Molecular and functional characterization of vacuolar-ATPase from the American dog tick Dermacentor variabilis

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Abstract

Vacuolar (V)-ATPase is a proton-translocating enzyme that acidifies cellular compartments for various functions such as receptor-mediated endocytosis, intracellular trafficking and protein degradation. Previous studies in Dermacentor variabilis chronically infected with Rickettsia montanensis have identified V-ATPase as one of the tick-derived molecules transcribed in response to rickettsial infection. To examine the role of the tick V-ATPase in tick–Rickettsia interactions, a full-length 2887-bp cDNA (2532-bp open reading frame) clone corresponding to the transcript of the V0 domain subunit a of D. variabilis V-ATPase (DvVATPaseV0a) gene encoding an 843 amino acid protein with an estimated molecular weight of ∼96 kDa was isolated from D. variabilis. Amino acid sequence analysis of DvVATPaseV0a showed the highest similarity to VATPaseV0a from Ixodes scapularis. A potential N-glycosylation site and eight putative transmembrane segments were identified in the sequence. Western blot analysis of tick tissues probed with polyclonal antibody raised against recombinant DvVATPaseV0a revealed the expression of V-ATPase in the tick ovary. Transcriptional profiles of DvVATPaseV0a demonstrated a greater mRNA expression in the tick ovary, compared with the midgut and salivary glands; however, the mRNA level in each of these tick tissues remained unchanged after infection with R. montanensis for 1 h. V-ATPase inhibition bioassays resulted in a significant decrease in the ability of R. montanensis to invade tick cells in vitro, suggesting a role of V-ATPase in rickettsial infection of tick cells. Characterization of tick-derived molecules involved in rickettsial infection is essential for a thorough understanding of rickettsial transmission within tick populations and the ecology of tick-borne rickettsial diseases.

Keywords: Dermacentor variabilis, Rickettsia montanensis, vacuolar-ATPase.

Introduction

Ticks are important vectors of human pathogens, including bacteria, viruses and parasites (Sonenshine, 2005). Among the bacterial pathogens transmitted by ticks are Anaplasma spp., Ehrlichia spp., Francisella tularensis, and the obligate intracellular spotted fever group (SFG) Rickettsia. Acquisition of SFG Rickettsia by ticks can be either through an infectious bloodmeal from a vertebrate host or acquired via transovarial transmission. Although the kinetics of the infection process are not well-defined, transmission results in maintenance of SFG Rickettsia among tick populations. The tick-derived molecules associated with sustained transmission are relatively unknown,
but characterization of their function may lead to novel points of intervention for tick-borne rickettsial diseases.

At the cellular level, based primarily on studies in vertebrate hosts, the process of rickettsial infection includes induced endocytosis and phagosomal escape to facilitate intracytoplasmic living of *Rickettsia* (Walker & Ismail, 2008). Host-derived molecules essential for rickettsial invasion include KU70 (Martinez et al., 2005) and, in ticks, histone H2B is associated with rickettsial entry of tick cells *in vitro* (Thepparit et al., 2010); however, the specific molecular processes by which *Rickettsia* invade tick cells is yet to be defined. In order to understand the mechanisms of rickettsial survival in the arthropod, previous studies have used molecular techniques such as differential display and subtractive hybridization-PCR to identify several *Dermacentor variabilis*-derived molecules thought to be associated with tick response to rickettsial infection (Macaluso et al., 2003; Mulenga et al., 2003). Among the putatively identified molecules differentially transcribed in response to rickettsial infection is vacuolar-ATPase (V-ATPase).

The structure and function of V-ATPase consists of multiple subunits separated into the membrane-associated V0 and water-soluble V1 domains. The V0 complex contains the proton-translocating pore and the V1 complex uses ATP to drive proton translocation across the membrane (Nishi & Forgac, 2002). The subunit composition of V-ATPase domains is variable; for example, the Saccharomyces cerevisiae V0 domain consists of six different subunits and the V1 domain is composed of eight different subunits (Kane, 2006; Forgac, 2007). A similar V1 domain is present in the midgut of the tobacco hornworm, Manduca sexta, with a V0 domain containing only four unique subunits (Merzendorfer et al., 2000). Despite the variability in subunits that compromise the V0 and V1 unique subunits (Merzendorfer et al., 2000), the specific molecular processes by which *Rickettsia* invade tick cells is yet to be defined. In order to understand the mechanisms of rickettsial survival in the arthropod, previous studies have used molecular techniques such as differential display and subtractive hybridization-PCR to identify several *Dermacentor variabilis*-derived molecules thought to be associated with tick response to rickettsial infection (Macaluso et al., 2003; Mulenga et al., 2003). Among the putatively identified molecules differentially transcribed in response to rickettsial infection is vacuolar-ATPase (V-ATPase).

