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Identification and characterization of proteins in the *Amblyomma americanum* tick cement cone

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Abstract

The adaptation of hard ticks to feed for long periods is facilitated by the cement cone, which securely anchors the tick mouthparts onto host skin and protects the tick from being groomed off by the host. Thus, preventing tick cement deposition is an attractive target for the development of innovative tick control. We used LC-MS/MS sequencing to identify 160 *Amblyomma americanum* tick cement proteins that include glycine-rich proteins (GRP, 19%), protease inhibitors (12%), proteins of unknown function (11%), mucin (4%), detoxification, storage, and lipocalin at 1% each, and housekeeping proteins (50%). Spatiotemporal transcription analysis showing mRNA expression in multiple tick organs and transcript abundance increasing with feeding suggest that selected GRPs ($n=13$) regulate multiple tick feeding functions, being classified as constitutively expressed (CE), feeding induced (FI), and up-regulated with feeding (UR). We show that transcription of CE GRPs is likely under the control of tick appetite associated factors in that mRNA abundance increased several thousand fold in 1 week old adult ticks, the time period that coincides with tick attainment of appetite. Given the high number of targets, we synthesized and injected unfed ticks with combinatorial (co) double stranded (ds)RNA and disrupted GRP mRNA in clusters according to similar transcription patterns: CE ($n=3$), FI, ($n=4$), and UR ($n=6$) to streamline the work. Our data suggest that CE and FI GRPs are important for maintenance of the tick feeding site in that reddening and subsequent bleeding were observed around the mouthparts

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of CE and FI GRP co-dsRNA injected ticks during feeding. Furthermore, although not significantly different, indices for blood meal size and fecundity were apparently reduced in FI and UR ticks. We discuss our data with reference to *A. americanum* tick feeding physiology.

Graphical abstract

Keywords

Amblyomma americanum; Tick cement cone; Tick attachment; LC-MS/MS sequencing; Combinatorial RNAi silencing

1. Introduction

Amblyomma americanum is among the hard tick species of medical and veterinary importance. At the time of manuscript preparation, the United States Centers for Disease Control and Prevention (CDC) listed 15 human tick-borne diseases (TBD), five of which agents were transmitted by *A. americanum*. These TBD agents include *Borrelia lonestari*, the previously suspected causative agent of southern tick-associated rash illness (STARI) (James et al., 2001; More et al., 2003; Wormser et al., 2005); *Rickettsia amblyommii*, the causative agent of rickettsiosis of the spotted fever group (Varela et al., 2004; Apperson et al., 2008; Stramdahl et al., 2008; Barret et al., 2014); *Francisella tularensis*, the causative agent of tularemia in humans (Childs and Paddock, 2002; Mani et al., 2015); and *Ehrlichia chaffensis* and *Ehrlichia ewingii*, the causative agents of human ehrlichiosis (Anderson et al., 1993; Buller et al., 1999). Recently, Savage et al. (2013) pointed to the possible role of *A. americanum* in transmission of Heartland virus, previously reported as pathogenic for humans (McMullan et al., 2012; Godsey et al., 2016). In veterinary health, *A. americanum* is a competent vector for *Cytauxzoon felis* and *Theileria cervi*, affecting domestic cats and white-tailed deer, respectively (Childs and Paddock, 2002; Shock et al., 2014).

With the exception of a few cases in which TBD infections occurred due to exposure to contaminated materials (Mani et al., 2015; Hutt et al., 2017), transmission of most TBD agents requires successful tick feeding. From this perspective, a deeper understanding of how ticks feed is expected to provide opportunities to develop innovative tick control

methods. *Amblyomma americanum*, similarly to other hard tick species, completes feeding over several days. To feed, ticks penetrate host skin with chelicerae and insert their barbed hypostome, which helps anchor the tick into the skin. The chelicerae and hypostome form the outer walls of the pre-oral canal that allows host fluid intake, as well as secretion of tick saliva into the host (Kemp et al., 1982). Within minutes of penetrating host skin, the tick starts to deposit an amorphous adhesive substance, called “tick cement”, which fills the lateral space between the mouthparts and skin layers, forming the cement cone. The cement cone anchors the tick mouthparts into the host to prevent removal by grooming, seals the wound around the mouthparts, and protects the mouthparts from the host’s immune system (Alekseev et al., 1995). At least two layers of the tick cement cone have been documented. The first layer (cortex) forms the core of the cement cone that surrounds the hypostome and is secreted in tick saliva within the first 30 min post-attachment, and is formed within the first 24 h after attachment (Kemp et al., 1982). Ticks continue to secrete the second layer (cortical cement) for 96 h post-attachment, to further strengthen the cement cone (Sonenshine, 1993).

Deposition of the cortex layer within 5 to 30 min of the tick inserting its hypostome into host skin precedes transmission of the majority of TBD agents, which occurs after the tick has attached for more than 2 days (Piesman et al., 1987; Hodzic et al., 1998; Katavolos et al., 1998; Kjemtrup and Conrad, 2000; Des Vignes et al., 2001; Hojgaard et al., 2008; Shkap et al., 2009; Cook, 2014). Thus, interfering with tick cement formation has the potential to prevent TBD transmission. There is published evidence that host immunity to components of tick cement can reduce tick-feeding efficiency (Brown and Askenase, 1986; Shapiro et al., 1989). Studies have also shown that immunization of animals with recombinant putative tick cement proteins provoked strong anti-tick immunity that significantly affected tick feeding performance (Bishop et al., 2002; Havlikova et al., 2009). For instance, Zhou et al. (2006) immunized rabbits against *Rhipicephalus haemaphysaloides* with recombinant glycine-rich protein and found significantly higher mortality of nymph, and lower attachment rates of adult and nymph ticks. In a related study, rabbit immunity to an immunodominant *Haemaphysalis longicornis* 29 kDa putative tick cement protein significantly reduced engorgement weights in adult ticks and up to 40 and 56% mortality in larvae and nymphs, respectively (Mulenga et al., 1999). In another study, immunization of guinea pigs with the *Rhipicephalus appendiculatus* putative tick cement protein, 64TRP, resulted in increased mortality rates and caused the midguts of some of the ticks to rupture (Trimnell et al., 2002, 2005). In a follow up study, Labuda et al. (2006), demonstrated that vaccinating mice with 64TRP blocked tick-borne encephalitic virus (TBEV) transmission to co-feeding nymphal ticks and protected mice from fatal infection with the virus. In another study, cattle mounted a strong antibody response to *R. appendiculatus* 36 kDa tick cement protein, but no anti-tick vaccine efficacy was reported (Bishop et al., 2002).

Despite its importance, molecular identities of proteins that form the tick cement cones remain unknown. Early chemical analytic studies revealed that tick cement cones are composed primarily of protein, as well as lipid and carbohydrate bound to the proteins in the core and cortex, respectively (Kemp et al., 1982). The majority of tick cement proteins are glycine-rich and to date, annotations of putative tick cement proteins have been based on the content of glycine amino acid residues (Kemp et al., 1982; Bishop et al., 2002; Valenzuela et

al., 2002; Untalan et al., 2005; Ribeiro et al., 2006, 2017; Mulenga et al., 2007; Maruyama et al., 2010; Oliveira et al., 2013; Jiang et al., 2014; Tirloni et al., 2014; Karim and Ribeiro, 2015; de Castro et al., 2016; Kim et al., 2016). The limitation of currently annotated putative tick cement proteins in public databases is that those were not identified from the tick cement cone, and thus it is unclear whether those are associated with tick cement formation. In a recent study, LC-MS/MS sequencing of gel-digested peptides was used to identify 33 proteins in the tick cement cone that was collected from artificially fed and sheep fed *A. americanum* (Bullard et al., 2016a). In this study we have used LC-MS/MS sequencing of in solution digested peptides to identify 179 proteins in tick cement cones that were recovered from mouthparts of manually detached 24–120 h fed *A. americanum* ticks. We further investigated the roles of 13 glycine-rich proteins (GRP) in tick feeding. We have discussed our data in the context of *A. americanum* tick feeding physiology.

