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Silencing of three *Amblyomma americanum* (L.) insulin-like growth factor binding protein-related proteins prevents ticks from feeding to repletion

Albert Mulenga* and Rabuesak Khumthong
Texas A & M University, AgriLife Research, Department of Entomology, College Station, Texas 77843, USA
*Author for correspondence (a-mulenga@tamu.edu)

SUMMARY

The insulin-like growth factor (IGF) binding proteins (IGFBP) family is the regulatory arm of the IGF signaling system that control mitogenic and anabolic actions of IGF peptide hormones. This study describes cloning and biological characterization of three *Amblyomma americanum* (L.) (*Aam*) proteins that show amino-terminal sequence and secondary structure similarity to the IGFBP superfamily. The three molecules here provisionally identified as *Aam*IGFBP-rP1 and short (S) and long (L) *Aam*IGFBP-rP6 are expressed in multiple tick organs and are responsive to tick feeding activity with the former being upregulated and the latter being downregulated. We show that they regulate tick physiological functions that may be related to *A. americanum* tick feeding success as revealed by RNAi-mediated dual silencing of *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L or *Aam*IGFBP-rP1 alone, which caused a reduction in blood meal size compared to the controls. Additionally, in the case of *Aam*IGFBP-rP1 silencing, 47% of ticks died while attempting to feed and those that did survive and spontaneously detached from the host failed to lay eggs. Although *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L show overall identities of 49% and 59%, respectively, to *Rhipicephalus microplus* C protein, the identity level jumps to ~84% when the comparison is restricted to first 70 amino acids of the mature protein. Similarly, the *Aam*IGFBP-rP1 mature protein is ~72%, 87%, 88% and 92% identical to that of *Ixodes scapularis* S, *R. microplus*, *R. appendiculatus* N and *A. variegatum* F, respectively. The observed across-tick-species conservation suggests that the three molecules (*Aam*IGFBP-rP1, *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L) represent target for development of vaccines to protect animals against multiple tick species. The data are discussed with reference to advances in tick molecular biology and the potential of the three proteins as targets for immunizing animals against tick feeding.

Key words: *Amblyomma americanum*, tick feeding, insulin-like growth factor binding proteins.

INTRODUCTION

Ticks are among the most important arthropods affecting humans and animals (Sonenshine, 1993). Direct tick feeding activity can cause damage to their hosts, paralysis, irritation and allergy. However, ticks are mostly known for their role as vectors of numerous tick borne disease (TBD) agents: rickettsiae, protozoa, spirochetes and viruses (Sonenshine, 1993; Jonejan and Uilenberg, 2004). Until the 1980s and early 1990s, when many emerging human TBD agents were described (Spach et al., 1993; Azad and Beard, 1998; Bratton and Corey, 2005; Perlman et al., 2006), ticks were mostly regarded as a veterinary problem. Management and treatment of major animal TBD agents, *Babesia* spp. (babesioses), *Theilleria* spp. (theillerioses), *Anaplasma* spp. (anaplasmoses) and *Ehrlichia ruminatium* (heartwater) continue to be a source of significant monetary losses in the livestock industry especially in tropical and sub-tropical countries (Jongejan and Uillenberg, 2004). In public health, cases of human TBDs, Lyme disease (*Borrelia burdoferi*), human granulocytic anaplasmosis (*A. phagocytophilum*) and human monocytic ehrlichiosis (*Ehrlichia chaffensis*) have been on the rise (Branton and Corey, 2005).

With the exception of *Francisella tularensis* (Petersen et al., 2009) and *Coxiella burnetti* (Simor et al., 1984; Reimer, 1993), which can be acquired through other means, all TBD agents require successful tick feeding to be transmitted. Thus, the avoidance of tick bites through suppression of tick vector populations is the method of choice to prevent TBD transmission. Tick vector populations have traditionally been controlled by acaricide application. Acaricides are essential in the short term, but they do not offer a permanent solution because of disadvantages such as widespread tick resistance to acaricides and contamination of the environment and food chain (Frisch, 1999). Vaccination of animals against tick feeding has been shown to be a practical and sustainable alternative (Frisch, 1999; Willadsen, 2004; Willadsen, 2006; de la Fuente et al., 2007). We are interested in understanding the molecular mechanisms that underlie the regulation of early stage tick feeding. Understanding the feeding process will lead to discovery of tick proteins that are important to pathogen transmission by ticks.

Tick feeding involves multiple behavioral changes that begin with hunger and ends with satiation (Walade and Rice, 1982). The sequence of events leading to successful tick feeding were categorized into nine behavioral changes: (1) attainment of appetence, (2) engaging the host, (3) exploring the host for a feeding site, (4) penetration, (5) attaching onto host skin and establishing the feeding site, (6) ingestion of host blood, (7) engorgement, (8) detaching from the host and (9) disengaging from the host (Walade and Rice, 1982). Identifying tick proteins that regulate various phases of the feeding cycle should enable us to develop novel strategies for controlling ticks and tick-borne pathogens. Several studies have focused on characterizing proteins expressed by ticks during the slow feeding phase (3- to 5-day fed ticks) (Mulenga et al., 2001; Mulenga et al., 2003; Valenzuela et al., 2002; Ribeiro, 2006) primarily because of the ease of obtaining their tissues, in that they are larger ticks. Multiple bioactive enzymes, anti-inflammatory agents, anti-complement, and anti-coagulants, have been identified in this manner (Ribeiro et al., 2006). We are interested in unfed tick proteins that may regulate the preparatory feeding phase as this precedes the key facets of tick parasitism, blood meal feeding and pathogen transmission. We previously identified 40 genes that were differentially upregulated or induced in unfed *Amblyomma americanum* ticks that were stimulated to start feeding (Mulenga et

al., 2007). While analyzing these sequences, we identified two sequence fragments that showed significant amino-terminal structural similarity to the insulin-like growth factor (IGF) binding protein (IGFBP) family.

Members of the IGFBP family are evolutionary conserved cysteine-rich extracellular proteins that have been identified in a wide range of organisms from humans, fish and chickens (Rodgers et al., 2008; Kamangar et al., 2006) to, fruit fly, army fall worm and shrimps (Castellanos et al., 2008; Honegger, 2008; Alic and Partridge, 2008; Andersen et al., 2000). In vertebrates where most studies have been conducted, they are the regulatory arm of the IGF signaling system, a complex interaction that includes IGF-I and IGF-II peptide hormones, IGF receptors and IGFBP proteases (Hwa et al., 1999; Burger et al., 2005; Delaney, 2005). The IGFBP family include six proteins, IGFBP-1 to IGFBP-6 that bind IGF peptide hormones with high affinity and a group of 10 IGFBP-related proteins (IGFBP-rP-1 to -10) that show significant amino-terminal sequence similarity to IGFBP-1 to -6 but bind IGF peptide hormones with low affinity (Hwa et al., 1999; Burger et al., 2005; Delaney, 2005). The six high-affinity binding proteins, IGFBP-1 to IGFBP-6 control anabolic and mitogenic functions of the IGF peptide hormones through sequestration, and indirectly by acting as their transporters, protecting them from degradation, limiting their binding to receptors and maintaining a reservoir of biologically inactive IGFs (Hwa et al., 1999; Delaney, 2005). In other studies, some IGFBPs and IGFBP-rPs have been shown to have intrinsic cell proliferation and tissue growth regulatory functions that are independent of their ability to bind IGF proteins (Hwa et al., 1999; Burger et al., 2005; Delaney, 2005; Sato et al., 2007; Ahmed et al., 2003). Although at the sequence level IGFBP-like proteins have been identified in arthropods, very few functional studies have been reported. A 27 kDa protein that shows sequence similarity to vertebrate IGFBP-rP1 and can bind insulin and IGF peptide hormones has been characterized in *Spodotera frugiperda* (Anderson et al., 2000) and *Drosophila melanogaster* (Honegger, 2008; Alic and Partridge, 2008). The *Drosophila* protein, ImPL-2 was recently shown to negatively regulate growth similar to vertebrate IGFBP-rP1 protein (Alic and Partridge, 2008). In ticks, two indirect studies that reported insulin-like reactivity in the synganglia of *Ornithodoros parkeri* (Zhu and Oliver, 1991) and nymphal and adult *Dermacentor variabilis* ticks (Davis et al., 1994), but there are no reports of the IGFBP family in ticks. In this study we have, for the first time, characterized three tick IGFBP sequences that we have provisionally identified as *A. americanum* (*Aam*) short (S) and long (L) IGFBP-rP6 and *Aam*IGFBP-rP1. We provide evidence suggesting that biological functions regulated by the three molecules are important to *A. americanum* tick feeding success as revealed by RNAi-mediated silencing of the three genes, which caused ticks to obtain significantly smaller blood meals. Most interestingly, *Aam*IGFBP-rP1 silencing also caused 47% mortality, and ticks that did survive and spontaneously detached from the host failed to lay eggs. The data are discussed with reference to advances in tick molecular biology and the potential of the three molecules as target anti-tick vaccine antigens.