Several bacterial pathogens require host cell V-ATPase to facilitate intracellular living. For example, in *Listeria monocytogenes*, phagosomal escape relies on acidification of the phagosome mediated by V-ATPase (Portnoy et al., 1992). V-ATPase is also suspected to have a role in the invasion of tick cells by *Anaplasma marginale* (Kocan et al., 2009). Although acidification of the vacuole is not definitively required for SFG *Rickettsia* infection (Welch et al., 2012), clathrin-coated pits that concentrate V-ATPases are probably an integral part of host cell invasion (Munderloh & Kurtti, 1995); however, the role of tick V-ATPase and the influence of SFG *Rickettsia* on this molecule remains to be elucidated. Based on the observed differential transcription of V-ATPase in SFG *Rickettsia*-infected ticks, we report here the molecular characterization of the full-length transcript and functional V-ATPase inhibition bioassays to further delineate the interaction between SFG *Rickettsia* and their tick hosts.

Results

Cloning and sequence analysis of DvVATPaseV0a

A full-length cDNA clone corresponding to the *D. variabilis* transcript of the V-ATPase V0 subunit (DvVATPaseV0a) was isolated using the gene-specific primers designed from differential display PCR (Macaluso et al., 2003). Using MacVECTOR, sequence analysis revealed a 2887-bp full-length cDNA with a 2532-bp open reading frame (ORF; GenBank accession number HM185485). The deduced amino acid sequence comprised 843 residues with an estimated molecular weight of 96 kDa. Amino acid sequence analysis of DvVATPaseV0a using a web-based multiple sequence alignment, multiple sequence comparison by log-expectation (muscle), showed 77, 66, 44 and 40% identity to VATPaseV0a from *Ixodes scapularis*, *Drosophila melanogaster*, *Homo sapiens*, and *S. cerevisiae*, respectively, as shown in Fig. 1. When analysed using the NETNGLYC 1.0 Server, an Asn-Xaa-Ser/Thr (asparagine-any amino acid-serine/threonine) sequon contained a potential N-glycosylation site at the asparagine (N358). Similarly to other organisms, DvVATPaseV0a possesses eight segment transmembrane portions predicted by TOPPRED software (Fig. 2).

Expression of recombinant DvVATPaseV0a, antibody generation, and detection of VATPaseV0a in tick tissues

Full-length recombinant (r)DvVATPaseV0a was produced as a hexahistidine (6xHis) fusion protein using the baculovirus expression system in Sf9 insect cells. The size of the recombinant protein produced corresponded to an estimated molecular weight analysis of full-length cDNA sequence ~96 kDa. A polyclonal antibody against rDvVATPaseV0a was generated in BALB/c mice. Western blotting analysis showed the rDvVATPaseV0a polyclonal antibody specifically recognizing three rDvVATPaseV0a protein bands, but not the His-tagged protein standard. The occurrence of multiple protein bands, after purifying and concentrating, may result from self-binding in the more concentrated protein preparation. To exclude the possibility of non-rDvVATPaseV0a protein contamination, the ~96 kDa of purified rDvVATPaseV0a was excised from the gel and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The results, again, showed three bands appearing in the same positions as the first purification, confirming that self-binding occurred in the concentrated protein preparation (data not shown).
Transcriptional analysis of the putative DvVATPaseV0α identified mRNA expression in tick salivary glands, midgut and ovary (Macaluso et al., 2003). To assess DvVATPaseV0α expression in tick tissues, protein extracts of salivary glands, midgut, and ovary were assessed by Western blot using antibodies to rDvVATPaseV0α. The result of the Western blot analysis shown in Fig. 3 revealed a detectable DvVATPaseV0α protein band in the ovary. The apparent molecular mass of this detected band was a little bigger than a 96-kDa band of the rDvVATPaseV0α which reflected the presence of post-translational modification on the protein in tick ovary. Although the same amount of protein was loaded (30 μg), no band was detected in the midgut and salivary glands, indicating a much lower amount of DvVATPaseV0α in the midgut and salivary glands.

Expression of VATPaseV0α mRNA in backless Dermacentor variabilis

To determine a transcriptional profile of VATPaseV0α in D. variabilis tissues (midgut, ovary and salivary glands) in
response to an early stage of rickettsial infection, backless ticks were generated and exposed to *Rickettsia montanensis*. After 1 h, the tissues were dissected from ticks, extracted for total RNA and measured for \( \text{DvVATPaseV0a} \) mRNA level by quantitative reverse transcription (qRT)-PCR. As shown in Fig. 4, mRNA was detectable in all tick tissues and in both unexposed and *R. montanensis*-exposed ticks. Correlating with the protein expression pattern, \( \text{DvVATPaseV0a} \) mRNA was expressed more highly in the ovary than in the other tissues, with a significant difference in uninfected ovaries compared with the midgut (\( P = 0.0154 \)) and salivary glands (\( P = 0.0155 \)); however, in all tissues the mRNA level remained unchanged in *R. montanensis*-exposed ticks compared with the unexposed control.