2. Materials and methods

2.1. Ethics statement

All animal work was conducted under humane conditions approved by Texas A&M University, USA, Institutional Animal Care and Use Committee (AUP 2014-0311).

2.2. Tick feeding, dissections and total RNA extraction

Ticks (nymphs and adults) used in this study were purchased from the Oklahoma State University tick laboratory (Oklahoma State University, Stillwater, OK, USA). Ticks were fed on rabbits and chickens. To feed on rabbits and to avoid ticks getting into the rabbit ear canal, ticks were confined to the top of rabbit ears. Ticks were restricted to feeding on top of the rabbit ear using a cotton stockinet tick containment cell that was glued onto the ears with Kamar Adhesive (Kamar Inc, Zionsville, IN, USA). To feed nymphs on rabbits, the cotton stockinet tick containment cell was lined with pantyhose material to prevent nymphs from escaping. Larvae (hatched in-house) and nymphs were also fed on chickens confined to a cage. To infest chickens, ticks were sprinkled onto feathers of chickens that were restricted in the cage. Engorged ticks that dropped off the chickens were collected from the bottom of the cage.

For dissections, female adult ticks that were partially fed on rabbits for 24, 48, 72, 96 and 120 h were manually detached and processed for dissection of salivary glands (SG), midguts (MG), and carcass (CA, tick remnant after removal of SG and MG) as described (Kim et al., 2014). Ticks for dissection were washed in diethylpyrocarbonate (DEPC)-treated water and then placed on a clean glass slide, ventral side down. A sterile razor blade was used to trim mouthparts around the basis capituli and the lateral edges around the tick. The tick was placed on a concave well slide with DEPC water and the dorsum was lifted and removed to expose the tick organs. Three biological replicates of tick organs (SG, MG and CA) that were pooled from five ticks were placed in 1 mL of the Trizol total RNA extraction reagent (Life Technologies, Waltham, MA, USA).

To extract total RNA, tick organs were homogenized in the Trizol reagent using a tissue dismembrator (Thermo Fisher Scientific, Waltham, MA, USA). For whole ticks (nymphs

and adults), individual animals were minced in Trizol using scissors and then homogenized as above. Total RNA was extracted according to the protocol provided by the manufacturer of the Trizol reagent. Total RNA was quantified spectrophotometrically using a Nano drop plate in a Synergy H1 plate reader (BioTek, Winooski, VT, USA).

Template cDNA was synthesized from 200 or 500 ng of total RNA using a Verso cDNA synthesis kit (Thermo Fisher Scientific). The Verso cDNA synthesis kit contains a reverse transcriptase enhancer reagent, which prevents genomic DNA carryover into the synthesized cDNA. However, to validate whether this was consistent, we included a “no reverse transcriptase” cDNA synthesis control to confirm depletion of genomic DNA (gDNA) during the cDNA synthesis step (not shown).

2.3. LC-MS/MS identification of proteins from tick cement cones

2.3.1. Collection of tick cement cones—Tick cement cones were collected, using soft tissue forceps, from mouthparts of manually detached adult *A. americanum* ticks (approximately 20 ticks) that had fed on rabbits for 24, 48, 72, 96 and 120 h. To extract proteins, tick cement cones were disrupted by sonication in urea buffer (100 mM Tris-HCl, 500 mM NaCl, 8 M urea, pH 7.4) followed by overnight incubation at room temperature. Following incubation, the tick cement cone lysate was centrifuged, and the supernatant was collected and dialyzed against 1X PBS, pH 7.4, overnight at 4°C. After dialysis, some tick cement proteins precipitated out in PBS. We separated the precipitates (pellet, PBS insoluble) from the solution (PBS soluble) fraction by centrifugation. The pellet (re-dissolved in urea buffer) and the PBS soluble fractions were submitted for LC-MS/MS sequencing at the laboratory of Dr. John Yates III at the Scripps Institute (La Jolla, CA, USA). Total protein content was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer’s instructions.

2.3.2. Protein digestion and sample preparation—Tick cement fractions were dissolved into 8 M urea/0.1 M Tris, pH 8.5, reduced with 5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma-Aldrich, St Louis, MO, USA) and alkylated with 25 mM iodoacetamide (Sigma-Aldrich). Proteins were digested overnight at 37°C in 2 M urea/0.1 M Tris pH 8.5, 1 mM CaCl₂ with trypsin (Promega, Madison, WI, USA) at a final ratio of 1:20 (enzyme:total protein). Digestion reactions, at a final concentration of 0.15 µg/mL, were quenched with formic acid (5% final concentration) and centrifuged for debris removal.

2.3.3. Pre-column and analytical columns—Reverse phase pre-columns were prepared by first creating a Kasil frit at one end of a deactivated 250 µm ID/360 µm OD capillary (Agilent Technologies, Santa Clara, CA, USA). Kasil frits were prepared by dipping a 20 cm capillary in 300 µL of Kasil 1624 (PQ Corporation, Malvern, PA, USA) and 100 µL of formamide solution, curing at 100 °C for 3 h and adjusting the length. Pre-columns were packed in-house with 2 cm of 5 µm ODS-AQ C18 (YMC America, INC., Allentown, PA, USA) particles from particle slurries in methanol. Analytical reversed phase columns were fabricated by pulling a 100 µm ID/360 µm OD silica capillary (Molex Polymicro Technologies™, Austin, TX, USA) to a 5 µm ID tip. The same packing material

was packed up to 20 cm directly behind the pulled tip. Reversed phase pre-columns and analytical columns were connected using a zero-dead volume union (IDEX Corp., Upchurch Scientific, Oak Harbor, WA, USA).

2.3.4. LC-MS/MS sequencing—Peptide mixtures were analyzed by nanoflow liquid chromatography mass spectrometry using an Easy NanoLC II and a Q-Exactive mass spectrometer (Thermo Scientific). Peptides eluted from the analytical column were electrosprayed directly into the mass spectrometer. Mobile phases were 5% acetonitrile/0.1% formic acid as solution A and 80% acetonitrile/0.1% formic acid as solution B. The flow rate was set to 400 nL/min. Cement samples (1.5 µg per injection) were separated in 155 min gradients as follows: 1–10% B for 10 min, 10–40% B for 100 min, 40–50% B for 10 min and 50–90% B for 10 min. The column was held at 90% B for 10 min, reduced to 1% B and re-equilibrated prior to the next injection. The mass spectrometer was operated in a data-dependent mode, collecting a full MS scan from 400 to 1,200 m/z at 70,000 resolution and an AGC target of 1×10^6 . The 10 most abundant ions per scan were selected for MS/MS at 17,500 resolution and AGC target of 2×10^5 and an underfill ratio of 0.1%. Maximum fill times were 20 and 120 ms for MS and MS/MS scans, respectively, with dynamic exclusion of 15 s. Normalized collision energy was set to 25.