MATERIALS AND METHODS

Tick dissections and total RNA isolation

Adult female ticks used in this study were obtained from a colony of *A. americanum* that is maintained on cattle and chickens for use in the laboratory of Dr Pete Teel, in our department. Tick dissections were routinely done as previously described (Mulenga et al., 2003). Briefly, 10 ticks that were partially fed for 24, 48, 72, 96 and 120 h on cattle were rinsed in 70% ethanol. Subsequently ticks were held onto a glass slide with a pair of soft tissue forceps and then the alloscutum edges were trimmed off using a sharp and sterile razor blade. Under a dissection microscope, the dorsal cuticle flap was lifted and tick organs, salivary glands (SG), midgut (MG) and ovary (OV) were teased out using an 18-gauge needle and a soft tissue forceps. Tick organs including the carcass (CA) representing the tick remnant after removal of SG, MG and OV pooled in groups of three or four were stored in RNAlater (Ambion, Austin, TX, USA) at –80°C until used for RNA extraction.

Extraction of total RNA from whole ticks and dissected tick organs was done using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, within the first hour of being detached from the host, whole ticks that were partially fed for 24–120 h were rinsed in 70% ethanol, dried between paper towels, pulverized in liquid nitrogen, and transferred to TRIzol reagent for total RNA extraction. Similarly, for dissected tick organs, they were rinsed in DEPC water to remove the storage solution and then transferred to the TRIzol reagent for RNA extraction. Tissue lysis was accomplished either by repeated pipetting (SG, MG and OV) or homogenization (CA and whole ticks) using a sonic dismembrator, model 100 (Fisher Scientific, Pittsburgh, PA, USA). Extracted total RNA was reconstituted in RNase-free water and stored at –80°C until used.

Cloning and sequence analysis of full-length *Amblyomma americanum* insulin-like growth factor binding proteins-related proteins

Templates for 3′ and 5′ RACE were synthesized from 5 μg of total RNA extracted from whole ticks that were partially fed for 5 days using the RLM RACE cDNA synthesis kit (Ambion) according to the manufacturer's protocol. Primers based on the short (S) and long (L) partial *Aam*IGFBP-rP6 cDNA sequences that were identified in a previous study (Mulenga et al., 2007) were used with 3′ and 5′ RACE templates to amplify full-length cDNAs. TBLASTN scanning of the tick ESTs in the Gene Indices database at the computational biology and functional genomics laboratory (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) using *Aam*IGFB-rP6S or *Aam*IGFB-rP6L cDNAs as query, retrieved a tick EST sequence that is highly conserved in *A. variegutum* (accession no. TC737), *Rhipicephalus microplus* (accession no. TC17305), *R. appendiculatus* (accession no. TC5751) and *Ixodes scapularis* (accession no. TC43412). RACE PCR primers based on consensus sequences of these ESTs were used to clone the third molecule. PCR products were routinely cloned in TOPO TA cloning vectors (Invitrogen) and sequenced using M13 TOPO plasmid-specific primers.

Sequence and bioinformatics analyses

DNA and deduced protein sequences were routinely analyzed using the Vector NTI (Invitrogen) or the MacVector (Accelrys, San Diego, CA, USA) software packages. For comparison with other proteins and provisional identification, DNA and deduced protein sequences were scanned against known entries in GenBank or the Gene Indices database at the computational biology and functional genomics laboratory using the BLASTX, BLASTP and TBLASTN homology search program. Additionally deduced amino sequences were submitted to the ExPASY Proteomics Server (http://ca.expasy.org/) for prediction of signal peptides, and identification of domains and amino acid motifs.

Comparative modeling

To gain insight into secondary structures of *Aam*IGFBP-rPs, the crystal structure of human IGFBP-4 complexed with human IGF-I

[2DSP chain B (Sitar et al., 2006)] was chosen as a molecular template based on PDB-BLAST. Structure-based sequence alignment was performed using Expresso (Armougom et al., 2006) with some slight manual adjustment. Comparative modeling was performed by MODELLER 9v1 (Sali and Blundell, 1993) using sequence alignment in PIR format (generated by GeneDoc) and 2DSP chain B (human IGFBP-4, chain B subtracted by WebLab Viewer as input data. To assess the quality, the *Aam*IGFBP model was evaluated using Verify3D (Luthy et al., 1992) and secondary structures visualized by PyMol 0.99 (DeLano, 2002).

Expression analyses by RT-PCR

To determine temporal and spatial expression patterns, oligo(dT) primed cDNA templates of whole ticks that were fed for 24, 48, 72, 96 and 120 h and tick organs, SG, MG, OV and CA of 120 h of ticks fed ticks were subjected to standard two-step RT-PCR. About 5 μg of total RNA was used to synthesize template cDNA using the VersoMax cDNA synthesis kit (ThermoScientific, New York, NY, USA). Prior to synthesis of cDNA, total RNA was treated with RQ1 DNAse according to the manufacturer's instructions (Promega, Madison, WI, USA) to remove genomic DNA contamination. A 1 μl aliquot of template cDNA was used in a PCR reaction with gene-specific PCR primers. PCR products were electrophoresed on a 2% agarose gel containing 1 μg ml^{-1} ethidium bromide. The tick actin gene (Mulenga et al., 2009) was used for PCR template load control. To determine mRNA abundance, densitograms of amplified PCR bands were determined using the web based ImageJ software (http://rsb.info.nih.gov/ij/). To correct for variations in PCR template concentrations, PCR band densities were normalized as previously described (Mulenga et al., 2008).

RNA interference-mediated gene silencing

To validate their significance in tick feeding, RNA interference (RNAi)-mediated silencing of *AamIGFBP-rP6S* and *AamIGFBP-rP6L* together, or *Aam*IGFBP-rP1 alone, was conducted as previously published (Mulenga et al., 2008). Briefly, double stranded (dsRNA) was synthesized *in vitro* using the MegaScript RNAi kit according to instructions by the manufacturer (Ambion). The rationale to silence *AamIGFBP-rP6S* and *AamIGFBP-rP6L* mRNA together in the same tick was based on their high sequence similarity. Templates for dsRNA synthesis were amplified from cloned plasmid DNA using gene-specific PCR primers (*AamIGFBP-rP6*S/L, For: 5′-GGAACCGCACTGCGAGGGCGTGA-3′, Rev: 5′-CCGGGA-AGAACCCTCAGCGAGG-3′; *AamIGFBP-rP1*, For: 5′-GACG-GGCCGTGCGGCGAGGA-3′, Rev: 5′-CCAGTAGGTGGCCGT-GTCCTCTGG-3′ with added T7 promoter sequence (5′-TAAT-ACGACTCACTATAGGG-3′). Green fluorescent protein (*GFP*, For: 5′-TCACGAACTCCAGCAGGACCATGTGATC-3′, Rev: 5′-ACGTAAACGGCCACAAGTTCAGCGTGTC-3′) PCR primers with added T7 promoter sequence were used to amplify templates for control dsRNA synthesis. Following purification of dsRNA and adjustment of concentration, three groups of 25 unfed *A. americanum* female ticks were microinjected with ~1 μl (~2–3 μg μl^{-1} in TE buffer pH 8.0) of a mixture of *AamIGFB-rP6S* and *AamIGFB-rP6L* dsRNA, *AamIGFBP-rP1* dsRNA alone or *GFP* dsRNA *via* the groove between the basis capituli and the scutum using half-inch 33 gauge needles attached to a 10 μl gas-tight syringe (Hamilton, NV, USA). Injected ticks were kept overnight at 22°C to observe any mortality due to injection injury. Injected ticks that were alive the next morning (*AamIGFBP-rP1*-dsRNA-injected *N*=21, *AamIGFBP-rP6S/L*-dsRNA-injected, *N*=22) and control ticks (no-injected, *N*=25, *GFP*-dsRNA-injected, *N*=22) were placed on cattle to feed. Ticks were confined to feeding area in cells that were secured on the back of the calf using livestock identification cement (Nasco, Ft Atkinson, WI, USA). Ticks were allowed to feed until they naturally detached from the host. Prior to placing female ticks in cells, male ticks (25 per cell) were pre-fed for 3–4 days in to stimulate the release of aggregation pheromones. We have observed that this arrangement synchronizes the female tick feeding cycle.