**Involvement of tick V-ATPase in *Rickettsia montanensis* infection**

To assess the function of tick V-ATPase in response to *R. montanensis* infection, V-ATPase inhibition assays were performed in the *D. variabilis*-derived cell line DVE1. Tick cells were treated for 2 h with either 5, 0.5, or 0.05 \( \mu \)M of V-ATPase inhibitor, bafilomycin A1, prior to infection with *R. montanensis* at a multiplicity of infection (MOI) of 10. After 1 h, removal of *R. montanensis* from the cells occurred before washing cells twice with phosphate-buffered saline (PBS), followed by low-speed centrifugation to exclude the possibility of collecting extracellular rickettsiae. Genomic DNA (gDNA) was then extracted from the cells and the percentage of rickettsial infection in comparison with control cells was assessed by quantitative PCR (qPCR). As shown in Fig. 5, inhibition of \( \text{DvVATPaseV0a} \) in DVE1 cells reduced percent relative invasion compared with the untreated control by 27% at 5 \( \mu \)M (\( P = 0.0005 \)) and 0.5 \( \mu \)M (\( P = 0.0005 \)) and by 13% at 0.05 \( \mu \)M (\( P = 0.0877 \)).

**Discussion**

The present study provides novel sequence and functional analyses for the \( \text{VATPaseV0a} \) from *D. variabilis*. A full-length cDNA of \( \text{DvVATPaseV0a} \) was isolated and multiple sequence alignment showed highest similarity in amino acid sequences to other arthropod species, including the available sequence from another ixodid tick, *I. scapularis*. The composition of V-ATPase is known to vary across species, yet enzymatic activity appears conserved, independent of subunit variations (Kane, 2006; Forgac, 2007; Wieczorek *et al.*, 2009). Amino acid sequence analysis revealed transmembrane segments and also a potential site for N-glycosylation which is consistent with the studies of coated vesicle (Adachi *et al.*, 1990) and yeast (Ryan *et al.*, 2008) \( \text{VATPaseV0a} \), in which the proteins were illustrated as transmembrane glycoproteins. Nevertheless, further investigation is needed to...
ovary was expressed at a higher level compared with observed transcriptional pattern (both in unexposed and not in the midgut and salivary glands corresponds to the untreated control cells (*P = 0.0005).

The expression and function of V-ATPase in tick cells were examined to understand the role of this enzyme in tick biology. To better characterize the role of V-ATPase in rickettsial infection, DVE1 cells were treated for 2 h with bafilomycin A1 (BAF) prior to infection with R. montanensis at a multiplicity of infection of 10. After 1 h, Rickettsia was removed. The cells were washed twice with phosphate-buffered saline and collected by low-speed centrifugation. Genomic DNA was then isolated and percent relative invasion was assessed by quantitative PCR. Data shown are mean percent relative invasion from two independent experiments. Error bar represents SEM values. The asterisks mark significant difference from untreated control cells (*P = 0.0005).

Figure 5. Effect of V-ATPase inhibitor on Rickettsia montanensis infection of DVE1 cells. DVE1 cells were treated for 2 h with bafilomycin A1 (BAF) prior to infection with R. montanensis at a multiplicity of infection of 10. After 1 h, Rickettsia was removed. The cells were washed twice with phosphate-buffered saline and collected by low-speed centrifugation. Genomic DNA was then isolated and percent relative invasion was assessed by quantitative PCR. Data shown are mean percent relative invasion from two independent experiments. Error bar represents SEM values. The asterisks mark significant difference from untreated control cells (*P = 0.0005).

