. 389–390.

22) R.O. Drummond and T.M. Whetstone, Oviposition of the Gulf Coast Tick, *J. Econ. Entomol.* **63** (1970), pp. 1547-1551.

23) U. Pal, X. Li, T. Wang, R. Montgomery, N. Ramamoorthi, A. deSilva, F. Bao, X. Yang, M. Pypaert, D. Pradhan, F.S. Kantor, S. Telford, J.F. Anderson and E. Fikrig, TROSPA, an Ixodes scapularis Receptor for Borrelia burgdorferi**,** *Cell*, **119** (2004), pp. 457-468.

24) N. Ramamoorthi, S. Narasimhan, U. Pal, F. Bao, X. F. Yang, D. Fish, J. Anguita, M. V. Norgard, F. S. Kantor, J. F. Anderson, R. A. Koski and E. Fikrig, *Nature*, **436** (2005), pp. 573-577.

25) J. de la Fuente, C. Almazán, E. F. Blouin, V. Naranjo and K. M. Kocan, Reduction of tick infections with *Anaplasma marginale* and *A. phagocytophilum* by targeting the tick protective antigen subolesin, *Parasitol. Res.* (2006) (Epub. ahead of print).

26) K.M. Kocan, J. de la Fuente, E. F. Blouin and J.C. Garcia-Garcia, Anaplasma marginale (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia**,** *Parasitol,* **129** (2004), pp. 5285-5300.

27) J.R. Sauer, R.C. Essenberg and A.S. Bowman, Salivary glands in ixodid ticks: control and mechanism of secretion, *J. Insect Physiol.* **46** (2000), pp. 1069-1078.

28) P.E. Guenther, D.M. Barker and J.R. Sauer, Sodium, chloride and water balance in feeding gulf coast tick, *Amblyomma maculatum* (KOCH), *Ann. Entomol. Soc. Am* **73** (1980), PP. 485-488.

29) H. Tabara, A. Grishok and C.C. Mello, RNAi in C. elegans: soaking in the genome sequence, *Science* **282** (1998), pp. 430-431.

30) B. Boisson, J. Jacques, V. Choumet, E. Martin, J. Xu, K. Vernick, C. Bourgouin, Gene silencing in mosquito salivary glands by RNAi, *FEBS Letters* **580** (2005), pp. 1988-1992.

Figure legends

Figure 1: Sequence identity of cloned cDNA fragments of A) AQP-7, B) NBC and C) ClC-2 from tick salivary glands.

Figure 2: RNAi mediated silencing as measured by qRT-PCR of A) AQP-7 transcript in AQP-7 dsRNA soaked ticks. B) NBC transcript in NBC dsRNA soaked ticks and C) ClC-2 transcript in ClC-2 dsRNA soaked ticks.

Figure 3: Days of repletion of A) Control and dsRNA AQP-7 soaked ticks. B) Control and dsRNA NBC soaked ticks and C) Control and dsRNA ClC-2 soaked ticks.

Figure 4: Replete tick weights of A) Control ticks and AQP-7 dsRNA soaked ticks. Average weight of control ticks: 670mg, minimum tick weight: 440mg. Average weight of AQP-7 dsRNA soaked ticks: 613mg, minimum tick weight: 206mg. B) Control ticks and NBC dsRNA soaked ticks. Average weight of control ticks: 807mg, minimum tick weight: 632mg. Average weight of NBC dsRNA soaked ticks: 714mg, minimum tick weight: 434mg. C) Control ticks and ClC-2 dsRNA soaked ticks. Average weight of control ticks: 807mg, minimum tick weight: 706mg. Average weight of AQP-7 dsRNA soaked ticks: 781mg, minimum tick weight: 491mg.

Figure 5: Egg mass/ replete weights of control, AQP-7 dsRNA treated, NBC dsRNA treated and ClC-2 dsRNA treated groups of ticks.