2.3.5. Data analysis—Protein and peptide identification were done using the Integrated Proteomics Pipeline – IP2 (Integrated Proteomics Applications). Tandem mass spectra were extracted from raw files using RawExtract 1.9.9.2 (McDonald et al., 2004) and searched with ProLuCID (Xu et al., 2015) against the *A. americanum* transcriptome (BioProject accession number [PRJNA226980](#)) concatenated with *Oryctolagus cuniculus* from Uniprot (www.uniprot.org) and reverse sequences of all entries. The search space included all fully-tryptic and half-tryptic peptide candidates. Carbamidomethylation on cysteine (+57.02146) was used as a static modification. Data were searched with 50 ppm precursor ion tolerance and 600 ppm fragment ion tolerance. Identified proteins were filtered using DTASelect (Tabb et al., 2002). A minimum of one peptide per protein, at least one tryptic terminus for each peptide and less than 1% false discovery rate (FDR) were required.

2.4. Spatiotemporal reverse transcriptase (RT)-PCR expression analysis of selected GRPs

Transcription analysis of randomly selected GRPs (Table 1) was done in two steps. In the first preliminary step, we used semi-quantitative RT-PCR analysis to quickly confirm whether encoding mRNAs were expressed during tick feeding. Routine semi-quantitative RT-PCR using specific primers (Table 1) was done using cDNA of dissected SGs, MGs, and CA of unfed adult female ticks and those that were partially fed for 24–120 h, as described (Chalaise et al., 2011). Actin primers (Table 1) were used as internal sample normalization controls (Chalaise et al., 2011).

In the second step, we used quantitative (q) RT-PCR analysis to accurately determine mRNA expression patterns in dissected SG as described (Kim et al., 2014, 2016). The reaction mixture contained 1.2 or 2.4 µL of each forward and reverse primer (150/150 or 300/300 nM ratio Table 1), 4 µL of template cDNA (10-fold dilution of template cDNA that was synthesized from ~200 ng of total RNA), and 20 µL of SYBR green master mix in a 40 µL

reaction volume. qRT-PCR was performed on a BioRad CFX96 Real-Time System (Hercules, CA, USA; TAMU Genomics Center) as follows: one cycle: 50°C for 2 min, 95°C for 10 min, and 40 cycles: 95°C for 15 s, 60°C for 1 min followed by a melt curve from 65°C to 95°C, incremented 0.5°C every 5 s (please note that for some of targets, the temperature for step 4 was 55°C). The specificity of the qRT-PCR was monitored by melting curve analysis in the Biorad® software. The comparative delta delta Ct method (Livak and Schmittgen, 2001) with ribosomal protein S4 (RPS4) (NCBI accession number **GAGD01011247.1**) as an internal reference control (Koci et al., 2013) was used to determine relative mRNA abundance as previously described (Kim et al., 2014; 2016).

Template cDNA that was used in both semi-quantitative and qRT-PCR was synthesized using a Verso kit, which eliminates genomic DNA contamination during the synthesis step. To confirm this, qualitative PCR using tick actin primers and “no reverse transcriptase” cDNA were used to validate that gDNA was depleted during the cDNA synthesis step. Please also note that in both semi-quantitative and qRT-PCR, we included a no template negative control for each primer set to check for template contamination.

2.5. qRT-PCR expression analysis during the “pre-appetence to appetite attainment” transition phase

Newly hatched larvae or ecdysed nymph and adult ticks transition from the “pre-appetence phase” with little or no desire to feed, to the “appetence phase” with an increased desire to feed (Gladney et al., 1970; Tukahirwa, 1976; Davey, 1987; Anderson et al., 1998). Preliminary semi-quantitative RT-PCR analysis showed that encoding transcripts for three (Aam-16245, Aam-179667 and Aam-178006) of the selected GRPs appeared to be constitutively expressed. We were curious to determine whether the three genes were also associated with tick molting. Template cDNA of larvae and nymphs that engorged on chickens were sampled at day 0 (day dropped off the host), during molting at days 3, 7, 11, 14, 21 and 28, and newly ecdysed (nymphs and adult ticks) at 1 and 2 weeks post-ecdysis were subjected to qRT-PCR using primers listed in Table 1. The rationale for the sampling timeline was based on published evidence that newly ecdysed ticks attain appetite within 7–21 days post-ecdysis (Gladney et al., 1970; Tukahirwa, 1976; Davey, 1987; Anderson et al., 1998). Total RNA extraction, cDNA synthesis, and qRT-PCR were done using the same procedure outlined in Section 2.4.

2.6. Target validation of selected GRPs using combinatorial (co)-RNA interference (RNAi) silencing

Given the high number of targets, we used co-RNAi silencing (Kramer et al., 2015; Shi et al., 2015) to streamline work. Three co-double stranded (ds) RNAs were synthesized according to preliminary semi-quantitative RT-PCR expression profiles. These were: (i) apparently constitutively expressed (CE, Aam-16245, Aam-179667 and Aam-178006) for those that were strongly expressed in both unfed and partially fed tick SG, (ii) feeding induced (FI, Aam-177101, Aam-177916, Aam-177785 and Aam-326) for those transcripts that were appeared to be undetectable in unfed tick SG, and (iii) up-regulated with feeding (UR, Aam-177792, Aam-15, Aam-179800, Aam-181586, Aam-42453 and Aam-21) for those that appeared to be weakly expressed in unfed tick SG but increased with feeding (not

shown). Template DNA for co-dsRNA synthesis was custom made by splicing unique ~200 bp of each GRP in the construct using MacVector (MacVector Inc., Apex, NC, USA) (Supplementary Fig. S1). Spliced sequences were unique to each GRP sequence to avoid non-target effects. To identify unique sequence regions, GRP sequences were searched against entries in GenBank and Uniprot, and portions of sequences that did not show similarity to other sequences were selected. Assembled template DNA was custom synthesized at Life Technologies (Thermo Fisher Scientific). An Ambion Megascript RNAi Kit (Thermo Fisher Scientific) was used to synthesize co-dsRNA according to the manufacturer's instructions. Templates for co-dsRNA synthesis were amplified using plasmid DNA and PCR primers with an added T7 promoter sequence (Table 1). GFP primers with an added T7 promoter were used to amplify the template for the control group. Approximately 2 µg of purified template DNA was used for co-dsRNA synthesis.

RNAi silencing was done twice. In the first round, unfed female ticks ($n = 20$) were injected with 0.5–1 µL (1 µg/µL) of co-dsRNA or GFP-dsRNA ventrally behind the fourth leg coxa using a 33 gauge needle as described (Chalaire et al., 2011; Mulenga et al., 2013; Kim et al., 2016). In the first RNAi silencing experiment, we had observed bleeding around mouthparts of CE and FI co-dsRNA-injected ticks. To rule out the possibility that the observed bleeding was caused by overcrowding of ticks, we repeated the RNAi silencing experiment with fewer ticks ($n = 15$).

Ticks injected with co-dsRNA or GFP-dsRNA were incubated at 25°C with relative humidity set to 85% and a 12:12 h dark:light cycle for 3 weeks before assessing the effects of RNAi silencing on tick feeding. The 3 week incubation period was empirically pre-determined as the length of time that was needed to substantially deplete GRP mRNA that predated induction of RNAi silencing in unfed ticks. Following the 3 week incubation, ticks were fed on rabbits (as described in Section 2.2) to assess the effects of RNAi silencing. The effects of RNAi silencing were evaluated based on the following tick feeding parameters: (i) tick attachment rate onto host skin, (ii) time to feed to repletion, (iii) engorgement weight (EW), index for amount of imbibed blood), (iv) fecundity (ability to lay eggs), (v) egg mass conversion ratio (EMCR; i.e. ability to utilize blood meal to produce eggs), and (vi) mortality rates. Tick attachment rates were determined 24 h after ticks were placed on the rabbit to feed, while the EW was determined by weighing replete-fed ticks upon spontaneous detachment. Fecundity was determined by documenting numbers of ticks that laid eggs and weighing egg clutches. Lastly, EMCR was determined by dividing the weight of the egg clutch over the engorgement mass.