confirm the presence of N-glycosylation of tick-derived VATPaseV0a. V-ATPase is ubiquitously found in and is responsible for acidification of a variety of intracellular organelles in eukaryotic cells, such as endosomes and lysosomes. The enzyme is essential for several biological processes, e.g. protein sorting, protein processing and degradation, coupled transport of small-molecule, receptor-mediated endocytosis, and receptor recycling (V-ATPase; Nishi & Forgac, 2002). To further characterize tick, the protein was expressed, and the polyclonal antibody raised against \textit{rDVATPaseV0a} was then used to confirm the presence of V-ATPase in tick tissues. The results showed a detectable protein band with a molecular weight slightly greater than the predicted 96 kDa, reflecting post-translational modification only in the tick ovary. Previous characterization of the C subunit of V-ATPase from \textit{Amblyomma americanum} embryos and salivary glands identified a role for V-ATPase in salivary fluid secretion (water balance), but V-ATPase was not essential to the process (McSwain \textit{et al.}, 1997); thus, it is interesting to investigate alternate roles for V-ATPase at the molecular and functional level. The detection of \textit{DVATPaseV0a} protein in the tick ovary but not in the midgut and salivary glands corresponds to the observed transcriptional pattern (both in unexposed and \textit{Rickettsia}-exposed ticks) in which the mRNA in the tick ovary was expressed at a higher level compared with other tissues. The inability to detect V-ATPase in the salivary glands and midgut, combined with abundant expression in the ovary might signify an essential role for this molecule in the ovary. The increased expression of V-ATPase in the tick ovary might be associated with receptor-mediated endocytosis and protein transport, the two processes that are important for ovulation and embryogenesis in which V-ATPase is involved. In many organisms, such as \textit{Caenorhabditis elegans} (Grant & Hirsh, 1999), \textit{D. melanogaster} (Schonbaum \textit{et al.}, 1995), \textit{Aedes aegypti} (Sappington \textit{et al.}, 1995), and also \textit{D. variabilis} (Mitchell \textit{et al.}, 2007), vitellogenin, a yolk precursor protein, is taken up through the receptor-mediated endocytosis pathway during oocyte growth. Studies using RNA interference in \textit{C. elegans} showed that V-ATPase is required for ovulation and oogenesis. Specifically, the inhibition of \textit{C. elegans} V1 subunit C and V0 subunit a (Oka & Futai, 2000; Oka \textit{et al.}, 2001) induced embryonic lethality. Moreover, studies of \textit{wnt/β}-catenin signalling, a cascade important for numerous biological functions including embryonic development, revealed an involvement of V-ATPase in acidification of the pathway (Cruciat \textit{et al.}, 2010). Taken together, these findings suggest that V-ATPase is physiologically important in the ovary, but the mechanisms by which V-ATPase functions in the tick ovary needs to be further elucidated.

As tick tissues including the salivary glands, midgut and ovary are essential for both horizontal and vertical transmission of SFG \textit{Rickettsia} (Munderloh & Kurti, 1995), the role of V-ATPase was then investigated during \textit{R. montanensis} invasion of \textit{D. variabilis}. Using a backless tick bioassay, no difference of \textit{DVATPaseV0a} transcript levels were observed between unexposed and \textit{Rickettsia}-exposed ticks. These results differ from previous analyses in which ticks chronically infected with \textit{R. montanensis} had upregulated V-ATPase transcription compared with \textit{R. montanensis}-uninfected control ticks (Macaluso \textit{et al.}, 2003). Comparison between the studies is difficult because of the point of assessment of infection in the current study (1 h) differs from chronically infected ticks. It is interesting to speculate that increased V-ATPase in chronically infected ticks, which maintain rickettsiae vertically through transovarial transmission, might be associated with increased vitellogenin processing or oocyte production; thus chronic infection is associated with an increased fitness advantage for an infected tick.

To better characterize the role of V-ATPase in rickettsial infection of tick cells, the \textit{D. variabilis}-derived tick cell line, DVE1, was incorporated into V-ATPase inhibition bioassays. Bafilomycin A1 is a well characterized inhibitor for V-ATPase, and previous studies have shown that V0 domain subunits a (Crider \textit{et al.}, 1994; Zhang \textit{et al.}, 1994; Wang \textit{et al.}, 2005) and c (Rautiala \textit{et al.}, 1993; Bowman & Bowman, 2002; Bowman \textit{et al.}, 2006) are targets of the
inhibitor. In ticks, the V-ATPase in salivary glands was impaired by bafilomycin A1 (McSwain et al., 1997). In the bioassay developed in the present study, a significant dose-dependent decrease in percent relative invasion was observed in cells treated with V-ATPase inhibitor compared with the untreated control. Because the role of V-ATPase was examined in the early stage of infection, it is possible that V-ATPase might play a role in receptor-mediated endocytosis, a process by which Rickettsia enter the cells (Munderloh & Kurtti, 1995). In addition to intracellular compartments, V-ATPase in clathrin-coated vesicles is present at the cell surface and is involved in receptor-mediated endocytosis and functions in recycling receptors (Forgac, 2000). An alternative mechanism by which rickettsial infection might be dependent on V-ATPase is related to the rearrangement of host cytoskeletal components. For example, V-ATPase V1 subunits B (Holiday et al., 2000) and C (Vitavksa et al., 2003) bind to actin filament (F-actin); and, in M. sexta V-ATPase subunit C binds not only to F-actin but also to a monomeric G-actin (Vitavksa et al., 2005). The biological function of V-ATPase and F-actin interaction was speculated to be involved in the organization of actin dynamics (Wieczorek et al., 2009). As previously characterized in vertebrate cells, SFG Rickettsia manipulate host actin in order to invade host cells (Dramsi & Cossart, 1998; Gouin et al., 2004). While, the mechanisms by which tick V-ATPase functions in response to rickettsial infection, including actin rearrangement, is unknown; the current data suggest the possibility that V-ATPase is involved in rickettsial infection of tick cells via a receptor-mediated endocytosis process.

