Anaplasma phagocytophilum Inhibits Apoptosis and Promotes Cytoskeleton Rearrangement for Infection of Tick Cells

Nieves Ayllón,a Margarita Villar,a Ann T. Busby,b Katherine M. Kocan,b Edmou F. Blouin,b Elena Bonzón-Kulichenko,c Ruth C. Galindo,a Attilo J. Mangold,a Pillar Alberdi,a José M. Pérez de la Lastra,a Jesús Vázquez,a José de la Fuente,a,b Instituto de Investigación en Recursos Cinegéticos IREC-CSIC-UCLM-JCCM, Ronda de Toledo s/n, Ciudad Real, Spain; a Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma, USA; b Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Cantoblanco, Madrid, Spain; c Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela, Rafaela, Santa Fe, Argentina

Anaplasma phagocytophilum causes human granulocytic anaplasmosis. Infection with this zoonotic pathogen affects gene expression in both the vertebrate host and the tick vector, Ixodes scapularis. Here, we identified new genes, including spectrin alpha chain or alpha-fodrin (CG8) and voltage-dependent anion-selective channel or mitochondrial porin (T2), that are involved in A. phagocytophilum infection/multiplication and the tick cell response to infection. The pathogen downregulated the expression of CG8 in tick salivary glands and T2 in both the gut and salivary glands to inhibit apoptosis as a mechanism to subvert host cell defenses and increase infection. In the gut, the tick response to infection through CG8 upregulation was used by the pathogen to increase infection due to the cytoskeleton rearrangement that is required for pathogen infection. These results increase our understanding of the role of tick genes during A. phagocytophilum infection and multiplication and demonstrate that the pathogen uses similar strategies to establish infection in both vertebrate and invertebrate hosts.

MATERIALS AND METHODS

Cultured tick cells. The ISE6 tick cell line, originally derived from I. scapularis embryos (provided by U. G. Munderloh, University of Minnesota), was cultured in L15B medium as described previously (22). The ISE6 cells were inoculated with the NY18 isolate of A. phagocytophilum propagated in HL-60 cells and maintained according to the procedures of de la Fuente et al. (23). Uninfected cells were cultured in the same way, except with the addition of 1 ml of culture medium instead of infected cells. Uninfected and infected cultures (five independent cultures with approximately 106 cells each) were sampled at 6 days postinfection (dpi) (early infection; percentage of infected cells, 11% to 17% [average ± standard deviation {SD}, 13% ± 2%]) and 13 dpi (late infection; percentage of infected cells, 26% to 31% [average ± SD, 28% ± 2%]). Collected cells were centrifuged at 10,000 × g for 3 min, and cell pellets were frozen in liquid nitrogen until used for protein, DNA, and RNA extraction.

Ticks. I. scapularis ticks were obtained from the laboratory colony maintained at the Oklahoma State University (OSU) Tick Rearing Facility. Larvae and nymphs were fed on rabbits, and adults were fed on sheep. Off-host ticks were maintained in a 12-h light and 12-h dark photoperiod at 22 to 25°C and 95% relative humidity (RH). Adult male I. scapularis ticks were infected with A. phagocytophilum by feeding on a sheep inoculated intravenously with approximately 1 × 107 A. phagocytophilum (NY18 isolate)-infected HL-60 cells (90 to 100% infected cells) (24). In this model, over 85% of ticks become infected with A. phagocytophilum in both guts and salivary glands (24). Ticks were removed from the sheep at
10 days after infestation, held in the humidity chamber for 4 days, and dissected for DNA and RNA extraction from guts and salivary glands. For analysis of mRNA levels by real-time reverse transcription-PCR (RT-PCR) in different tick developmental stages, eggs (three batches of approximately 3,000 eggs each), larvae (three pools of 100 larvae each), nymphs (three pools of 100 nymphs each), and adult unfed and fed ticks (10 ticks each) were used for RNA extraction and analysis. The levels of mRNA of selected genes in individual infected and infected *I. scapularis* guts, salivary glands, and whole female ticks (*n* = 12 to 20) were characterized by real-time RT-PCR. Animals were housed and experiments were conducted with the approval and supervision of the OSU Institutional Animal Care and Use Committee.