2.7. Effect of injecting newly ecdysed adult *A. americanum* with CE co-dsRNA on tick feeding

Preliminary analysis of transcription data in newly ecdysed ticks (Section 2.5) revealed that mRNA of CE GRPs was up-regulated several thousand fold at 1 week post-ecdysis. We were curious to investigate whether injecting CE co-dsRNA into day 0 newly ecdysed adult ticks could prevent accumulation of mRNA, and consequently prevent CE GRP proteins being formed. At the time of drafting this manuscript there were no reports of whether or not the RNAi silencing pathway was active in molting ticks or newly ecdysed adult ticks. Thus,

prior to RNAi silencing, we determined the expression of the dicer gene as an indicator of an active RNAi silencing pathway. Template cDNA of replete-fed nymphs during molting and newly ecdysed adult ticks that was synthesized in Section 2.5 was subjected to qRT-PCR using dicer-specific primers (Table 1). PCR primers were designed based on the *Amblyomma triste* dicer sequence (NCBI accession number **GBBM01005393**).

After confirmation of expression of the dicer, day 0 newly ecdysed female ticks were injected with CE co-dsRNA. Due to ecdysis occurring on different days, two RNAi experiments were conducted. In the first round, 11 ticks were each injected with CE co-dsRNA or GFP dsRNA as described in Section 2.6. In the second round, six ticks were used. Injected ticks were incubated for 7–14 days. This incubation period was empirically pre-determined as the time period during which accumulation of CE GRP mRNA was reduced by nearly 100%. Following incubation, ticks were placed on rabbits and effects of RNAi silencing on tick feeding assessed as described in Section 2.6.

2.8. Validation of RNAi silencing

Prior to assessing the effects of RNAi silencing on *A. americanum* tick feeding, disruption of target mRNA in co-dsRNA injected ticks was validated by qRT-PCR using primers listed in Table 1. Three ticks per treatment were sampled at 24 h post-attachment and individually processed for total RNA extraction, cDNA synthesis, and qRT-PCR as described (Kim et al., 2014, 2015). Relative quantification (RQ) of target mRNA was determined using the comparative delta delta Ct method (Livak and Schmittgen, 2001). Target mRNA suppression levels were calculated using the formula, $S = 1 - (RQ^T/RQ^C \times 100)$ where S = mRNA suppression, RQ^T and RQ^C = RQ in tissues of co-dsRNA and GFP-dsRNA injected ticks, respectively. Statistical significance was determined using ANOVA in the PRISM software package (Graphpad Software, La Jolla, CA, USA).

3. Results

3.1. *Amblyomma americanum* tick cement protein composition is complex

We successfully dissolved *A. americanum* tick cement cones in 8 M urea buffer. When dialyzed against PBS, some proteins, which we have called “PBS insoluble (PBS-I)” precipitated out, and other proteins that we have called “PBS soluble (PBS-S)” remained in solution. Both fractions were successfully digested with trypsin and peptides subjected to LC-MS/MS sequencing. Peptide sequences were searched against an in-house database of translated *A. americanum* using the IP2 proteomics pipeline (Xu et al., 2015) to identify 160 unique tick cement proteins (Supplementary Table S1. The in-house *A. americanum* database, assembled based on Sequence Read Archive (SRA) BioProject ID number PRJNA226980 (Porter et al., 2015) was collapsed with rabbit host proteins.

When searched against acari sequence entries in GenBank and/or Uniprot, 95% (152/160) of tick cement proteins in this study showed identity to hard tick (*Amblyomma* and other) species, with the rest showing identity to soft tick and mite sequences (Supplementary Table S1). In a recent study, 33 proteins were described in tick cement cones of *A. americanum* that were artificially fed through membranes and naturally on sheep (Bullard et al., 2016a).

Comparative sequence analysis show that three of the 33 tick cement proteins identified in Bullard et al. (2016a) were also found in this study. The three proteins include two GRPs, similar to *R. appendiculatus* GRP RIM36, NCBI accession numbers ACG76243.1 and JAG92718.1 that show 95% and 94% identity to Aam-178006 and Aam-179667, and JAG91718.1, a ribosomal protein that is 100% identical to Aam-175803 in this study (not shown).

Annotations based on matches to entries in GenBank and Uniprot indicate that the 160 tick cement sequences include glycine-rich proteins (19%, 30/160), tick cement proteins of miscellaneous or unknown function (11%, 18/160), protease inhibitors (12%, 18/160), lipocalins (1%, 2/160), storage proteins (1%, 3/160), detoxification proteins (1%, 2/160), mucin-like (4%, 6/160) and housekeeping-like proteins (50%, 80/179) (Fig. 1A, Supplementary Table S1). As summarized in Supplementary Table S1, (i) protease inhibitors include serpin (serine protease inhibitor), TIL domain ($n=4$), and Kunitz ($n=18$) serine protease inhibitors, (ii) storage proteins include ferritin, vitellogenin and calmodulin ($n=3$), and (iii) detoxification proteins include sulfotransferase and thioredoxin ($n=2$). The 80 housekeeping-like proteins are classified into nine functional classes (Fig. 1B, Supplementary Table S1). These include cytoskeletal (actin, alpha and beta tubulin, 21%, 17/80), nuclear regulation (histone and GTPase, 19%, 15/80), energy and carbohydrate metabolism (F₀F₁ type ATP synthase alpha, NAD-dependent malate dehydrogenase, glyceraldehyde 3 phosphate dehydrogenase, pyruvate kinase, glucose 6 phosphate isomerase, and NAD-dependent isocitrate dehydrogenase) that account for 10% (8/80). Others include protein export associated (heat shock proteins and vesicle coat protein clathrin heavy chain, 1%, 1/80), proteasome associated (ubiquitin/40S ribosomal protein S27A fusion, 23%, 18/80), transcription machinery (ATP dependent RNA helicase, polyadenylate-binding protein) and transporter (ADP/ATP transporter, sodium/potassium transporting ATPase) associated each accounting for 5% (4/80), and signal transduction associated (Ras-related proteins, 6%, 5/80). Please note that our housekeeping-like proteins are not host proteins; those are tick proteins that show high identity to mammalian housekeeping proteins.

The identification-compare analysis in Integrated Proteomics Pipeline (IP2) revealed that the majority of identified tick cement proteins were found in the PBS-I fraction. We found that 41% and 59% of tick cement proteins were identified in PBS-S and PBS-I fractions of tick cement extracts, respectively (Supplementary Tables S1 and S2, Fig. 2). As expected, the majority (93%) of GRPs were exclusively identified in the PBS-I fraction since these proteins have biochemical characteristics that induce aggregation (Fig. 2) (Condit et al., 1990; Bullard et al., 2016a). It is important to note that more than 500 rabbit proteins that were associated with the tick cement cone were also identified and those host proteins were removed from this analysis. Please note that peptides that matched tick cement proteins are listed in Supplementary Tables S2 and S3.