Several bacterial pathogens use V-ATPase as a mechanism for invasion and survival in host cells. In L. monocytogenes, V-ATPase is required for the activation of the pore-forming haemolysin, essential for phagosomal escape (Portnoy et al., 1992). A dual role for V-ATPase in Chlamydia infection favours bacterial infection and defends the host cell against infection through autophagy (Yasir et al., 2011). With respect to tick-borne bacterial pathogens, V-ATPase is thought to play a role in F. tularensis phagosomal escape (Chong et al., 2008; Santic et al., 2008); however, the essentiality of V-ATPase is debatable (Clemens et al., 2009). Similarly to the R. montanensis and D. variabilis model, V-ATPase was upregulated in D. variabilis exposed to A. marginale (de la Fuente et al., 2007) and a role for the molecule in cell invasion and pathogen development was identified (Kocan et al., 2009). The essential role of V-ATPase in tick physiology, both feeding and oogenesis, make this molecule a viable target for exploitation by rickettsiae, and may provide insight into the maintenance of SFG Rickettsia in tick populations. Further studies to investigate the mechanism(s) by which Rickettsia manipulate tick V-ATPase to facilitate either horizontal or vertical transmission are required.

**Experimental procedures**

**Tick dissection and tissue preparation**

As previously described by Macaluso et al. (2001), Rickettsia-free D. variabilis originally derived at Old Dominion University were routinely maintained at Louisiana State University. After feeding for 3–5 days, uninfected female ticks were removed from host animals, washed twice with 70% ethanol and rinsed with distilled water. Tick tissues, including midgut, ovary, and salivary glands, were removed from the ticks and washed in sterile PBS (0.137 mM NaCl, 2.7 mM KCl and 8 mM Na2HPO4, pH 7.4) or 0.1% diethyl pyrocarbonate (DEPC)-treated water. Ovaries were transferred into microtubes containing RNAlater (Ambion, Austin, TX, USA) for RNA extraction. For protein preparation, all organs were placed in protease inhibitor cocktail (PIC, Roche, Indianapolis, IN, USA). Tissues were immediately processed or stored at –80 °C until used for extraction.

**Protein and mRNA extraction from tick tissues**

Tick tissue from at least five D. variabilis were rinsed with DEPC-treated water before total RNA and mRNA were extracted from using the NucleoSpin RNA II and NucleoTrap mRNA Mini kits (Clontech, Mountain View, CA, USA), respectively, according to the manufacturer’s instructions. For protein extraction, the tissue samples were thawed on ice for 15 min and washed once with PBS supplemented with PIC. The tissues were then lysed by adding 100 μl of lysis buffer (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, pH 8.0), homogenizing with plastic pestle for 15 min and sonicating in a bath sonicator (Crest Ultrasonics, Trenton, NJ, USA) for 10 min. The lysate was centrifuged at 16 000 × g for 10 min at 4 °C. The supernatant was then transferred to 1.7-ml centrifuge tubes. The protein solution was diluted two-fold and the protein concentration was measured by DC protein assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Then, 30 μg of protein from the midgut, ovary and salivary glands was analysed using SDS-PAGE and Western immunoblot.

**Cloning of the tick V-ATPase V0 subunit a full-length cDNA**

The full-length cDNA for DvATPaseV0a was generated using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer’s protocol. Briefly, 1 μg of mRNA extracted from ovary was used to create 3′ and 5′ rapid amplification of cDNA ends-ready cDNA. Gene-specific primers designed from a partial sequence of DwATPaseV0a gene (Macaluso et al., 2003) were used to amplify the 3′ and 5′ end fragments. PCR products were cloned into TOPO TA cloning vectors (Invitrogen, Carlsbad, CA, USA) and the plasmids were isolated using the SV miniprep kit (Promega, Madison, WI, USA) according to the manufacturers’ protocols. The cloned plasmid inserts were sequenced by the dye terminator method on a 373 automated fluorescence sequencing system (Applied Biosystems, Carlsbad, CA, USA) and the plasmids were routinely maintained at the University of Maryland, Baltimore. For DNA sequence analysis, the MacVECTOR software program (Accelrys, San Diego, CA, USA) was used. Similarity comparison of DNA sequence was carried out.
out against the protein database in GenBank using BLASTX. Amino acid sequence analyses were conducted using web-based software suites. MUSCLE was used to create a sequence alignment file and calculated percent identity (pairwise alignment). The alignment output was created using GeneDoc software. The transmembrane regions were predicted using TopPred. A potential N-glycosylation site was obtained using NetNGlyc 1.0 Server.