To validate whether the injection of dsRNA triggered the disruption of target gene mRNA, three ticks per treatment were detached after 48–60 h. The 48–60 h time point was chosen to ensure that the ticks that were sampled for the assay were alive and had a chance to attempt feeding. These ticks were individually processed for total RNA extraction. Total RNA was treated with RQ1 DNAse (Promega, Madison, WI, USA) to remove genomic DNA contamination and then subjected to standard two-step semi-quantitative RT-PCR. To forestall the possibility of re-amplifying the injected dsRNA during validation of target gene silencing, PCR primers were set to cDNA regions flanking the domain that was used for dsRNA synthesis.

Photographs of ticks that had spontaneously detached from the host were used to document the observed physical phenotypes. To assess the effects of gene silencing on the ability of ticks to complete feeding, tick feeding parameters, numbers of ticks that attached at 24 h following placement of ticks on the animal, mortality during feeding, time to spontaneous detachment from the host and engorgement masses (EM; as an index of amount of blood imbibed) were evaluated. For statistical analysis, the observed EMs were subjected to one way analysis of variance (ANOVA) followed by pair-wise comparison of means using the Tukey HSD (honestly significant differences) test with confidence levels set by default to studentized range statistic (Q) of 0.05 and 0.01 using the web-based statistical calculator, VassarStats (http://faculty.vassar.edu).

RESULTS

cDNAs cloning, provisional identification, sequence analysis and comparative modeling

We cloned three ~1300–1400 base pair full-length cDNAs encoding 152, 168 and 248 amino acid residue polypeptides that we have provisionally identified as *Aam*IGFBP-rP6S (accession number GU907778), *Aam*IGFBP-rP6L (accession no. GU907779; Fig. 1A) and *Aam*IGFBP-rP1 (accession no. GU907780; Fig. 1B), respectively. Provisional identification was based on conservation of the IGFBP domain (InterPro pattern 'PS51323', ExPASY, http//ca.expasy.org), which characterizes the amino terminus region of the IGFBP superfamily (first 70–80 amino acid residues of the mature protein) and the carboxyl-terminal domain structures described by Hwa et al. (Hwa et al., 1999). As shown in Fig. 1B, the carboxyl-terminal regions of *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L do not show any similarity to known domains or protein motifs, consistent with human IGFBP-rP6 (Hwa et al., 1999). Additionally, similar to human *IGFBP-rP6* cDNA, which is characterized by a long 3′ untranslated region (UTR) (Lassalle et al., 1996), both *AamIGFBP-rP6S* and *AamIGFBP-rP6L* cDNAs have ~800 bp 3′ UTR (not shown). However, unlike human *IGFBP-rP6* 3′ UTR, which has a series of AU rich elements, tick sequences do not to contain any (results not shown). Furthermore human *IGFBP-rP6* has an open reading frame of 552 (Lassalle et al., 1996) compared to 459 and 507 for *AamIGFBP-rP6S* and *AmIGFBP-rP6L*, respectively (not shown). The short and long designation of *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L is based on the fact that they are highly similar and the major difference is the 14 amino acids (Fig. 1A) or 42 nucleotides (not shown) deletion in the C-terminal