3.2. Selected GRP mRNAs are expressed in multiple tick organs and are responsive to blood meal feeding

To gain further insight into functional role(s) in tick feeding physiology, selected GRPs (Table 1) were subjected to transcription analysis in dissected SG, MG and CA of unfed ticks and those that were fed for 24, 48, 72, 96 and 120 h. In a preliminary study, we used semi-quantitative RT-PCR analysis to confirm transcription of the selected GRPs during tick feeding (not shown). This analysis showed that mRNA of all selected GRPs was expressed in all tested organs except for three (Aam-15, Aam-42453 and Aam-177785) and two (Aam-177792 and Aam-177785) that were not detected in MG and CA, respectively (not shown). Subsequently we used qRT-PCR analysis in SG cDNA to accurately relate mRNA expression to the tick feeding process. We observed five general expression profiles across the 12 targets (Fig. 3). In the first profile (Fig. 3A–C), GRP transcript abundance increased by more than 20 fold by the 24 h feeding time point and remained at this level until the 120 h feeding time point. In the second profile, transcript abundance increased, respectively, by more than five (Fig. 3D, E), 100 (Fig. 3F), 200 (Fig. 3G, H), and 300 (Fig. 3I) fold at the 24 h time point but then declined with feeding, reaching a near steady state level by the 120 h feeding time point. In the third profile (Fig. 3J), mRNA abundance was highest in unfed tick SG but decreased by two fold at the 24 to 72 h time points, before it increased by two fold at 96 h and declined by three fold at the 120 h feeding time point. In the fourth profile, transcript abundance increased at 24 h and decreased until the 96 h time point before increasing again at 120 h of feeding (Fig. 3K). Lastly in the fifth profile, transcript level was at a steady state until the 96 h feeding time point before increasing by more than 100 fold by the 120 h time point (Fig. 3L).

Please note that template cDNA that was used in Fig. 3 and Figs. 4 and 6 was synthesized using a Verso kit, which eliminates gDNA contamination. No amplification in “no reverse transcriptase (RT)” cDNA synthesis confirmed this (not shown).

3.3. Transcripts of constitutively expressed GRP are weakly expressed during molting but up-regulated several thousand fold at 1 week post-ecdysis

Newly hatched larvae and newly ecdysed nymph and adult ticks show little appetite (pre-appetence) to feed until after 7–21 days post-ecdysis (Gladney et al., 1970; Tukaira, 1976; Davey, 1987; Anderson et al., 1998). We were interested to determine the transcription profile of CE-GRPs during the tick's transition from pre-appetence to the appetite phase. Fig. 4 demonstrates that while mRNA of all CE GRPs was expressed during molting (larvae and nymphs), transcript abundance significantly increased post-ecdysis. With the exception of Aam-178006 in molting larvae (Fig. 4Ea) for which expression was at a steady state until day 14 of molting, transcript expression levels for all other GRPs were higher in engorged ticks at day 0 before dropping to low levels from day 7 of molting (Fig. 4Aa and Ca). In molting nymphs, transcript abundance in engorged nymph (day 0) transcript dropped by ~6500, 1500 and ~4500 fold by the third day of molting and remained at this level before significantly increasing several thousand fold immediately after ecdysis (Fig. 4Ba, Da, Fa). In newly ecdysed nymphs (Fig. 4Ab, Cb, and Eb), transcript abundance increased by more than 10,000, 3000, and 3500 fold by the first week post-ecdysis. Similarly, in newly ecdysed

adult ticks, transcript abundance increased significantly between 30,000–100,000 fold by 1 week post-ecdysis (Fig. 4Bb, Db, Fb).

3.4. Disruption of CE and FI GRP transcripts caused reddening and subsequent bleeding around tick attachment sites

We successfully synthesized and injected ticks with co-dsRNA to disrupt GRP transcripts in clusters according to similarity in transcription patterns: (CE) Aam-16245, Aam-179667 and Aam-178006), (ii) FI (Aam-177101, Aam-177916, Aam-177785 and Aam-326), and (iii) UR (Aam-177792, Aam-15, Aam-179800, Aam-181586, Aam-42453 and Aam-21). Ticks that were injected with co-dsRNA or GFP-dsRNA were allowed to feed to repletion and tick feeding physical phenotypes were documented at 24 h intervals as summarized in Fig. 5. Our analyses indicate that RNAi silencing of GRP genes did not affect the ability of ticks to attach onto skin and start feeding. However, 4 days after the ticks had attached, intense reddening was observed around attachment sites of CE and FI co-dsRNA injected ticks (not shown). In CE co-dsRNA injected ticks, the reddened attachment sites started to bleed by 7 days post-attachment (Fig. 5A, arrowhead). Leakage of blood became so intense that on day 9, veterinarians advised halting the experiment for the well-being of the animal. Blood leakage also appeared around the mouthparts of FI injected ticks at day 9 and peaked around day 11, but it was not so intense as to cease tick feeding (arrowhead in Fig. 5A). To rule out the possibility that the observed bleeding was caused by many ticks crowding together, RNAi silencing of CE and FI GRP genes was repeated with fewer ticks per rabbit ear ($n=15$ compared with $n=20$ in round one). It was interesting to note that, similar to observations in Fig. 5A, reddening and subsequent leakage of blood was observed in the second round of RNAi silencing starting from days 8 (CE) and 10 (FI) post-attachment (arrowhead in Fig. 5B). However, bleeding was not as intense as in the first round and thus the experiment was not stopped for CE co-dsRNA injected ticks. It is important to note that dried blood was observed on control ticks at day 8, however there was not continuous bleeding (Fig. 5B).

One-way ANOVA of EW (index for amount of blood taken by the tick) determined significant differences among mean EWs ($F(3, 45) = 3.44$, $P = 0.0245$). However Tukey's multiple comparison of means revealed that mean EW of CE (0.37 ± 0.041 g, $P = 0.0211$) and UR (0.35 ± 0.047 g, $P = 0.0255$), and FI (0.49 ± 0.051 g, $P = 0.405$) co-dsRNA injected ticks, although apparently smaller, were not significantly different from GFP-dsRNA injected ticks (0.53 ± 0.043 g) (not shown). Similarly, one-way ANOVA analysis of the egg mass conversion ratio (ability to convert blood meal to eggs) revealed no significant differences between treatments and control ticks (not shown). The effects of RNAi silencing in the second round were comparable with those observed in the first.

Prior to assessing the effects of RNAi silencing, we sampled three ticks per treatment at 24 h post attachment to validate RNAi silencing. Transcripts of the three GRPs in CE co-dsRNA, Aam-16245, Aam-179667 and Aam-178006, were, respectively, suppressed by 80–90%, 80–100% and 60–100% in two of the three tested ticks (not shown). However, validation of RNAi silencing in FI and UR co-dsRNA injected ticks was inconclusive.

3.5. Injection of CE co-dsRNA into newly ecdysed (day 0) adult ticks prevents accumulation of CE GRP transcripts and causes bleeding around tick attachment sites

In Section 3.3, qRT-PCR analysis revealed that mRNA of CE GRP, which was at a steady state level during molting of nymphs, increased several thousand fold in 1 week old newly ecdysed adult ticks. Thus, we reasoned that if newly ecdysed ticks (day 0) were injected with CE co-dsRNA, accumulation of transcripts could be prevented, which in turn might have dramatic RNAi silencing effects. Consistent with this assumption, injection of CE co-dsRNA into newly ecdysed ticks prevented accumulation of CE GRP mRNA by up to 100% in 1 to 2 week old adult ticks (not shown).