RNAi in ISE6 tick cells and ticks. Oligonucleotide primers homologous to *I. scapularis* genes containing T7 promoters (see Table S1 in the supplemental material) were used for *in vitro* transcription and synthesis of double-stranded RNA (dsRNA) as described previously (25), using an Access RT-PCR system (Promega, Madison, WI) and a Megascript RNA interference (RNAi) kit (Ambion, Austin, TX). *I. scapularis* subolesin (11) and the unrelated Rs86 (26) dsRNAs were synthesized using the same methods described previously and used as positive and negative controls, respectively. The dsRNA was purified and quantitated by spectrophotometry.

RNAi experiments were conducted in cell cultures by incubating ISE6 tick cells with 10 μl dsRNA (5 × 10¹¹ to 5 × 10¹³ molecules/μl) and 90 μl L15B medium in 24-well plates, using 10 wells per treatment (12). Control cells were incubated with subolesin dsRNA and the unrelated Rs86 dsRNA. After 48 h of dsRNA exposure, tick cells were infected with cell-free *A. phagocytophilum NY18* obtained from approximately 5 × 10¹⁰ infected HL-60 cells (90 to 100% infected cells) and resuspended in 24 ml culture medium, resulting in 1 ml/well (27), or mock infected by adding culture medium alone. Cells were incubated for an additional 72 h, collected, and used for DNA and RNA extraction. RNA was used to analyze gene knockdown by real-time RT-PCR with respect to the level of expression of the Rs86 control. DNA was used to quantify the *A. phagocytophilum* infection levels by major surface protein 4 gene (*msp4*) PCR.

Unfed *I. scapularis* adult ticks (*n* = 10 females per group for the tick feeding experiment; *n* = 12 to 20 females per group for the effect on *A. phagocytophilum* infection) were injected with approximately 0.5 μl dsRNA (5 × 10¹⁰ to 5 × 10¹¹ molecules/μl) in the lower right quadrant of the ventral surface of the exoskeleton of the ticks (11). The injections were done using a 10-μl syringe with a 1-in., 33-gauge needle (Hamilton, Bonaduz, Switzerland). Control ticks were injected with subolesin dsRNA or the unrelated Rs86 dsRNA or were left un.injected. After completion of the experiments, ticks were dissected and whole-body tissues (tick feeding experiment) or separated guts and salivary glands (*A. phagocytophilum* infection experiment) were used for DNA and/or RNA extraction. RNA was used to analyze gene knockdown by real-time RT-PCR with respect to the level of expression of the Rs86 control, DNA was used to quantify the *A. phagocytophilum* infection levels by *msp4* PCR.

**Tick feeding experiment.** After dsRNA injection, female ticks were held in a humidity chamber for 1 day, after which they were allowed to feed on a sheep with 10 male ticks per tick feeding cell. The weight of individual female ticks collected after completion of feeding was determined. Two ticks per group were dissected for RNA extraction to analyze gene knockdown by real-time RT-PCR.

**Analysis of mRNA levels by real-time RT-PCR.** Total RNA was extracted from cultured ISE6 tick cells and tick samples using TRIReagent (Sigma, St. Louis, MO) following the manufacturer’s recommendations. Real-time RT-PCR was performed on tick RNA samples with gene-specific primers (see Table S2 in the supplemental material) using an iScript one-step RT-PCR kit with SYBR green and an iQ5 thermal cycler (Bio-Rad, Hercules, CA) following the manufacturer’s recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently over the same temperature range for every sample (28). The mRNA levels were normalized against the tick 16S rRNA level (15) using the genNorm method (where *C*ₚ is the threshold cycle) implemented by the Bio-Rad iQ5 standard edition, version 2.0 (29).