A

Signal peptide | IGFBP superfamily domain

```
AamIGFBP-rP6L   1 MKIALVFFLLAAATVCVYATSEPHCEGVTC 30
AamIGFBP-rP6S   1 MKIALAFFLLAAAAVCVYATSEPHCEGVTC 30
RmIGFBP-rP6S    1 MKIVLAFLLIALAAVCVSATSAPNCEGVTC 30
HumIGFBP-rP6    1 MKSVLLLTTLLVPAHLVAAWS--NNYAVDC 28

AamIGFBP-rP6L  31 DPETCPKHEC----HCG-SHKDACGCCDFC 55
AamIGFBP-rP6S  31 DPETCPKHEC----HCG-SHKDACGCCDFC 55
RmIGFBP-rP6S   31 DPETCPKHEC----QCG-SHKDACGCCDFC 55
HumIGFBP-rP6   29 -PQHCDSSECKSSPRCKRTVLDDCGCCRVC 57

AamIGFBP-rP6L  56 NKCANEECHP--EHND--KCAEGHHCVVKA 81
AamIGFBP-rP6S  56 NKCANEECHP--EHND--KCAEGHHCVVKP 81
RmIGFBP-rP6S   56 NKCLEEECHP--EHND--RCAEGLHCVIG- 80
HumIGFBP-rP6   58 AAGRGETCYRTVSGMDGMKCGPGLRCQPSN 87

AamIGFBP-rP6L  82 AGDDHGHATGTGHCE--PVE-------SSTG 103
AamIGFBP-rP6S  82 AGDDHGHATG--HCE--PVE-------SSTG 101
RmIGFBP-rP6S   81 ----EDATAG--HCK--PGG-------ASTA 96
HumIGFBP-rP6   88 GEDPFGEEFG--ICKDCPYGTFGMDCRETC 115

AamIGFBP-rP6L 104 HDGAHGHDEAHG-------------HNETHG- 121
AamIGFBP-rP6S 102 HDGTHGHDGAHG-------------HNETHG- 119
RmIGFBP-rP6S   97 TPEVNVPD-----------------SEAP-- 108
HumIGFBP-rP6  116 NCQSGICDRGTGKCLKFPFFQYSVTKSSNR 145

AamIGFBP-rP6L 122 HDGAHGHDETHGHAAAPDSEAPVQDAHHHA 151
AamIGFBP-rP6S 120 HDGAHGHDETHGHAATPDSETPAQD----- 144
RmIGFBP-rP6S  109 VDEHHAHHEGGEHAKT-------------- 124
HumIGFBP-rP6  146 FVSLTEHDMASGDGNIVREEVVKEN----- 170
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*Aam*IGFBP-rP6S deleted peptide

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AamIGFBP-rP6L 152 EEGSEHGHSGGDSAHHE----- 168
AamIGFBP-rP6S 145 ---------GGDSAHHE----- 152
RmIGFBP-rP6S  125 ----------GEEAHHE----- 131
HumIGFBP-rP6  171 --------AAGSPVMRKWLNPR 184
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B

Signal peptide | IGFBP superfamily domain

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AamIGFBP-rP1    1 -----MRAVHCTP-LLLLALCGLLAPASSRKECGPCDLDRCEPPS 39
AvarIGFBP-rP1   1 -----MSAVHCAPLLLLLALCGLLAPASTRKECGPCDLGRCEPPS 40
RmIGFBP-rP1     1 -----MSSTVHPTVLLLVLLAAAASAA--RHECGPCDLDRCEPPR 38
RapIGFBP-rP1    1 -----MSSIAHATLLLLMLLVAAASAA--RRECGPCDPDRCEPPR 38
IxscIGFBP-rP1   1 -----------MKRVLLFVLSAPWPPWP-PDECGPCDPDACRTP- 32
HumIGFBP-rP1    1 MERPSLRALLLGAAGLLLLLLPLSSSSS-SDTCGPCEPASCPPLP 44

AamIGFBP-rP1   40 -GQCLAGIVQDACGCCMVCGQREGRRCYHRSVKGSLVDGPCGEDL 83
AvarIGFBP-rP1  41 -GQCLAGIVQDSCGCCMVCGQREGQRCYHRSVKGSLVDGPCGEDL 84
RmIGFBP-rP1    39 -GQCLSGIVKDACGCCMVCGQREGQRCYHRSVKGSLADGPCGEDL 82
RapIGFBP-rP1   39 -GQCLSGIVQDACGCCMVCGQREGQRCYHRSVKGSLADGPCGEDL 82
IxscIGFBP-rP1  33 -DSCLAGLVKDACGCCMVCGQREGHRCYHRSVAGSWEHGPCGEDL 76
HumIGFBP-rP1   45 PLGCLLGETRDACGCCPMCARGEGEPCGG----GGAGRGYCAPGM 85

AamIGFBP-rP1   84 ECRL---RRD----LAPGDP-AEALCVCSRQEPVCGTDGVTYDNV 120
AvarIGFBP-rP1  85 ECRL---RRD----LAPTDP-AEALCVCSRPEPVCGTDGVTYDNV 121
RmIGFBP-rP1    83 ECRP---RRD----LAPGDP-AVALCVCSRPEPVCGSDGVTYDNV 119
RapIGFBP-rP1   83 ECRP---RRD----LAPGDP-AEALCVCSRPEPVCGSDGVTYDNV 119
IxscIGFBP-rP1  77 DCRP---RAD----LAPGDP-AEALCVCNKAWPMCGSDGVTYDSV 113
HumIGFBP-rP1   86 ECVKSRKRRKGKAGAAGGPGVSGVCVCKSRYPVCGSDGTTYPSG 130
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Kazal-type serine proteinase inhibitor domain

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AamIGFBP-rP1  121 CQLTEARYRLRN-G---LEAASRGPCYSAPRVVTAPENTRNRTGG 161
AvarIGFBP-rP1 122 CQLTEARYRLRN-G---LEAASRGPCYSAPKVVTAPENTRNRTGG 162
RmIGFBP-rP1   120 CQLTEQRYKMRN-G---LEAASRGPCYTAPRVVTPPENTRNRTGG 160
RapIGFBP-rP1  120 CQLTEQRYKMRN-G---LEAASRGPCYTAPRVVTPPENTRNRTGG 160
IxscIGFBP-rP1 114 CQLTEARYSRRD-G---LEAASRGPCMAAPRVVTPPEHTRNYTGG 154
HumIGFBP-rP1  131 CQLRAASQRAESRGEKAITQVSKGTCEQGPSIVTPPKDIWNVTGA 175
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Immunoglobulin domain

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AamIGFBP-rP1  162 RAAMTCEVSGFPVPTIEW-RVDRGDG---PLKSLPTDSSRINVQS 202
AvarIGFBP-rP1 163 RAAMTCEVSGFPVPTIEW-RVDHGNG---PLKPLPTDSSRINVQS 203
RmIGFBP-rP1   161 RAAMTCEVTGYPVPTVEW-RVDRGEG---PLKGLPTDSSRISVQS 201
RapIGFBP-rP1  161 RAAMTCEVTGYPVPTVEW-RVDHGEG---PLKALPTDSSRISVQS 201
IxscIGFBP-rP1 155 RAAMSCEVMGYPVPSVEW-RVDRGNG---VQQALPTDDPRLSVQT 195
HumIGFBP-rP1  176 QVYLSCEVIGIPTPVLIWNKVKRGHYGVQRTELLPGDRDNLAIQT 220

AamIGFBP-rP1  203 RGGPDSFEVTSWLQLLDPRPEDTATYWCAPANQNAEPSAAAKLNV 247
AvarIGFBP-rP1 204 RGGPESYEVTSWLQLLDLRPEDTATYWCVGANENGEVSAAAKLNV 248
RmIGFBP-rP1   202 RGGPDSFEVTSWLQLLDLRPEDTATYWCVGSNENGETSAAAKLNV 246
RapIGFBP-rP1  202 RGGPDSFEVTSWLQLLDLRPEDTATYWCVGANENGETSAAAKLNV 246
IxscIGFBP-rP1 196 RGGPNSYELTSWLQLLSLTAADTATYWCVAANELGEASAAAKLNV 240
HumIGFBP-rP1  221 RGGPEKHEVTGWVLVSPLSKEDAGEYECHASNSQGQASASAKITV 265

AamIGFBP-rP1  248 LP----------------- 249
AvarIGFBP-rP1 249 LP----------------- 250
RmIGFBP-rP1   247 LP----------------- 248
RapIGFBP-rP1  247 LP----------------- 248
IxscIGFBP-rP1 241 IR----------------- 242
HumIGFBP-rP1  266 VDALHEIPVKKGEGAEL 282
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Fig. 1. See next page for legend.

Fig. 1. Multiple sequence alignment analysis. (A) ClustalW was used to align predicted *A. americanum* (*Aam*) IGFBP-rP6S and *Aam*IGFBP-rP6L proteins with the translational product of *Rhipicephalus microplus* (*Rm*) EST no. TC890 and human IGFBP-rP6 protein (NM_007036). (B) Alignment of putative *Aam*IGFBP-rP1 with *A. variegutum* (*Avar*, accession no. TC737), *R. microplus* (*Rm,* accession no. TC17305), *R. appendiculatus* (*Rap*, accession no. TC5751), *I. scapularis* (*Ixsc*, accession no. TC43412) and human (Hum) IGFBP-rP1 (accession no. NM_001553). The signal peptide is indicated by a line with an arrowhead and the IGFBP superfamily consensus amino acid motif is indicated by a dashed line above. Within the IGFBP domain, filled diamonds denote the 12 consensus cysteine residues whereas the consensus 'CGCCXXC' amino acid motif is boxed. In A, the 14 amino acid deletion in *Aam*IGFBP-rP6S is indicated by a solid line above. In B, the Kazal-type serine proteinase inhibitor domain is indicated by a solid line below. Within the Kazal-type serine proteinase inhibitor domain, the filled circles denote the consensus cysteine residues. The immunoglobulin domain in *Aam*IGFBP-rP1 (B) is indicated by a dotted line above. Please note that shading denotes conserved amino acid residues. Additionally, note that HumIGFBP-rP6 sequences that are deleted in ticks are also shaded gray.

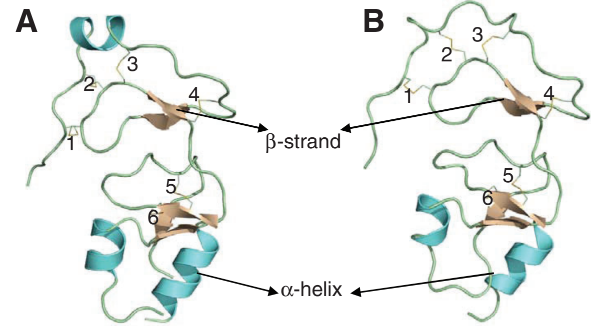

Fig. 2. Models of the N-terminal domain of *Aam*IGFBP-rP6L. The human IGFBP-4 (2DSP) structure (A) (Sitari et al., 2006) was used as template to develop the model of the amino-terminal domain of *A. americanum* IGFBP-rP6L (B) by comparing their molecular templates. Six pairs of conserved disulfide bridges are shown and numbered 1 to 6.

region. In the case of *Aam*IGFBP-rP1 (Fig. 1B) its C-terminal domain possess the Kazal-type serine proteinase inhibitor (InterPro pattern 'PF076648') and immunoglobulin-like (InterPro pattern 'PS50835') domain (Fig. 1B), consistent with human IGFBP-rP1 sequence features (Hwa et al., 1999). Sequence alignments shown in Fig. 1A,B, show that the 12 cysteine residues and the signature amino acid motif 'CGCCXXC' that characterize the amino terminus region of the IGFBP superfamily (Hwa et al., 1999) are conserved in ticks (Fig. 1A,B). Scanning of all three putative proteins on the SignalP server (http://www.cbs.dtu.dk/services/SignalP/) revealed a 19 amino acid signal peptide (SP) in *Aam*GFBP-rP6S and *Aam*IGFBP-rP6L, and a 23 SP in *Aam*IGFBP-rP1.