In preliminary analyses, we determined that injection of CE co-dsRNA into day 0 newly ecdysed adult ticks depleted GRP transcript up to 100% by day 14 after co-dsRNA injection. Thus we proceeded to inject two groups of newly ecdysed adult ticks: groups A ($n = 11$) and B ($n = 6$) were injected with CE co-dsRNA or GFP dsRNA and incubated for 2 and 1 week, respectively, before feeding ticks on rabbits to assess the effects of RNAi silencing. Before assessing the effects of RNAi silencing, we confirmed that CE GRP mRNA was ~100% suppressed in adult ticks that had attached for 24 h, except for Aam-179667 which was up to ~85% (Fig. 6A and 6B). Despite the observed ~100% suppression of CE GRP mRNA, ticks were still able to attach and feed. Similar to observations in Fig. 5, reddening and subsequent bleeding was observed around mouthparts of both group A and B ticks that were injected with CE co-dsRNA but not GFP dsRNA treated ticks (Fig. 6C, D). Additionally, an expanding hematoma was observed on the underside of a single attached female tick (Fig. 6Ea, Eb). Analysis of indices for blood meal size and egg laying indicated that despite the fact that mRNA of CE GRP was prevented from accumulating, the ability of ticks to take a blood meal and reproduce were not affected (not shown).

4. Discussion

The adaptation of hard ticks to feed for long periods is facilitated by deposition of the tick cement cone, and thus, there has been a concerted effort toward understanding tick cement formation (Kemp et al., 1982; Alekseev et al., 1995; Sonenshine, 2013). The attraction is that the tick cement cone protects the tick from being groomed off by the host, and that deposition of the tick cement cone within 5–30 min of the tick inserting its hypostome (Sonenshine, 2013) precedes transmission of major tick-borne disease agents (Piesman et al., 1987; Hodzic et al., 1998; Katavolos et al., 1998; Kjemtrup and Conrad, 2000; Des Vignes et al., 2001; Hojgaard et al., 2008; Shkap et al., 2009; Cook, 2014). This makes preventing deposition of tick cement an attractive target for development of innovative tick control. This study makes a contribution to this goal by describing LC-MS/MS identification and characterization of 160 proteins in *A. americanum* tick cement cones. It is notable that consistent with published chemical analysis studies (Kemp et al., 1982), the largest cluster of proteins identified in this study is the GRPs. Data here builds on a recent study that described 33 proteins in the tick cement cones of *A. americanum* ticks that fed on sheep and artificially fed on sheep blood through silicone reinforced nitrocellulose membranes (Bullard et al., 2016a). It is important to note that our study and Bullard et al. (2016a) share three proteins. Thus, this study makes a significant contribution in describing an additional 160

proteins not previously known to be directly associated with the *A. americanum* tick cement cone. The low number of proteins shared between the two studies could be explained by the fact that two different approaches were used: in solution digestion in this study, and in gel digestion in Bullard et al. (2016a).

The observation that the 160 tick cement proteins identified in this study were classified into 18 functional classes points to the complexity of tick cement formation. Functional roles in tick cement formation for the majority of tick cement proteins in this study cannot be discerned with the possible exception for GRPs and putative mucins. Given that they form the largest class of proteins in the tick cement cone and their adhesive and tensile characteristics, and association with pliable structures such as silk (Maruyama et al., 2010), GRPs are thought to play essential roles in tick cement formation. Similarly, mucin-like tick cement proteins can be envisioned to participate in tick cement formation. In mammals, some secreted mucin proteins form gels that are associated with innate immune responses (Linden et al., 2008; Kane, 2010). Whether or not the gel forming property is conserved among putative tick cement mucins is not known. It is possible that mucins could form the initial scaffolding gel that is strengthened by structural proteins such as the actin, beta and alpha tubulin-like proteins also identified in our study. In addition to tick cement formation, some of the tick cement proteins reported here could have other functions at the tick-feeding site. For instance, serine protease inhibitors could play roles in mediating tick evasion of the host's first line of defense against tick feeding. The tick feeding style of disrupting host tissue and then sucking up host blood that bleeds into the feeding lesion is expected to trigger serine protease host defense pathways such as blood clotting, inflammation and complement activation, all of which are controlled by protease inhibitors (Huntington et al., 2000; Gettins, 2002; Huntington, 2013). It is interesting to note that one of the protease inhibitors identified in *A. americanum* tick cement is a trapping serine protease inhibitor (serpin), *A. americanum* serpin AAS27, previously described in Porter et al. (2015). Trapping serpins are central to regulating inflammation, blood clotting and complement activation (Huntington et al., 2000; Gettins, 2002). Could AAS27 be among the first tick proteins that stop the host defense against tick feeding? Another interesting observation in our data is that 50% of tick cement proteins in this study are putative housekeeping proteins. Whether or not putative housekeeping tick cement proteins are functional remains to be determined. It will be interesting to understand the exogenous effects of tick-derived housekeeping proteins on the host defense response to tick feeding. Indirect evidence in mammals suggests that the presence of some of the house keeping proteins in tick cement could be beneficial to tick feeding. For instance, extracellular ribosomal and heat shock proteins have anti-inflammatory effects (Filip et al., 2009; Lv et al., 2013; Khandia et al., 2016). Given that to complete feeding ticks must evade or modulate the host inflammation response, the presence of ribosomal and heat shock proteins could be regarded as pro-tick feeding (Rodriguez-Mallon et al., 2015). Similarly, mammalian extracellular histones have antimicrobial effects (Hirsch, 1958; Rose et al., 1998; Lee et al., 2009), and thus tick secretion of these proteins might be part of the tick's mechanism to prevent its feeding site from being colonized by microbes. Our data suggested that some of the *A. americanum* tick cement proteins could be hydrophobic as suggested by majority of these proteins precipitating in an aqueous PBS solution. In biological solutions, hydrophobic proteins bind

onto chaperone proteins such as heat shock proteins to fold in such a way that the hydrophobic regions are hidden (Sun and MacRae, 2005; Zhang et al., 2015). There is a likelihood that heat shock proteins identified in this study acted as chaperones of the apparently hydrophobic tick cement proteins to help those remain in solution. Findings of housekeeping-like proteins in our data is not unique to this study, as several other studies have identified tick saliva proteins that show high amino acid identities to the host's housekeeping proteins (Radulovic et al., 2014; Lewis et al., 2015; Tirloni et al., 2015; Kim et al., 2016).

Due to the high number of tick cement proteins identified in this study, studies to determine significance are expected to be challenging. Our approach was to determine the significance of selected candidate GRP tick cement proteins as a functional cluster. Spatiotemporal transcription analysis as used in this study is routinely used as the first step to understand functions of tick genes in tick feeding physiology (Mulenga et al., 2008, 2009; de Castro et al., 2016; Kim et al., 2016). Tick cement proteins are expressed in tick SG (Sauer et al., 1995; Kemp et al., 2013), and thus there is an expectation that encoding mRNAs are transcribed in the SG. Consistent with this, encoding mRNAs of all GRPs investigated here were expressed in SG in addition to other tick organs. The apparent ubiquitous expression of GRP transcripts in both unfed and partially fed ticks may suggest that GRPs in this study could be associated with tick cement deposition and other yet unknown tick feeding physiology functions. At least two layers of the tick cement cone, cortex and cortical layers have been documented (Kemp et al., 1982). Deposition of the first cortex layer of the tick cement cone starts within 5–30 min of the tick inserting its hypostome, and the second, cortical layer begins from 24 through 96 h post-attachment (Kemp et al., 1982). The observation that transcript abundance of GRPs significantly increased at 24 h post-attachment might suggest these tick cement proteins may be associated with formation of the cortical layer, with the exception of one candidate GRP for which transcript abundance decreased at 24 h, which might be associated with the cortex layer. Given that tick cement deposition is completed within 96 h of attachment, one would expect the expression of tick cement transcripts to be confined to this time period. The observation that some of the transcripts were highly expressed throughout the 5 day tick feeding period tested here, could indicate that candidate GRP genes were associated with other functions of tick feeding physiology. Whereas in tick research GRPs are mostly known for their potential roles in tick cement formation (Kemp et al., 1982), indirect evidence in other organisms suggests that these proteins could be involved in a myriad of other functions including anti-microbial activity (Alekseev et al., 1995; Carlier et al., 2015; Rahman et al., 2017), innate immunity in mammals (Tam et al., 2012), wound healing, plant defense and stress responses (Mangeon et al., 2010; Lin and Chen, 2014).