**Determination of *A. phagocytophilum* infection levels by real-time PCR.** DNA was extracted from ISE6 tick cells and tick samples using the TRIReagent (Sigma, St. Louis, MO) following the manufacturer’s recommendations. *A. phagocytophilum* infection levels in ISE6 tick cells were characterized by real-time RT-PCR with normalization against the level of tick 16S rRNA as described previously (11) but using oligonucleotide primers MSP4-L (5’-CTTCGGTGCGCACACCATGCTG-3’) and MSP4-R (5’-TGGCTGTGGTGCTGACGCCG-3’) and PCR conditions of 5 min at 95°C and 35 cycles of 10 s at 95°C, 30 s at 55°C, and 30 s at 60°C.

**Flow cytometry.** Recombinant *I. scapularis* voltage-dependent anion-selective channel (VDAC) or mitochondrial porin (T2) (GenBank accession no. XP_002408065) proteins and *A. phagocytophilum* NY18 MSP4 (GenBank accession no. AAV67031) proteins and *A. phagocytophilum* NY18 MSP4 (GenBank accession no. AF54957) proteins were expressed in Escherichia coli (Champion pET 101 directional TOPO expression kit; Carlsbad, CA) and purified using an Ni-nitrilotriacetic acid affinity column chromatography system (Qiagen Inc., Valencia, CA) following the manufacturer’s recommendations. Purified proteins were used to immunize rabbits, and IgGs from preimmune and immunized animals were purified (Montage antibody purification kit and spin columns with PROSEP-A medium; Millipore, Billerica, MA) and used for analysis. Anti-spectrin alpha chain or anti-alpha-fodrin (CG8) rabbit polyclonal IgGs (catalog no. S5381-03B) were purchased from US Biological Co. (Marblehead, MA) and used as recommended by the manufacturer. For flow cytometry analysis, ISE6 tick cells from early and late *A. phagocytophilum* infection and uninfected control ISE6 tick cells were washed in phosphate-buffered saline (PBS), fixed, and permeabilized with an Intracel fixation and permeabilization kit (Immunocept, Salamanca, Spain) following the manufacturer’s recommendations. After permeabilization, the cells were washed in PBS and incubated with primary unlabelled antibody (preimmune IgG isotype control, MSP4, subolesin, T2, and CG8 antibodies; 50 μg/ml) washed in PBS, and incubated in 100 μl of PBS with fluorescein isothiocyanate (FITC)–goat anti-rabbit IgG (Sigma, Madrid, Spain)-labeled antibody (diluted 1/500) for 15 min at 4°C. Finally, the cells were washed with PBS and resuspended in 500 μl of PBS. All samples were analyzed on a FACScan flow cytometer equipped with CellQuest Pro software (BD Biosciences, Madrid, Spain). The viable cell population was gated according to forward-scatter and side-scatter parameters. The level of MSP4, subolesin, T2, and CG8 in the viable cells was determined as the geometric median fluorescence intensity (MFI) of the test labeled sample minus the MFI of the isotype control (30).

**Immunofluorescence in uninfected and *A. phagocytophilum*-infected ISE6 tick cells.** Antibodies against subolesin, T2, CG8, and MSP4 were used for immunofluorescence in uninfected and *A. phagocytophilum*-infected (30% infection) ISE6 tick cells. Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Tick cell smears were prepared using a cytospin centrifuge. The cells were permeabilized with 0.3% Triton X-100 in PBS for 30 min, blocked with 2.5% bovine serum albumin in PBS for 1 h at room temperature, and incubated overnight at 4°C with purified antibodies (subolesin, 50 μg/ml; CG8 and T2, 18 μg/ml; MSP4, 24 μg/ml). After washing with PBS, slides were incubated in 100 μl PBS with FITC–goat anti-rabbit IgG (Sigma)-labeled antibody (diluted 1/160) for 75 min at room temperature. Finally, the slides were washed with PBS and mounted with ProLong Gold antifade reagent (Invitrogen, OR). Images were acquired on a Nikon Eclipse Ti-U microscope with a ×100 oil immersion objective and a Nikon Digital Sight DS V1 camera.