Given this conservation, we thought to develop comparative models of *Aam*IGFBPs based on the first 90 amino acids of the mature protein. The comparative model (Fig. 2) was confirmed to be of high quality as determined by a 'verify3D' score of +0.11 to +0.52 for 86 of the 90 residues. Verify3D scores below +0.1 are indicative of serious problems in the model (Janusz et al., 2002), which in this case accounted for only 4 of the 90 residues involved in the model (not shown). Based on the model in Fig. 2A, *Aam*IGFBP amino-terminal domain has secondary structures that are similar to the human IGFBP-4 (Sitar et al., 2006), except that the tick has two α-helices whereas human IGFBP-4 has three (Sitar et al., 2006).

AamIGFBP-rPs are conserved in other ticks

When compared with each other, putative *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L proteins are ~86 and 89% identical at the amino acid and nucleotide (not shown) levels, respectively. The identity levels drops to ~20% and 28% when both *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L are compared with human IGFBP-rP6 (accession no. NM_007036) and *Aam*IGFBP-rP1 (not shown), respectively. When scanned against tick EST sequences on the Gene Indices database (http://compbio.dfci.harvard.edu), *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L showed 49% and 59% overall amino acid identity to a *Rhipicephalus microplus* (*Rm*) EST (TC890) translational product (Fig. 1A). However, when sequence comparison was restricted to the first 70 amino acid residues, the identity levels between *Aam*IGFBP-rP6S or *Aam*IGFBP-rP6L and *Rhm*IGFBP-rP6S sequences jumped to 84% and 82%, respectively (not shown). The TC890 EST is provisionally identified as *Rm*IGFBP-rP6S in this study because of closer similarity to *Aam*IGFBP-rP6S (Fig. 1A) and the 27 amino acid deletions in its carboxyl-terminal region (Fig. 1A). In the case of *Aam*IGFBP-rP1, it shows ~92, 88, 87, 72 and 34% amino acid identity to *A. variegutum* (accession no. TC737)*, R. microplus* (accession no. TC17305), *R. appendiculatus* (accession no. TC5751), *Ixodes scapularis* (accession no. TC43412) and human IGFBP-rP1 (accession no. NM_001553), respectively.

AamIGFBPs mRNAi expression analyses by RT-PCR

In a previous study, we used real-time RT-PCR analyses to determine that both *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L were expressed in the SG, MG, OV and CA (the remaining carcass) and that their transcript abundance decreased with tick feeding (Mulenga et al., 2007). In this study, results summarized in Fig. 3A show that *Aam*IGFBP-rP1 is expressed in SG, MG, OV and CA as revealed by semi-quantitative RT-PCR. *Aam*IGFBP-rP1 is expressed to a similar degree in SG, MG and CA; in the OV, however, the expression level was about one sixth that of the other tissues (Fig. 3B). At the temporal level, *Aam*IGFBP-rP1 is expressed in ticks that were fed for 1–5 days (3C) with transcript abundance apparently increasing at least fourfold by day 5 of feeding (Fig. 3D).

RNAi-mediated dual silencing of *AamIGFBP-rP6S* and *AamIGFBP-rP6L* or *AamIGFBP-rP1* alone caused ticks to obtain significantly smaller blood meals

Qualitative two-step RT-PCR expression analysis summarized in Fig. 4 was used to validate whether or not the injection of dsRNA into ticks triggered disruption of the target gene mRNA. The expectation was that if the mRNA of the target gene, was completely disrupted, its cDNA would not be amplified. On this basis, both *AamIGFBP-rP6S* and *AamIGFBP-rP6L* mRNA were partially disrupted as faint PCR bands were detectable (Fig. 4). However, in the case of *Aam*IGFBP-rP1, its mRNA was apparently completely silenced as no bands were amplified (Fig. 4). In order to assess the effects of silencing, ticks were allowed to feed on cattle until spontaneous detachment. At 24 h after ticks were placed on cattle to feed, we observed that both control and treatment ticks were attached to host skin, indicating that silencing of the three molecules did not affect the ability of ticks to start feeding (not shown). However, results summarized in Figs 5 and 6 show that dual silencing of *AamIGFBP-rP6S* and *AamIGFBP-rP6L* mRNA or *AamIGFBP-rP1* mRNA alone caused ticks to obtain smaller blood meals. Fig. 5 documents the observed physical phenotypes. During the first 4 days of feeding, phenotypes of treatment groups were

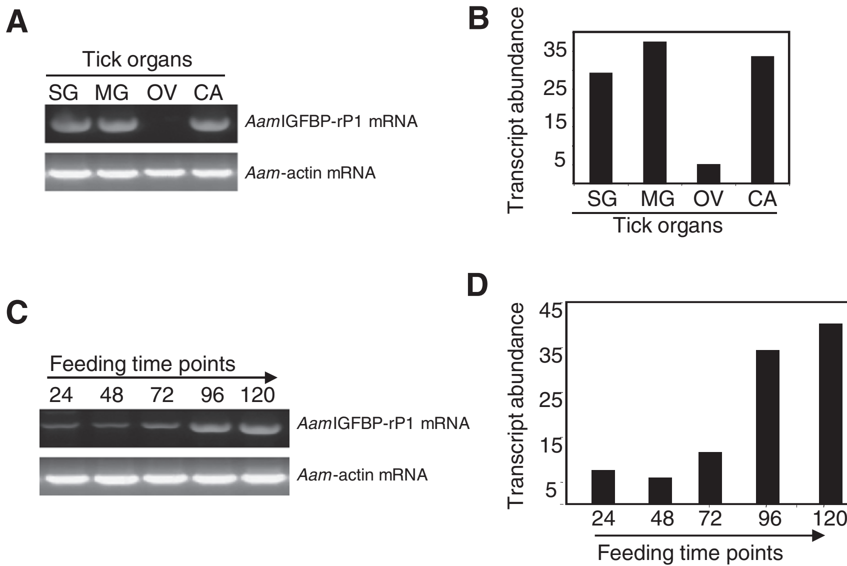

Fig. 3. Temporal and spatial RT-PCR expression analyses of *Aam*IGFBP-rP1. Oligo(dT) primed cDNA templates of whole ticks that were partially fed for 24–120 h, and of the dissected organs: salivary glands (SG), midgut (MG), ovary (OV) and the tick remnant carcass (CA), of 5-day fed ticks were subjected to two-step RT-PCR (A,C). Densities of visualized PCR bands were determined using the ImageJ software and then normalized (B,D) against tick actin, as previously described (Mulenga et al., 2008). Tick actin primers (Mulenga et al., 2009) were used for sample loading control. Results shown here are representative of three individual tick replicates.

similar to controls. However, from day 5 of feeding, *AamIGFBP-rP1*-dsRNA-injected ticks assumed a darkish coloration, which became progressively darker (* in Fig. 5). To measure the effects of silencing on the tick's ability to take in host blood, engorgement mass (EM) was determined. The range of EMs, 780–940 mg (N=12) and 750–1070 (N=11) for non-injected and *GFP*-dsRNA-injected controls, 270–640 (N=11) and 285–541 (N=8) for *AamIGFBP-rP6S/L-* and *AamIGFBP-rP1*-dsRNA-injected, respectively. A one-way analysis of variance to examine differences among EMs revealed that there were significant differences among the four groups ($F_{3,38}$=63.72, P<0.0001). A *post-hoc* pair-wise comparison of mean EMs summarized in Fig. 6 with Tukey's HSD (α=0.05, Q=2.7) revealed that both the *AamIGFBP-rP6S* and *AamIGFBP-rP1*-dsRNA-injected group (512±34) and the *AamIGFBP-rP1*-dsRNA-injected (417±39) were significantly smaller than non-injected (883±12) and *GFP*-dsRNA-injected (830±27) control groups (P<0.01). Although apparently smaller, the mean EM of *AamIGFBP-rP1*-dsRNA-injected ticks was not statistically different from ticks injected with the dual mixture of *AamIGFBP-rP6S/L* and *AamIGFBP-rP1* dsRNA. When incubated at 25°C for egg laying, all *AamIGFBP-rp1*-dsRNA-injected ticks failed to lay eggs, whereas all of those injected with *AamIGFBP-rP6S* and *AamIGFBP-rP6L* dsRNA laid eggs (results not shown).

DISCUSSION

This study describes, for the first time, cloning, bioinformatics analyses and biological characterization of three *A. americanum* IGFBP-like proteins, here provisionally identified as *Aam*IGFBP-rP6L, *Aam*IGFBP-rP6S and *Aam*IGFBP-rP1. Based on data in this study, we are unable to conclusively determine whether or not the three molecules in this study are functionally similar to vertebrate proteins. However, the adoption of secondary structures (Sitar et

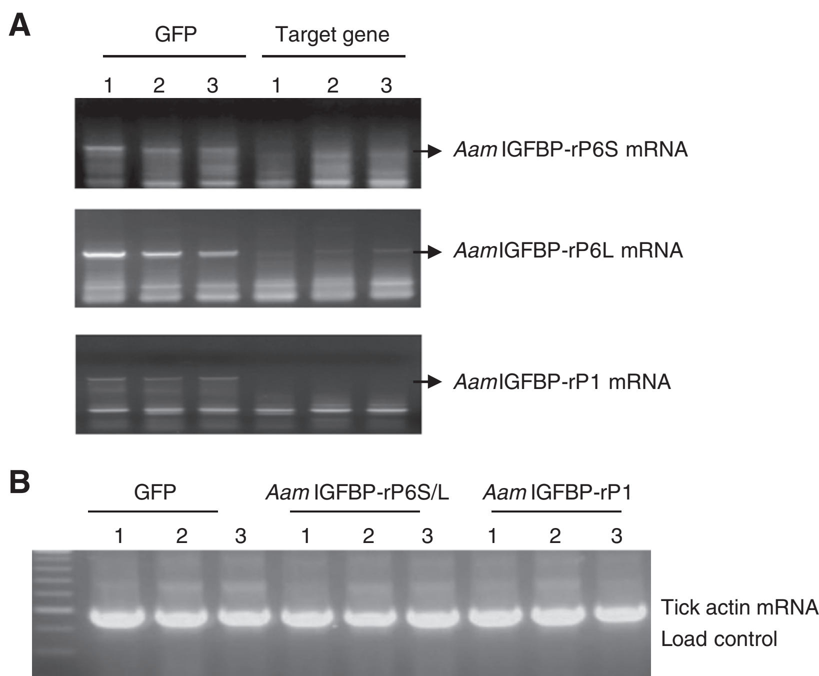

Fig. 4. Validation of RNAi-mediated silencing of *Aam*IGFBP-rP6S, *Aam*IGFBP-rP6L and *Aam*IGFBP-rP1. Three ticks were injected with double stranded RNA (dsRNA) GFP (control), and the dual mixture of *AamIGFBP-rP6S-* and *AamIGFBP-rP6L-* or *AamIGFBP-rP1*-dsRNA alone were manually detached at 48 h post attachment. These ticks were individually processed for total RNA using the TRIzol reagent and treated with DNAse to eliminate genomic DNA contamination. Total RNA was then subjected to two-step RT-PCR using PCR primers that were set on sequences flanking the dsRNA region. PCR products were analyzed on 2% agarose gels containing 1 μg ml^{-1} ethidium bromide. Arrowheads indicate the amplified target gene cDNA. Panels A and B represent validation of gene silencing and sample load control, respectively.

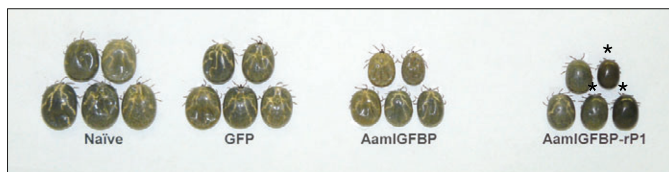