Another interesting observation in our data was that the transcription of CE GRPs might be under the control of yet unknown tick appetite associated factors as suggested by transcript abundance of CE GRPs increasing several thousand fold in 1 week old nymphs and adult ticks. Newly hatched larvae or newly ecdysed nymphs and adult ticks do not show appetite to feed until after the hardening phase (sclerotization) at approximately 7–14 days post-ecdysis (Gladney et al., 1970; Tukaira, 1976; Davey, 1987; Anderson et al., 1998). The host's defense response against tick feeding is expected to be swift, and thus to successfully

feed, the tick must be ready to counter the host defense (Mulenga et al., 2007). Could the observed increase in transcript abundance upon attainment of appetite be an indicator for the tick's molecular preparation to start feeding?

The long-term goal of our research is to find effective target antigens for tick vaccine development. Thus, another goal of this study was target validation to identify effective GRPs. Given the high number of candidate GRPs, we adopted the co-RNAi silencing approach (Kim et al., 2014; Shih et al., 2015; Kramer et al., 2015; Tchurikov et al., 2016) to streamline the work. We successfully synthesized co-dsRNA and disrupted GRP mRNA in clusters according to similar transcription profiles: constitutively expressed (CE, $n=3$), up-regulated with feeding (UR, $n=6$), and feeding-induced (FI, $n=4$). With the exception of the observed intense reddening and subsequent bleeding around mouthparts of CE and FI co-dsRNA, the effects of RNAi silencing on other tick feeding parameters were modest. The observed reddening and subsequent bleeding suggested that some of the CE and FI GRP proteins played important functions in maintaining the integrity of the tick-feeding site. Reddening at sites of injury or infection is an indicator for inflammation (Male et al., 2012; Abbas et al., 2016). Thus, we speculate that the observed inflammation was caused by failure of CE and FI co-dsRNA injected ticks to suppress inflammation at, and/or preventing microbial colonization of, the tick feeding site. Future work to determine whether or not CE and FI GRP proteins are functional anti-inflammatory or anti-microbial proteins is needed. Another interesting observation was that bleeding around CE co-dsRNA injected tick mouthparts was exacerbated by tick overcrowding in that bleeding was much more intense around crowded ticks compared with singly attached ticks.

The limitation of RNAi silencing is that the disruption of the cognate mRNA does not stop the functions of the cognate protein that was produced before the target mRNA was disrupted. The implication of this is that the effects of RNAi silencing might be masked. However, this scenario is ruled out in the case of candidate CE GRPs. We had observed that the abundance of CE GRP mRNA was low to near zero during molting but increased several thousand-fold at 1 week post-ecdysis. The limitation with our analysis was that the protein expression was not validated; nonetheless we reasoned that injecting CE co-dsRNA into day 0 newly ecdysed adult ticks could prevent the accumulation of CE mRNA and consequently protein. However, despite a nearly 100% suppression of CE GRP mRNA, ticks were still able to successfully attach and feed. This observation points to the possibility that CE GRP might not be associated with formation of the first layer of the tick cement cone. Additionally, recent studies by Bullard et al. (2016b) and Barnard et al. (2012) saw similar reductions in target mRNAs without preventing tick feeding. Of interest to this study, bleeding around attachment sites was observed, further confirming the importance of CE GRP proteins in maintaining the integrity of the tick attachment site. Although it was not a common occurrence, the observation of spreading hematoma on the underside of a singly attached CE co-dsRNA injected tick further suggested the importance of CE GRP proteins in maintenance of the tick feeding site. Whether or not the spreading hematoma is an indication of anti-coagulant activity for some of the CE GRP proteins remains to be established. We would like to caution here that although dsRNA was directed against sequence regions that did not show identity to other *A. americanum* genes, it is possible that

off-targets may be identified once the genome of this tick is sequenced. If this happens, the significance of CE GRP in tick feeding might need reevaluation.

In conclusion, this study has contributed towards our understanding of the molecular basis of tick cement formation. The next phase of the research is to conduct functional studies to identify proteins that are essential to cement formation. Given the high number of tick cement proteins identified in this study, functional analysis studies are expected to be laborious. The application of combinatorial RNAi silencing as demonstrated in this study will streamline the initial prioritization phase. Overall, data presented here set the foundation for in-depth molecular studies on formation of the tick cement cone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Tick cement composition is complex.
- Glycine-rich proteins (GRPs) are the most abundant class of proteins in tick cement.
- Thirteen selected GRPs have unique mRNA transcription patterns during tick feeding.
- Some of the GRPs are important to the integrity of the tick attachment site.

Fig. 1.

The *Amblyomma americanum* tick cement cone contains peptides from a variety of functional protein classes. Tick cement cones that were collected from mouthparts of manually detached ticks which were fed for 24–120 h were dissolved overnight in urea buffer as described in Section 2.3.1. Tick cement extracts were subjected to in solution digestion using trypsin and then subjected to LC-MS/MS sequencing. Peptides that were identified in LC-MS/MS were searched against a translated *A. americanum* transcriptome (Sequence Read Archive (SRA) BioProject ID number [PRJNA226980](#)) that was collapsed with rabbit proteins. To annotate *A. americanum* tick cement proteins, assembled DNA sequences were searched against a database of tick protein sequences downloaded from GenBank and Uniprot. Unique proteins per functional class are expressed as percentages of the total number of tick cement proteins identified. (A) Functional protein classes identified, and (B) breakdown of the 50% housekeeping protein-like functional classes.

Fig. 2.

Amblyomma americanum tick cement proteins found in both PBS-soluble (PBS-S, black bars) and PBS-insoluble fractions (PBS-I, grey bars) of the tick cement cone. Peptides were subjected to Identification Compare analysis in the Integrated Proteomics Pipeline (IP2). Counts of unique proteins for each functional class (Y axis) are represented as percentages of the total per functional class (X axis).

Fig. 3.

Encoding transcripts of selected *Amblyomma americanum* glycine-rich proteins (GRPs) are responsive to tick feeding. Template cDNA that was synthesized using total RNA from salivary glands that were dissected from unfed and 24, 48, 72, 96 and 120 h partially fed female ticks were subjected to quantitative Reverse Transcriptase (RT)-PCR analysis using the primers listed in Table 1. The relative abundance of transcripts (Y axis) was calculated using the formula 2^{-C_T} as described in Section 2.5. Both alphabetical (A–L) and corresponding GRP identification numbers have been included for ease of description in the text. Note: expression of Aam-326 could not be determined.

Fig. 4.