Construction of plasmid and expression of rDvVATPaseV0a
The Baculovirus Expression System with Gateway Technology (Invitrogen) was used to express rDvVATPaseV0a according to manufacturer's protocol. Briefly, the ORF of DvVATPaseV0a was subcloned into the pENTR/D-TOPO entry vector (Invitrogen) and then transferred into the cloning cassette of the pDEST10 vector (N-terminal His fusion vector, Invitrogen). The cloned ORF DvVATPaseV0a plasmid was transformed into DH10Bac E. coli (Invitrogen), which contains the baculovirus shuttle vector (bacmid), to produce a recombinant bacmid harbouring DvVATPaseV0a. The white colonies of recombinant bacmid DNA in a background of blue colonies containing the unaltered parent bacmid were selected and isolated from small-scale selective (50 μg/ml kanamycin, 7 μg/ml Gentamycin, 10 μg/ml tetracycline, 100 μg/ml Bluo-gal, and 40 μg/ml isopropyl-beta-D-thiogalactopyranoside) medium. These recombinant baculoviruses (1 μg) were used to transfect 9 × 10^6 Sf9 insect cells (Gibco, Carlsbad, CA, USA) using Cellfectin reagent (Invitrogen) according to the manufacturer's recommendation. The transfected cells were maintained at 27 °C and the culture medium containing the recombinant baculoviruses was collected at 7 days post-transfection as a primary viral stock (P1).

The P1 viral stock (40 μl) was used to infect fresh Sf9 (2 × 10^6) cells and incubated at 27 °C for 4 days. The medium containing baculoviruses was then collected and designated as P2 viral stock. The P2 stock was diluted tenfold from 10^3 to 10^4 and the viral titer was determined by endpoint dilution as described by O'Reilly et al. (1994). To express the protein, Sf9 cells were infected with rDvVATPaseV0a baculoviruses at MOI of 1 and maintained at 27 °C for 4 days. The cells were then harvested, washed once with PBS and kept at −20 °C until used for protein purification.

Purification of DvVATPaseV0a from polyacrylamide gel
The infected cells were resuspended in lysis buffer and stirred at room temperature for 1 h. The lysate was centrifuged at 16 000 × g for 30 min and the supernatant was removed. The pellet was then added with 1x NuPAGE LDS Sample Buffer (Invitrogen) supplemented with 100 mM dithiothreitol (DTT, Fisher Scientific, Pittsburgh, PA, USA) and loaded onto NuPAGE 4–12% Bis-Tris Zoom gels (Invitrogen). The gels were then negatively stained with E-zinc Reversible Stain Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The unstained band of rDvVATPaseV0a was then excised from the gel and the residual stain from the edges of the excised gel pieces was erased by soaking them in Tris-glycine buffer (25 mM Tris and 192 mM glycine). The excised gel pieces were crushed using a clean pestle and added with 0.5 ml elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5). The mixture was then incubated in a rotary shaker at 30 °C overnight and the protein was collected after centrifugation.

Protein identification
To confirm peptide sequences of purified rDvVATPaseV0a protein, SDS-PAGE coupled with mass spectrophotometry analysis was performed as described previously by Sunykakumithorn et al. (2008). Briefly, the protein band was excised using the Proteome Works Spot Cutter (Bio-Rad), and a MassPrep Station (Waters, Milford, MA, USA) was used as the digestion robot. The peptides were then extracted from the gel plugs and separated by liquid chromatography using an Atlantis dC18 column (75 μm by 100 mm; Waters/Micromass, Milford, MA, USA). For analysis, a Q-Tof (quadrupole time-of-flight) Micro (Waters/Micromass) hybrid mass spectrometer was used and electrospray analysis (positive mode) was performed. ProteinLynx Global Server, version 2.0 (Waters/Micromass) was used for data acquisition and analysis. Database comparative analysis with an online Mascot (Matrix Science, Boston, MA, USA) tandem mass spectrophotometry ion search against the NCBInr/Proteobacteria database was carried out. The resultant peaks of the ∼96-kDa band matched the V-ATPase protein.

Preparation of a polyclonal antibody specific for rDvVATPaseV0a
Polyclonal antibodies to purified rDvVATPaseV0a were generated in BALB/c mice as described by Mulenga et al. (2003). Briefly, three mice were immunized subcutaneously with rDvVATPaseV0a protein (30 μg protein per mouse) emulsified with equal volume of TiterMax Gold adjuvant (Sigma-Aldrich, St. Louis, MO, USA), followed by two more injections (60–80 μg protein per mouse) at 2-week intervals. Antiserum with reactivity toward rDvVATPaseV0a was collected 2 weeks after the third injection and stored at 4 °C for subsequent studies.