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Score = 36.6 bits (83), Expect = 0.37
Identities = 16/32 (50%), Positives = 22/32 (68%), Gaps = 0/32 (0%)
Frame = +3

Query -   *Amblyomma americanum*
Subject- *Arvicanthis niloticus*

Figure 1A

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Query  194   LVNGNLIKVPMEKYKEEQELPQINITEQLSQTGLWQAI  307
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Score = 73.6 bits (179), Expect = 5e-12
Identities = 37/98 (37%), Positives = 64/98 (65%), Gaps = 7/98 (7%)
Frame = +2

Query: *Amblyomma americanum*
Subject: *Strongylocentrotus purpuratus*

Figure 1B

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Subject  862  ISKKVQLPRERVIDMSAEDQKAWELEEMAKPIDVENLHIDP

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              APFQLVERT+++KVHS+FS
              APFQLVERTSILKVHSLFS 921

Query  267    MLGLQHAYVTAIGRLIGVVALKELRKAIEKMNSG  368
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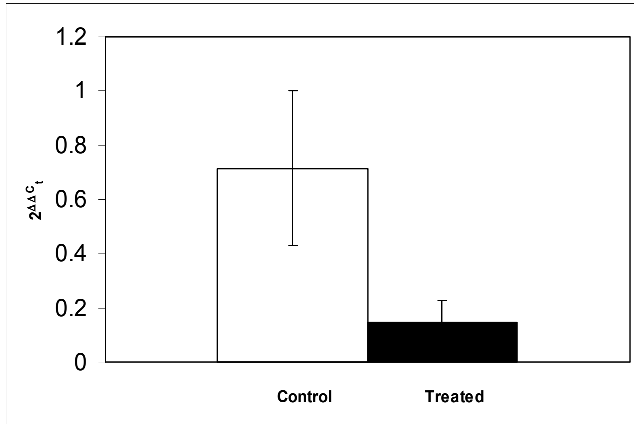
Score = 36.6 bits (83), Expect = 0.37
Identities = 16/32 (50%), Positives = 22/32 (68%), Gaps = 0/32
0%)Frame = +3

Query- *Amblyomma americanum*
Subject- *Aedes aegypti*

Figure 1C



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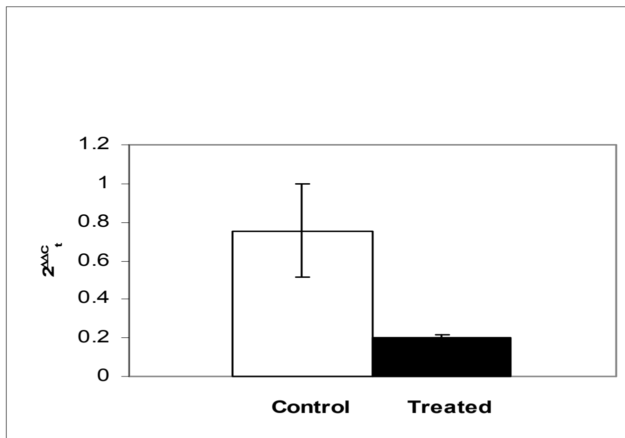