Transcript abundance of constitutively expressed (CE) *Amblyomma americanum* glycine-rich protein (GRP) increases several thousand fold post ecdysis. Three engorged larvae (A, C, E) and nymph stage ticks (B, D, F) that were incubated for molting were sampled at: days 0 (the day the ticks dropped off the host), 3, 7, 11, 14, 17, 21, the day of ecdysis, and at 1 and 2 weeks post-ecdysis (wk post). Ticks were individually processed for total RNA extraction that was used for cDNA synthesis which was then used for quantitative Reverse Transcriptase (RT)-PCR analysis. The relative abundance of transcripts (Y axis) was calculated using the formula 2^{-C_T} as described in Section 2.5. Each graph was split into two parts (a, b) due to low level transcription during tick molting compared with transcription levels post-ecdysis. (a) Transcription during molting and (b) transcription post ecdysis.

Fig. 5. Disruption of constitutively expressed (CE) and feeding induced (FI) *Amblyomma americanum* glycine-rich protein (GRP) mRNA caused bleeding around tick attachment site. (A) Approximately 1 μ g of GFP-double-stranded (ds)RNA (control) or combinatorial dsRNA that was constructed to disrupt GRP mRNA in clusters according to similar transcription patterns: CE ($n=3$), FI ($n=4$) and up-regulated with feeding (UR, $n=6$) was injected into unfed female *Amblyomma americanum* ($n=20$). dsRNA-injected ticks were maintained at 25°C with relative humidity set to 85% for 3 weeks before evaluating effects of RNA interference (RNAi) silencing on *A. americanum* feeding on rabbits. On day 9, CE ticks were removed from the ear for the well-being of the rabbit due to significant bleeding around the tick attachment sites. (B) To rule out the possibility that ticks attaching closely together (overcrowding) caused the observed effects of RNAi silencing of CE and FI GRP mRNA in the first round, the experiment was repeated with fewer ticks ($n=15$). In both rounds of the experiments, ticks were photographed every 24 h. Arrowheads denote tick attachment sites at which bleeding was observed.

Fig. 6.

Nearly 100% disruption of constitutively expressed (CE) *Amblyomma americanum* glycine-rich proteins (GRP) transcripts in newly ecdysed adult ticks did not prevent tick attachment but caused bleeding around the tick attachment sites. Newly (0 days old) ecdysed adult ticks ($n=11$ for round one, and $n=6$ for round two) were injected with $\sim 1 \mu\text{g}$ of double stranded (ds)-GFP or CE combinatorial (co)-dsRNA and incubated for 2 weeks (round one) or 1 week (round two) before assessing the effects of RNA interference (RNAi) silencing on tick feeding. Validation of RNAi silencing in the (A) first and (B) second round of RNAi silencing. Three ticks were sampled at 24 h after attachment and individually processed to validate disruption of CE mRNA as described in Section 2.8. The three CE GRP targets are noted. Tick feeding phenotypes (C, D) were documented every 24 h. Arrowheads indicate continuous bleeding. (Ea) A single attached female tick (arrowhead) for which an expanding hematoma (circled, Eb) was observed.

Table 1

All primers used in the study.

Semi-quantitative primers	I.D.	Forward primers 5'-3'	Reverse Primers 3'-5'	Annealing Temp. (°C)
Semi-quantitative primers	Aam -179667	CCAGTCGGCTGATGTCCTTG	GTACGACACGAACTGCTG	58
	Aam -16245	TGGCGGTACTTTGGTCGTA	GACGAGACAAGGTCCTGTAG	53
	Aam -178006	CCCTGGAGGATTCAGGCATTC	TGGGTGCGAACTGGGTGTG	56
	Aam -177101	TACTTCGGGTTTCACCTTCCGTG	GCATAGAAGCCACCTTGGGAAC	55
	Aam -177916	TGGTGGTGAAGACGGTCAG	GAGCCTCCATCATATCACTGC	53
	Aam -177785	ACTGGTCTTCTCGGAGGTAC	GCCTCCATAGAGACCTGAGACG	53
	Aam -326	GAATGGGATCAGCGGGTGGTTC	CTCCTTGCCTCCTGTATACAAACG	54
	Aam -177792	ACTGGTCTTCTCGGAGGTAC	CCGAATGATCCCCAAAGGACC	56
	Aam -15	CTTTGACAGTGGCAGCGGAAG	TCGTTCTACTGAGCGTCTCTCC	60
	Aam -179800	TGCAGGTTTTGTGGC	CTTGAAACCCAAACCGT	48
	Aam -181586	AAGCGTATTACGGTGGCTTG	GACCACCGAGACGTCCAAAG	52
	Aam -42453	CTCCAGGCTTGAGGAACC	AGGATCATTAGAGGGCTTCCA	53
	Aam -21	GGTGGTGACTTAGGAGGCAG	GCTGGAGGAACTGTCGGTAG	53
	Actin (AY255624)	GGACAGCTACGTGGGCGACGAGG	CGAATTCACGCTCAGCCGTGGTGG	60
	Dicer (GBBM01005393)	GTGGGTGACTACGTGCTCAA	GACCTCTGACACTCGATGA	60
	Aam-179667	ACTCGGAGGTCTTTTGACAC	CAGTGGATGGTCACTAAGC	55
	Aam-16245	TATGGAGGATACCCGAGGTT	AACCGCATAACCTGGATAC	60
	Aam-178006	TGGAGGATTCAGGCATTC	GGATCGCGTTGGCACTAG	60
	Aam-177101	AAACCAAAGCTGTGCAAG	TGGAGAATCAGGGCATAGAG	60
	Aam-177916	CGTAAACCATTTGTAGCAA	TCGCAATACCAACAGCAGAG	60
	Aam-177785	GGCTCATTTGGACATTGG	AATCCGCTTCCATACAAACC	55
	Aam-326	CTCCAACCAACCGCCGCC	GCCTAGGTGGAGGTTTCG	-
Quantitative reverse transcriptase-PCR primers	Aam-177792	TGGTTTCCCTGGAGTTTACG	CGAAAGGTCCGTAGGTATGG	60
	Aam-15	GTGGAAGTGGTCAAGGAGGA	CTGATTCCCGATAGTATGG	60
	Aam-179800	GCTCCTTTGCTTATGTTTCG	GTCCGAAAGATTCAATGCGT	60
	Aam-181586	GCCGCCCAAGCCACCAG	GGTGGCTTGTATGCGGAGG	55
	Aam-42453	GGGTAATCATGGTCATAG	GGCTCGTATGTTGTGTGG	60
	Aam-21	GAGGAACTGTCGGTAGCTTG	GGAAAGTGGAGGAATGGCAG	60
	RpS4 (GAGD01011247.1)	GGCGCCGAGGTGAAGAA	CCTTGCCGTCACCTTGAT	50;55;60

L.D.	Forward primers 5'-3'	Reverse Primers 3'-5'	Annealing Temp. (°C)
Combinatorial – double-stranded RNA primers	TAAACGACTCACTATAGGGGACATCTGTCTCCACCCGACCCG	TAAACGACTCACTATAGGGGGTGCAGAACTGGGTGTGGGA	65
	TAAACGACTCACTATAGGGGCTTTGTACGGAGGCTACCCAGGAG	TAAACGACTCACTATAGGGGCTTAGGAAAGTGGAGGAAITGGC	60
	TAAACGACTCACTATAGGGGAGATCTGGGCTGCTGGTAT	TAAACGACTCACTATAGGGGTGTGAGAGAGATCCTAG	59

CE, constitutively expressed; FI, feeding induced; UR, up regulated with feeding.