Fig. 5. Ticks were photographed after spontaneously detaching from their host. Ticks shown are representatives of non-injected (naïve; *N*=8), green fluorescent (GFP) dsRNA-injected control ticks (*N*=9), those injected with the dual mixture of *AamIGFBP-rP6S-* and *AamIGFBP-rP6L*-dsRNA (*N*=7) and *AamIGFBP-rP1*-dsRNA (*N*=8) that had spontaneously detached by day 10. The asterisks (*) indicate *AamIGFBP-rP1*-dsRNA-injected ticks that were moribund and assumed a blackish coloration from day 5 of feeding.

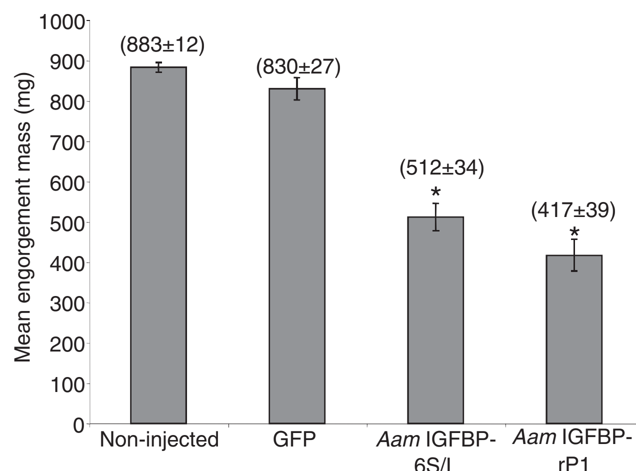

Fig. 6. The effect of RNAi-mediated silencing of *Aam*IGFBP-rP6S, *Aam*IGFBP-rP6L and *Aam*IGFBP-rP1 on *Amblyomma americanum* female tick feeding success. Control ticks, non-injected (naïve) and those injected with double stranded (dsRNA) GFP were fed on cattle along side those injected with the dual mixture of *AamIGFBP-rP6S-* and *AamIGFBP-rP6L*-dsRNA or *AamIGFBP-rP1*-dsRNA alone. After spontaneously detaching from the host, ticks were individually weighed. Mean engorgement masses (EMs; in mg) were used to plot the bar graph using Microsoft Excel software. The asterisks (*) denotes that mean EMs of indicated treatment groups were significantly smaller than the both control groups, $P<0.01$. Error bars indicate ± s.e.m.

al., 2006) and conservation of key amino acid residues that characterize the IGFBP protein superfamily (Hwa et al., 1999) as revealed by our comparative modeling and sequence alignment analyses data indicates that *Aam*IGFBP-rP6S, *Aam*IGFBP-rP6L and *Aam*IGFBP-rP1 proteins, are likely to regulate biological functions that are similar to or related to their mammalian counterparts. Temporal gene expression patterns in total RNA of ticks at different feeding phases have been used as valuable tool to gain insight on probable role(s) or establish a biological relationship to tick feeding regulation (Mulenga et al., 2001; Mulenga et al., 2008; Leboule et al., 2002). On the basis of our temporal mRNA expression analysis data we speculate that *Aam*GFBP-rP6S and *Aam*IGFBP-rP6L, which are highly expressed during the first 24 h of feeding, but are downregulated as ticks continue to feed (Mulenga et al., 2007), were associated with regulating early stage tick feeding events such as formation of the feeding site, whereas *Aam*IGFBP-rP1, which is upregulated in response to feeding may be associated with regulating continuous events such as blood meal feeding and/or maintenance of the tick-feeding lesion.

From the perspective of our long-term interest to find tick proteins that regulate feeding as targets for immunizing animals against tick feeding and prevent pathogen transmission, an important goal of this study was to investigate the effect of RNAi-mediated silencing of the three molecules on *A. americanum* feeding and reproduction. The substantially smaller blood meals obtained by ticks as a result of either dual silencing of *Aam*IGFBP-rP6L and *Aam*IGFBP-rP6S or *Aam*IGFBP-rP1 alone suggest that these three proteins may be associated with physiological functions that are related to the tick feeding pathway. Of particular interest to us was the observation of 47% mortality and a failure to lay eggs in *Aam*IGFBP-rP1-silenced ticks, all of which will lead to reduced tick populations and lower frequency of tick-borne disease transmission, the ultimate goal of any tick control program.

The observation that EMs of ticks that were injected with *Aam*IGFBP-rP1 dsRNA was not statistically significantly different from those injected with the dual mixture of *Aam*IGFBP-rP6L and *Aam*IGFBP-rP6S dsRNA, rules out the possibility that the failure to lay eggs in the former was caused by ticks taking in an insufficient amount of blood. Indirect evidence in rainbow trout in which IGFBP-rP1 is upregulated during vitellogenesis and oocyte maturation (Kamangar et al., 2006) suggest the possibility that *Aam*IGFBP-rP1 silencing may have disrupted the vittelogenesis and/or oocyte maturation process of ticks leading to failure to lay eggs. It is important to point out here that, based on our RT-PCR data, *Aam*IGFBP-rP1 is weakly expressed in the ovary compared with salivary glands and midguts. This may be explained by the fact that we tested *AamIGFBP-rP1* mRNA expression in RNA of ticks that were fed for up to 120 h. This period is well before the rapid tick feeding phase when vittelogenesis and oocyte maturation starts (Friesen and Kaufman, 2009; Coons et al., 1989). It is possible that similar to rainbow trout (Kamangar et al., 2006), *AamIGFBP-rP1* mRNA is upregulated from the rapid tick feeding phase, which was not investigated in this study.

The EM as used in this study is not an accurate measure of the amount of blood imbibed by the tick (Kaufman, 2007). The tick feeding process consists of alternating episodes of blood sucking and the tick secreting excess fluids through its saliva back into the host, which results in an approx. two- to threefold concentration of the final blood meal (Kaufman and Philips, 1973; Koch et al., 1974; Koch and Sauer, 1984). Thus it has been estimated that the total volume of whole blood taken in by the tick is somewhere between 200 and 300 times the unfed female body mass although the EM is only about 100 times the unfed mass (Kaufman, 2007). It is not known at present whether or not silencing of the three molecules affected the tick's ability to feed *per se*, or if there is some other physiological process that affects feeding indirectly by interfering with the uptake of blood in or the process to concentrate the blood meal. Being among the recently described members of the IGFBP family, very little is known about biological functions of IGFBP-rP6 (Sarrazin et al., 2006) and thus we unable to predict the probable role (s) of *Aam*IGFBP-rP6S and *Aam*IGFBP-rP6L in regulating tick feeding. However, there is potential for *Aam*IGBP-rP1 to be indirectly involved in regulating blood meal feeding by mediating the anti-coagulant and vasodilation functions of tick saliva. Vertebrate IGFBP-P1 is involved in several biological functions, including stimulating the secretion of prostacylin (Hwa et al., 1999; Burger et al., 2005). Prostacyclin (PGI_2) is a potent inhibitor of platelet aggregation and a vasodilator (Rosenblum and El-Sabban, 1979) that has been found in huge amounts in the saliva of *I.*

scapularis (Ribeiro et al., 1988). Similarly, Aljamali et al. (Aljamali et al., 2002) found 6-keto-PGF(1alpha), a breakdown product of PGI_2 in salivary glands of *A. americanum* PGI_2 is thought to facilitate tick feeding by blocking platelet aggregation to prevent blood coagulation and causing vasodilation to increase blood flow to the tick-feeding site (Ribeiro et al., 1988). The expected response to the tick feeding style of lacerating host tissue to create its feeding site and then sucking up blood from the hematoma that forms in the wounded area should stimulate vasoconstriction to slow the blood flow to wounded area and blood coagulation to stop further blood loss. Ticks successfully feed by secreting numerous molecules, such as PGI_2, that keep host blood in a fluid state at the tick-feeding site and in the gut. Studies have shown that platelet aggregation function, and not blood coagulation in general, was critical to controlling bleeding of small blood vessels such as those injured by ticks during tick feeding (Mustard and Packham, 1977; Packham, 1994). Thus it is not surprising that tick saliva contains a high titer of PGI_2 [535 $ng\,ml^{-1}$ in *I. scapularis* (Ribeiro et al., 1988) and 124–134 $ng\,ml^{-1}$ in *A. variegatum* (Martinez et al., 1993)]. PGI_2 can disaggregate clumped platelets at concentrations of as low as 1 $ng\,ml^{-1}$ (Randomski et al., 1987). Bowman et al. (Bowman et al., 1995) have proposed a possible pathway of PG synthesis in ticks from blood meal-derived arachidonic acid. However, specific mechanisms regulating PGI_2 secretion in ticks are not known. It will be exciting to investigate whether or not, similar to vertebrates, *Aam*IGFBP-rP1 is involved the secretion pathway of PGI_2 in ticks. The possibility of *Aam*IGFBP-rP1 being involved in the secretion pathway of PGI_2 raises exciting prospects to indirectly block the role of PGI_2 in tick feeding by immunizing animals against *Aam*IGFBP-rP1 function. The expectation is that during tick feeding, antibodies to recombinant *Aam*IGFBP-rP1 will bind and interfere with biological functions of native *Aam*IGFBP-rP1 in the tick to block PGI_2 secretion.