Figure 2B

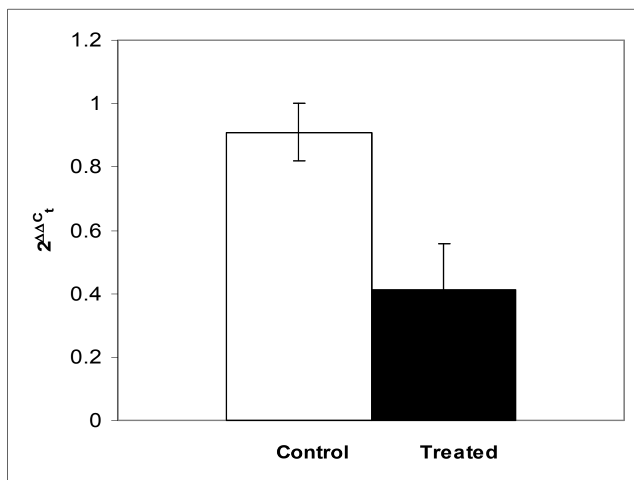


Figure 2C

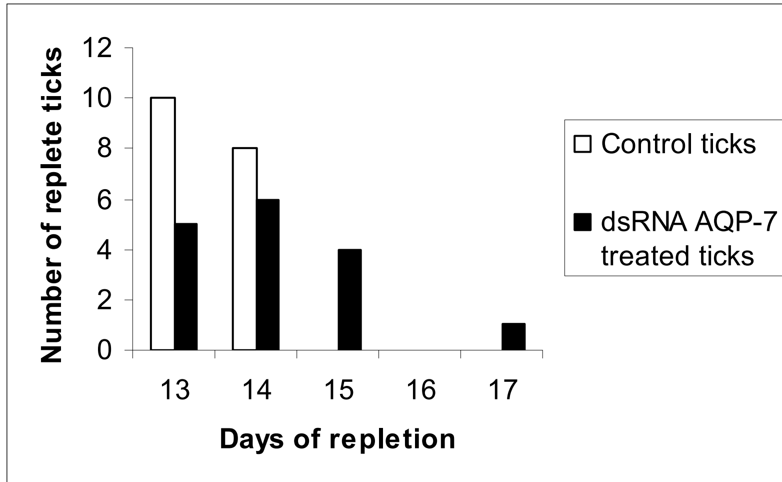
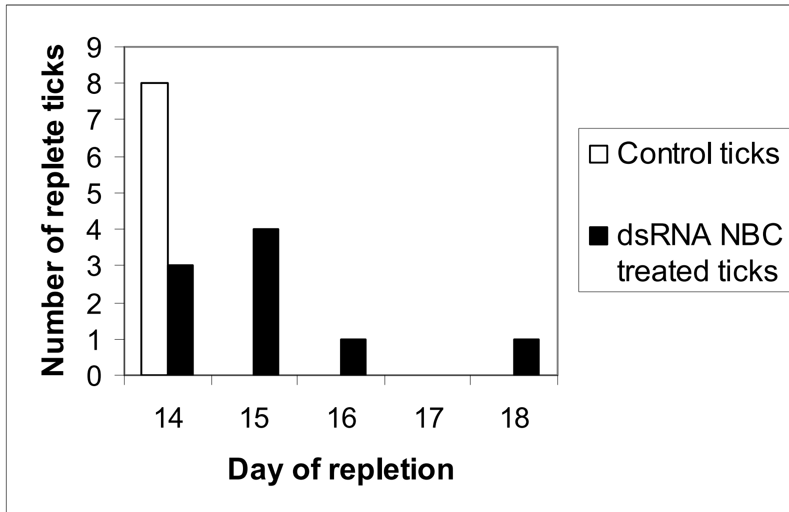


Figure 3A



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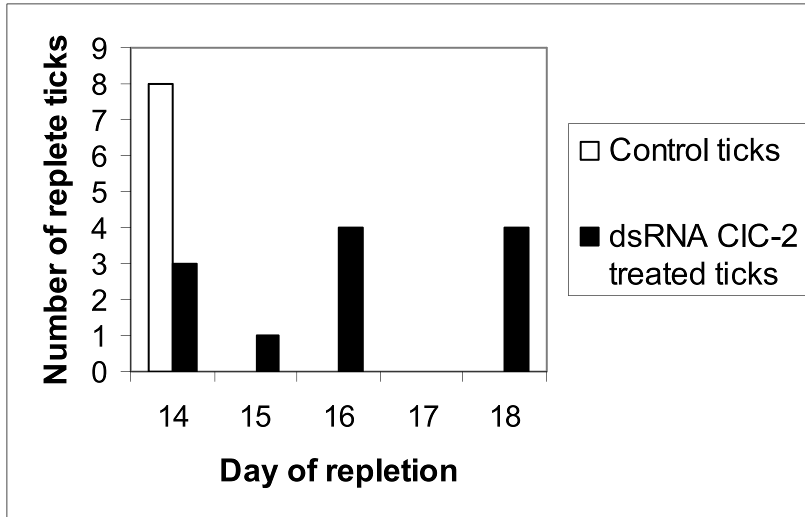