SDS-PAGE and Western blot analysis
Proteins were separated by NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electronically transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 5% (w/v) skim milk in tris buffer saline (TBS)-TWEEN (T) buffer (20 mM Tris-HCl, 500 mM NaCl and 0.1% (v/v) Tween-20; pH 7.5) at room temperature for 1 h and incubated with either 1: 5000 dilution of anti-6xHis monoclonal antibody (Clontech) or a 1: 200–500 dilution of polyclonal anti-rDvVATPaseV0a in TBS-T for 2 h. After three washes, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Pierce) diluted 1: 20 000 at room temperature for 1 h and then washed three times with TBS-T. The bound antibody complexes were detected using an enhanced chemiluminescent system (Pierce).

Cell culture
Sf9 cells (Gibco) were cultured in SF900 II serum-free medium (Gibco) supplemented with penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively, Gibco). DVE1 cells (derived from D. variabilis) were grown in L15C medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Waltham, MA, USA).
USA), 5% trypsete phosphate broth (Difco, Sparks, MD, USA), 0.1% lipoprotein-cholesterol concentrate (MP Biomedicals, Santa Ana, CA, USA), 6 mM HEPES solution (Sigma-Aldrich), and 0.06% sodium bicarbonate (Sigma-Aldrich). Vero cells were grown in DMEM high glucose (Invitrogen) containing 5% fetal bovine serum (Hyclone). For all cell lines, conditioned medium was replaced with new medium once a week. Si9 and DVE1 cells were passaged (1:5 and 1:3, respectively) approximately every 2 and 4 weeks, respectively. Vero cells were subcultured (1:6 or 1:12) every 2–3 weeks with 0.05% trypsin-EDTA (Invitrogen). Si9 and DVE1 cells were grown in a humidified incubator at 27 and 32 °C, respectively. Vero cells were maintained in a humidified 5% CO2 incubator at 34 °C.

**Rickettsia culture and purification**

*Rickettsia montanensis* were maintained in Vero cells as described by Sunyakumthorn et al. (2012). The isolation of *R. montanensis* from infected Vero cells was carried out as previously described by Weiss (1973) with minor modification. Briefly, *Rickettsia*-infected cells were detached by scraping and lysed by vortexing with sterile 3-mm borosilicate glass beads for 5 min (Sigma-Aldrich). The cell lysate was then transferred aseptically to 15-ml centrifuge tubes and centrifuged at 4 °C, 275 × g for 3 min to pellet cellular debris. The supernatant was transferred to a 10-ml syringe and filtered through a 2-

**Expression of VATPaseV0a mRNA in backless Dermacentor variabilis**

The backless *D. variabilis* was used as a model to study an expression of *DvVATPaseV0a* mRNA in tick tissues (midgut, ovary, salivary glands) in response to *R. montanensis* infection using a modified protocol of Bell (1980), as previously described by Sunyakumthorn et al. (2012). Briefly, six unfed female ticks were washed with 70% ethanol followed by 1% benzalkonium chloride solution and rinsed three times with sterile water. The ticks were air-dried and afterwards the mouthparts and the legs were excised. The ticks were then cut along the perimeter of alloscutum and the dorsal cuticle was removed. The backless ticks were individually transferred onto 96-well plate containing complete L15B medium and incubated at 34 °C. After 24 h, three ticks were infected with *R. montanensis* (2.4 × 10^8 per tick) for 1 h while the other three were kept in the medium without exposure to *Rickettsia*. Tick tissues were collected by removing and pooling the tissues from three different ticks. The tissues were placed in RNA later and the samples were kept at −20 °C until used for RNA extraction. The experiments were performed independently twice.

**Total RNA isolation and relative quantitative reverse transcription-PCR**

Total RNA from uninfected- and *R. montanensis*-infected tick tissues was extracted using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA), treated with TURBO DNase (Ambion), and purified using an RNA cleanup kit (Zymo Research, Irvine, CA, USA) as described in the manufacturers’ manuals. First-strand cDNA was synthesized from 40 ng total RNA using iScript reverse transcription kit (Bio-Rad) according to the manufacturer’s instructions. All qPCR reactions were performed in 96-well plates in a 35-μl volume containing 100 nM of each primer (5′-CTCCTGGCCCGTGATTGAT-3′ and 5′-GCTGCTCCGCTGTC-3′) for *V-ATPase*, 5′-CTCGTTCTGGGAGGTAAGG-3′ and 5′-CTGATCTCATTGTTGGAAG-3′ for *Actin*, DNaase/RNase-free water, 2 μl of cDNA template (samples) or water (negative control) and 2X iTaq SYBR Green Supermix with ROX (Bio-Rad). The mixtures were then aliquoted in triplicate 10-μl reactions onto 384-well plates and run on an ABI 7900HT unit (Applied Biosystems, Carlsbad, CA, USA) at Louisiana State University, School of Veterinary Medicine. No RT reaction (water was added instead of reverse transcriptase) was performed to confirm an absence of gDNA. Results were analysed using an ABI 7900HT sequence detection system (sds v2.3) software. Data are presented as the percent difference in threshold cycle (Ct) value (ΔCt = Ct * Actin – Ct * V-ATPase).