Although massive tick infestations can affect the productivity of animals, ticks cause more damage because of their role as vectors of tick-borne diseases (Jonejan and Uilenberg, 2004). Except for certain tick borne viruses such as Powassan (POW) virus that are transmitted into the host within minutes of the tick attaching onto host skin (Ebel and Kramer, 2004), there is clear evidence that numerous tick-borne pathogens such as agents of Rocky Mountain spotted fever (*Rickettsia rickettsii*), Lyme disease (*Borrelia burgdorferi*), human babesiosis (*Babesia microti*) and human granulocytic ehrlichiosis (*Anaplasma phagocytophilum*) require ticks to feed for at least 48 h before these agents are reactivated, start to replicate and are able to be transmitted to the vertebrate host (Katavolos et al., 1998; Spencer and Parker, 1923; Piesman and Spielman, 1980; Piesman et al., 1987).

From the perspective of ticks being important because of their role as vectors, an ideal target anti-tick vaccine antigen should be one that if disrupted blocks ticks from starting to feed and implicitly prevent tick-borne pathogen transmission. In this light one may argue against the potential of the three proteins in this study being suitable targets for anti-tick vaccine antigens as their silencing did not stop ticks from starting to feed. It is also noteworthy that the 47% tick mortality observed in *Aam*IGFBP-rP1 silencing occurred after 5 days of feeding, by which time most pathogens will have been transmitted. We are, however, interpreting these observations with caution, for two reasons. The first is that tick-feeding regulation is likely to be a multi-protein-regulated activity and preventing ticks from starting to feed may require knocking out multiple, yet unknown, tick proteins alongside the three described in this study. Secondly and most importantly, the RNAi methodology as used in tick research has limitations, as it does not block functions of proteins that are already expressed prior to silencing the target mRNA. The implication of this is that for genes that are expressed in unfed ticks such as the three molecules in this study, silencing of their mRNA will not block the functions of the pre-existing protein at the start of feeding and thus the effects of silencing will be delayed until the pre-existing protein become degraded. This means that, if any of the three molecules in this study played a role in regulating early-stage tick feeding events such as tick attachment onto host skin or formation of the tick feeding lesion, the RNAi-mediated silencing of the mRNA will not prevent ticks from starting to feed. This may explain why the effects of silencing the three molecules in this study were only observable from the fifth day of feeding. Given the diversity of tick species that infest animals in nature, tick vaccines targeting highly conserved antigens such as the three molecules described in this study are desirable. Studies are underway to evaluate the utility of the three proteins in this study as anti-tick vaccine antigens.

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REFERENCES

Ahmed, S., Yamamoto, K., Sato, Y., Ogawa, T., Herrmann, A., Higashi, S. and Miyazaki, K. (2003). Proteolytic processing of IGFBP-related protein-1 (TAF/angiomodulin/mac25) modulates its biological activity. *Biochem. Biophys. Res.* **310**, 612-618.

Alic, N. and Partridge, L. (2008). Stage debut for the elusive *Drosophila* insulin-like growth factor binding protein. *J. Biol.* **7**, 18.

Aljamali, M., Bowman, A. S., Dilwith, J. W., Tucker, J. S., Yates, G. W., Essenberg, R. C. and Sauer, J. R. (2002). Identity and synthesis of postaglandins in the lone star tick, *Amblyomma americanum* (L.), as assessed by radio-immunoassay and gas chromatography/mass spectrometry. *Insect. Biochem. Mol. Biol.* **32**, 331-341.

Andersen, S. A., Hansen, H. P., Schaffer, L. and Kristensen, C. (2000). A new secreted insect protein belonging to the immunoglobulin superfamily binds insulin and related peptides and inhibits their activities. *J. Biol. Chem.* **275**, 16948-16953.

Armougom, F., Moretti, S., Poirot, O., Audic, S., Dumas, P., Schaeli, B., Keduas, V. and Notredame, C. (2006). Expresso: automatic incorporation of structural information in multiple sequence alignments using 3D-Coffee. *Nucleic Acids Res.* **34**(Web Server issue):W604-608.

Azad, A. F. and Beard, C. B. (1998). Rickettsial pathogens and their arthropod vectors. *Emerging Infet. Dis.* **4**, 179-186.

Bowman, A. S., Dillwith, J. W. and Sauer, J. R. (1996). Tick salivary prostaglandins: presence, origin and significance. *Parasitol Today* **12**, 388-396.

Bowman, A. S., Sauer, J. R., Zhu, K. and Dillwith, J. W. (1995). Biosynthesis of salivary prostaglandins in the lone star tick, *Amblyomma americanum* (L.). *Insect Biochem. Mol. Biol.* **25,** 735-772.

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

Bratton, R. L. and Corey, R. G. (2005). Tick borne disease. *American Family Physician* **71**, 2323-2330.

Burger, A. M., Leyland-Jones, B., Banerjee, K., Spyropoulos, D. D. and Seth, A. K. (2005). Essential roles of IGFBP-3 and IGFBP-rP1 in breast cancer. *Eur. J. Cancer* **41**, 1515-1527.

Castellanos, M., Jiménez-Vega, F. and Vargas-Albores, F. (2008). Single IB domain (SIBD) protein from *Litopenaeus vannamei*, a novel member for the IGFBP family. *Comp. Biochem. Physiol. Genomics Proteomics* **3**, 270-274.

Coons, L. B., Lamoreaux, W. J., Roseel-Davis, R. and Tarnowski, B. I. (1989). Onset of vitellogenin production and vitellogenesis, and their relationship to changes in the midgut epithelium and oocytes in the tick Dermacentor variabilis. *Exp. Appl. Acarol.* **6**, 291-305.

Davis, H. H., Dotson, E. M. and Oliver, J. H., Jr (1994). Localization of insulin-like immunoreactivity in the synganglion of nymphal and adult *Dermacentor variabilis* (Acari: Ixodidae). *Exp. Appl. Acarol.* **18**, 111-122.

de la Fuente, J., Almazán, C., Canales, M., Pérez de la Lastra, J. M., Kocan, K. M. and Willadsen, P. (2007). A ten-year review of commercial vaccine performance for control of tick infestations on cattle. *Anim. Health. Res. Rev.* **8**, 23-28.

DeLano, W. L. (2002). *The PyMOL Molecular Graphics System.* San Carlos, CA, USA: DeLano Scientific, http://www.pymol.org.

Denley, A, Cosgrove, L. J., Booker, G. W., Wallace, J. C. and Forbes, B. E. (2005). Molecular interactions of the IGF system. *Cytokine Growth Factor Rev.* **16**, 421-439.

Ebel, G. D. and Kramer, L. D. (2004). Short report: duration of tick attachment required for transmission of powassan virus by deer ticks. *Am. J. Trop. Med. Hyg.* **71**, 268-271.

Friesen, K. J. and Kaufman, W. R. (2009). Salivary gland degeneration and vittelogenesis in the ixodid tick *Amblyomma hebraeum*: surpassing a critical weight is the prerequisite and detachment from the host is the trigger. *J. Insect Physiol.* **55**, 936-942.