Figure 3C

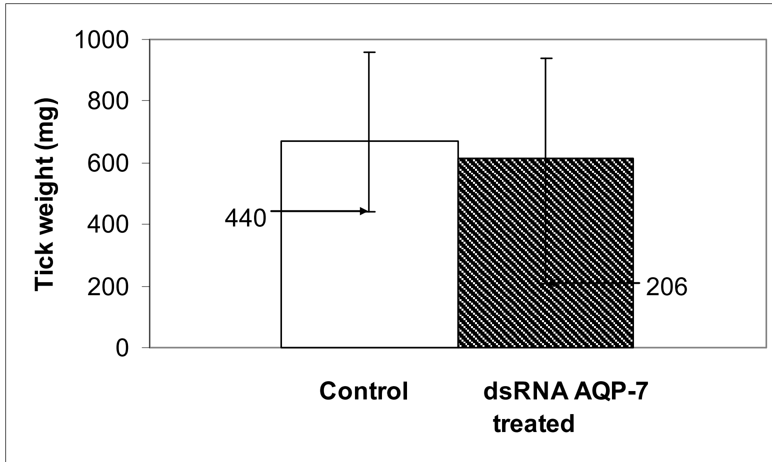

Figure 4A

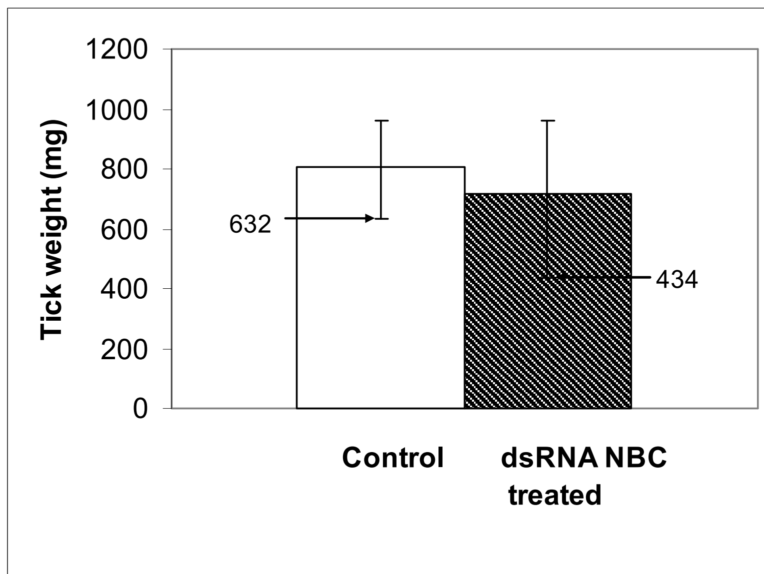

Figure 4B

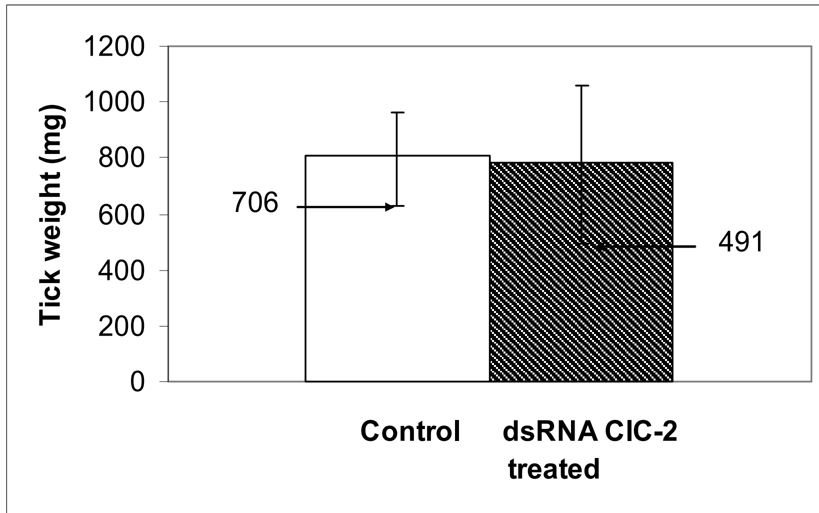

Figure 4C

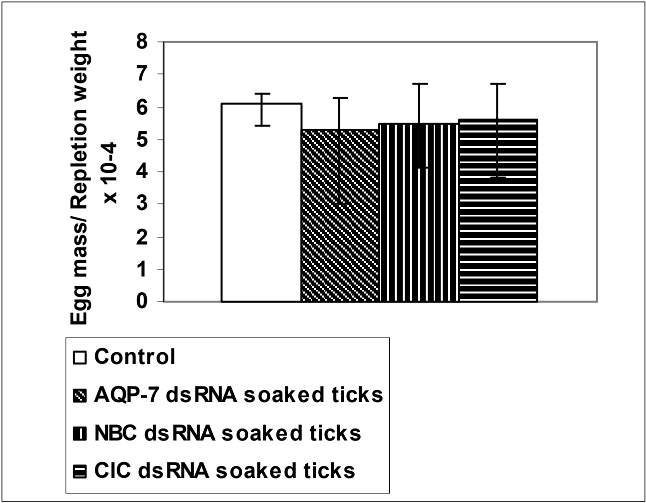

Figure 5

VITA

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