**DvV-ATPase inhibition assay**

DVE1 cells (1 × 10^5) were seeded onto 96-well plates (Greiner Bio-One, Monroe, NC, USA) and incubated at 32 °C for 48 h. The cells were treated with 5, 0.5, and 0.05 μM of the V-ATPase inhibitor, bafilomycin A1 (EMD Millipore, Billerica, MA, USA), or medium containing 0.1% dimethyl sulphoxide (inhibitor vehicle control) for 2 h. *R. montanensis* was then inoculated onto the treated cells at MOI of 10 and the plates were centrifuged at 700 × g for 2 min to facilitate the binding of *Rickettsia* to host cells. After 1 h, *Rickettsia* was removed and the cells were added with 150 μl PBS. The samples were centrifuged at 275 × g for 4 min to collect only infected host cells. After removal of supernatant, the cell pellet was washed with 1 ml PBS and centrifuged at 275 × g for 4 min. The samples were stored at −20 °C until used for gDNA isolation. According to the manufacturer’s instructions, gDNA was extracted from the samples using DNeasy Blood & Tissue Kit (Qiagen). Genomic DNA was eluted in 35 μl DNase/RNase free water. The number of *Rickettsia* and tick cells were then quantified by qPCR. The experiments were performed in quadruplicate for each concentration of the inhibitor used and the results were the combination of two independent experiments.

**Construction of a standard reference plasmid for qPCR**

A standard reference plasmid containing portions of *R. montanensis* ompB (*Rm*OmpB) and *D. variabilis* calreticulin (*Dv*CRT) genes was generated and used to create a standard curve in qPCR assays as described by Reif et al. (2008). Briefly, fragments of *DvCRT* (132 bp) and *Rm*OmpB (106 bp) were amplified using CTRDv/321F (5′-AAAAAACCTAGAAGGAG AAAACAGAAGGACTG-3′)/CTRDr/452R (5′CATGTTCTGGTC CTGCGTTG-3′) and OmpBRm/2832F (5′GGCCTGGTTGTCCTCA ATAC-3′)/OmpBRm/2937R (5′-AAAAAACACTACACATTG GAATTGTTAAGTCTGATG-3′) primer pairs, respectively. The amplicons were then digested with *XbaI* (New England BioLabs, Ipswich, MA, USA) and ligated together. The ligation product was amplified using OmpBRm/2832F (5′-GCCCTGGTTGTCCTCAAA) and
TAC-3') and CRTDw452R (5'-CAATGTCTGCTGCTGTG G-3') primers, cloned into pCR4-TOPO vector (Invitrogen) and sequenced.

Quantification of Rickettsia and tick cells by qPCR
To quantify copies of *R. montanensis* and DVE1 genes in samples, probe-based qPCRs were performed as described by Thepparit et al. (2011). Briefly, serial dilutions of plasmids containing *Rm*OMPb and *Dv*CRT vectors were used to create a standard curve. qPCR was performed using 0.3 μM each *Dv*CRT_TYE665 (TYE665/5'-TGGAAAGGGCTGCTGAGG C-3'/MabRSp) and *Rm*OMPb_HEX (HEX5'-CQGGGCAAAA GatGCCAGGGCTCAACCTGTA CCCCC-3'/IAbk FQ) probes and 0.2 μM each CRTDw321F (5'-AGGAAAGAACAGGAAGG ACTG-3'), CRTDw452R (5'-CAATGTTCGTCTGCTGTGCT-3'), *Rm*OMPb2832F (5'-CGGTTGGA TGTCTCATTAC-3') and *Rm*OMPb2937R (5'- CCTAAGTGTATAGTCTGTA CGT-3') primers. Percent relative invasion was calculated by comparing the ratios of rickettsial gene to tick gene between treated and untreated control groups.

Statistical analysis
ANOVA was conducted using the SAS statistical package (Version 9.3) GLM procedure. For mRNA level measurement in backless ticks, the same tests were used to elucidate a role of the Rickettsia effects of relative mRNA expression of VATPaseV0a between unexposed and *Rickettsia*-exposed ticks. For V-ATPase inhibition assays, the same tests were used to elucidate a role of the V-ATPase inhibitor, bafilomycin A1, on rickettsial invasion of tick cells. *P*-values of ≤0.05 were considered to indicate statistical significance.

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