Frisch, J. E. (1999). Towards a permanent solution for controlling cattle ticks. *Int. J. Parasitol.* **29**, 57-71.
Honegger, B., Galic, M., Köhler, K., Wittwer, F., Brogiolo, W., Hafen, E. and Stocker, H. (2008). Imp-L2, a putative homolog of vertebrate IGF-binding protein 7, counteracts insulin signaling in *Drosophila* and is essential for starvation resistance. *J. Biol.* **7**, 10.
Hwa, V., Oh, Y. and Rosenfeld, R. G. (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr. Rev.* **20**, 761-87.
Janusz, B., Leszek, R. and Daniel, F. (2002). Fold-recognition detects an error in the Protein Data Bank. *Bioinformatics* **18**, 1391-1395.
Jonejan, F. and Uilenberg, G. (2004). The global importance of ticks. *Parasitology* **129 Suppl.**, S3-S14.
Kamangar, B. B., Gabillard, J. C. and Bobe, J. (2006). Insulin-like growth factor-binding protein (IGFBP)-1, -2, -3, -4, -5, and -6 and IGFBP-related protein 1 during rainbow trout post vitellogenesis and oocyte maturation: molecular characterization, expression profiles, and hormonal regulation. *Endocrinology* **147**, 2399-2410.
Katavolos, P., Armstrong, P. M., Dawson, J. E. and Telford, S. R., III (1998). Duration of tick attachment required for transmission of granulocytic ehrlichiosis. *J. Infect. Dis.* **177**, 1422-1425.
Kaufman, W. R. (2007). Glutony and sex in female ixodid ticks: How do they compare to other blood-sucking arthopods? *J. Insect. Physiol.* **53**, 264-273.
Kaufman, W. R. and Philips, J. E. (1973). Ion water balance in the ixodid tick, *Dermacentor andersoni* I. Routes of ion and water excretion. *J. Exp. Biol.* **58**, 523-536.
Koch, H. G. and Sauer, J. R. (1984). Quantity of blood ingested by four species of hard ticks (Aacri: Ixodidae) fed on domestic dogs. *Ann. Entomol. Soc. Am.* **77**, 142-146.
Koch, H. G, Sauer, J. R. and Hair, J. A. (1974). Concentration of the ingested meal in four species of hard ticks. *Ann. Entomol. Soc. Am.* **67**, 861-866.
Lassalle, P., Molet, S., Janin, A., Heyden, J. V., Tavernier, J., Fiers, W., Devos, R. and Tonnel, A. B. (1996). ESM-1 is a novel human endothelial cell-specific molecule expressed in lung and regulated by cytokines. *J. Biol. Chem.* **271**, 20458-20464.
Leboulle, G., Rochez, C., Louahed, J., Ruti, B., Brossard, M., Bollen, A. and Godfroid, E. (2002). Isolation of Ixodes ricinus salivary gland mRNA encoding factors induced during blood feeding. *Am. J. Trop. Med. Hyg.* **66**, 225-233.
Luthy, R, Bowie, J. U. and Eisenberg, D. (1992). Assessment of protein models with three-dimensional profiles. *Nature* **356**, 83-85.
Martinez, D., Aumont, G., Moutoussamy, M., Gabriel, D., Tatareau, A. H., Barre, N., Vallee, F. and Mari, B. (1993). Epidemiological studies on dermatophilosis in the Carribean. *Rev. Elev. Med. Vet. Pays. Trop.* **46**, 323-327.
Mulenga, A., Sugimoto, C., Ingram, G., Ohashi, K. and Misao, O. (2001). Characterization of two cDNAs encoding serine proteinases from the hard tick *Haemaphysalis longicornis. Insect Biochem. Mol. Biol.* **31**, 817-825.
Mulenga, A, Misao, O and Sugimoto, C. (2003). Three serine proteinases from midguts of the hard tick *Rhipicephalus microplus*; cDNA cloning and preliminary characterization. *Exp. Appl. Acarol.* **29**, 151-164.
Mulenga, A., Blandon, M. and Khumthong, R. (2007). The molecular basis of the *Amblyomma americanum* tick attachment phase. *Exp. Appl. Acarol.* **41**, 267-287.
Mulenga, A., Khumthong, R., Chalaire, K. C., Strey, O. and Teel, P. (2008). Molecular and biological characterization of the *Amblyomma americanum* organic anion transporter polypeptide. *J. Exp. Biol.* **211**, 3401-3408.
Mulenga, A., Khumthong, R. and Chalaire, K. C. (2009). Ixodes scapularis tick serine proteinase inhibitor (serpin) gene family; annotation and transcriptional analyses. *BMC Genomics* **10**, 217.
Mustard, J. F. and Packham, M. A. (1977). Normal and abnormal haemostasis. *Br. Med. Bull.* **33**, 187-191.
Packham, M. A. (1994). Role of platelets in thrombosis and hemostasis. Can *J. Physiol. Pharmacol.* **72**, 278-284.
Perlman, S. J., Hunter, M. S. and Zchori-Fen, E. (2006). The emrging diversity of Rickettsia. *Proc. R. Soc. B.* **273**, 2097-2106.
Petersen, M. J., Mead, P. S., Martin, E. and Schriefer, M. E. (2009). *Francisella tularensis:* an arthropod-borne pathogen *Vet. Res.* **40**, 7-15.
Piesman, J. and Spielman, A. (1980). Human babesiosis on Nantucket Island: prevalence of *Babesia microti* in ticks.. *Am. J. Trop. Med. Hyg.* **29**, 742-746.
Piesman, J., Mather, T. N., Sinsky, R. J. and Spielman, A. (1987). Duration of tick attachment and *Borrelia burgdorferi* transmission. *J. Clin. Microbiol.* **25**, 557-558.
Radomski, M. W., Palmer, R. M. J. and Moncada, S. (1987). The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.* **92**, 639-646.
Reimer, L. G. (1993). Q fever. *Clin. Microbiol. Rev.* **6**, 193-198.
Ribeiro, J. M., Makoul, G. T. and Robinson, D. R. (1988). *Ixodes dammni:* evidence for salivary prostacylin secretion. *J. Parasitol.* **74**, 1068-1069.
Ribeiro, J. M., Alarcon-Chaidez, F., Im, B. F., Mans, B. J., Mather, T. N. and Wikel, S. K. (2006). An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem. Mol. Biol.* **36**, 111-129.
Rodgers, B. D., Roalson, E. H. and Thompson, C. (2008). Phylogenetic analysis of the insulin-like growth factor binding protein (IGFBP) and IGFBP-related protein gene families. *Gen. Comp. Endocrinol.* **155**, 201-207.
Rosenblum, W. I. and El-Sabban, F. (1979). Topical prostacylin (PGI2) inhibits platelet aggregation in pial venules of the mouse. *Stroke* **10**, 399-401.
Sali, A. and Blundell, T. L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779-815.
Sarrazin, S., Adam, E., Lyon, M., Depontieu, F., Motte, V., Landolfi, C., Lortat-Jacob, H., Bechard, D., Lassalle, P. and Delehedde, M. (2006). Endocan or endothelial cell specific molecule-1 (ESM-1): a potential novel endothelial cell marker and a new target for cancer therapy. *Biochim. Biophys. Acta* **1765**, 25-37.
Sato, Y., Chen, Z. and Miyazaki, K. (2007). Strong suppression of tumor growth by insulin-like growth factor-binding protein-related protein 1/tumor-derived cell adhesion factor/mac25. *Cancer Sci.* **98**, 1055-1063.
Simor, A. E., Brunton, J. L., Salit, I. E., Vellend, H., Ford-Jones, L. and Spence, L. P. (1984). Q fever: hazard from sheep used in research. *Can. Med. Assoc. J.* **130**, 1013-1016.
Sitar, T., Popowicz, G. M., Siwanowicz, I., Huber, R. and Holak, T. A. (2006). Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins. *Proc. Natl. Acad. Sci. USA* **103**, 13028-13033.
Sonenshine, D. E. (1993). *Biology of Ticks.* Oxford, UK: Oxford University Press.
Spach, D. H., Liles, W. C., Campbell, R. E., Anderson, D. E. and Fristsche, T. R. (1993). Tick-borne diseases in the United States. *New Eng. J. Med.* **329**, 936-947.
Spencer, R. R. and Parker, R. R. (1923). Rocky Mountain spotted fever: infectivity of fasting and recently fed ticks. *Public Health Rep.* **38**, 333-339.
Valenzuela, J. G., Francischetti, I. M., Pham, V. M., Garfield, M. K., Mather, T. N. and Ribeiro, J. M. (2002). Exploring the sialome of the tick *Ixodes scapularis. J. Exp. Biol.* **205**, 2843-2864.
Walade, S. M. and Rice, M. J. (1982). The sensory basis of tick feeding behavior. In *Physiology of Ticks*. Oxford UK: Pergamon Press.
Willadsen, P. (2004). Anti-tick vaccines. *Parasitol.* **129 Suppl.**, S367-S387.
Willadsen, P. (2006). Tick control: thoughts on a research agenda. *Vet. Parasitol.* **138**, 161-168.
Zhu, X. X. and Oliver, J. H., Jr (1991). Immunocytochemical localization of an insulin-like substance in the synganglion of the tick *Ornithodoros parkeri* (Acari: Argasidae). *Exp. Appl. Acarol.* **13**, 153-159.