**Apoptosis (CASP9) assay.** The apoptosis assay was conducted using a caspase-9 (CASP9) colorimetric assay kit (GenScript, Piscataway, NJ). The assay for CASP9 is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate LEHD-pNA. The CASP9 activity in uninfected and *A. phagocyto-
Protein and mRNA levels in ISE6 tick cells in response to A. phagocytophilum

<table>
<thead>
<tr>
<th>Protein (sample identifier)</th>
<th>GenBank accession no.</th>
<th>Ratio of level for infected/uninfected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein hu-li tai shao, adducin (CG2)</td>
<td>B7P1C8, ISCW000621</td>
<td>Early infection: NS</td>
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<tr>
<td>Spectrin alpha chain, cytoskeletal protein (CG8)</td>
<td>B7P1U8, ISCW000012, XP_002433506</td>
<td>Late infection: −2.17</td>
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<td>Beta-tubulin (CG10)</td>
<td>B7PA92, ISCW017133</td>
<td>Early infection: −1.27</td>
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<tr>
<td>Na+/K+ ATPase, alpha subunit (T1)</td>
<td>B7PP4, ISCW002538</td>
<td>Early infection: −2.14</td>
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<tr>
<td>Voltage-dependent anion-selective channel (mt) (T2)</td>
<td>B7P5X8, ISCW000781, XP_002408065</td>
<td>Late infection: −1.33</td>
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<tr>
<td>Fatty acid-binding protein (FABP) (T3)</td>
<td>B7QMW0, ISCW015316</td>
<td>Late infection: −1.34</td>
</tr>
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*Protein levels were determined by proteomics analysis using protein one-step in-gel digestion, peptide iTRAQ labeling, isoelectric focusing fractionation, and liquid chromatography-tandem mass spectrometry analysis for peptide identification (unpublished results). mRNA levels were determined by real-time RT-PCR. Abbreviations and symbols: CG, cell growth; T, transport; mt, mitochondrial; − (negative values), lower levels in infected cells; NS, no significant differences between infected and uninfected cells (P > 0.05).

**RESULTS**

The expression of tick genes encoding cell growth and transport proteins is downregulated in response to *A. phagocytophilum* infection. The expression of tick genes encoding proteins in cell growth and transport biological processes was analyzed in ISE6 tick cells from early and late infection (Table 1). The results showed differences between protein and mRNA levels. However, the mRNA and protein levels were lower for genes encoding cell growth proteins hu-li tai shao, adducin (CG2), spectrin alpha chain or alpha-fodrin (CG8), and beta-tubulin (CG10) and transport proteins Na+/K+ ATPase, alpha subunit (T1), voltage-dependent anion-selective channel or mitochondrial porin (T2), and fatty acid-binding protein (T3) in *A. phagocytophilum* cells from early and/or late infection.

Changes in mRNA levels for selected genes in uninfected ISE6 tick cells and ticks. The expression of CG2, CG8, CG10, T1, T2, and T3 was characterized in uninfected ISE6 tick cells and tick developmental stages (Fig. 1). The results revealed differences in gene expression. For CG2, CG8, and CG10, the highest mRNA levels were observed in uninfected male ticks (Fig. 1). For T1, T2, and T3, the highest mRNA levels were observed in ISE6 tick cells, except for T2 mRNA levels, which were similar between tick cells.
and fed female ticks (Fig. 1). In ticks, CG2, CG8, CG10, and T2 mRNA levels increased from eggs to adult males, suggesting a role for these genes during male tick development (Fig. 1). The mRNA levels of all selected genes except the CG8 gene increased with female tick feeding, suggesting a possible role for these genes during blood meal ingestion/digestion (Fig. 1).

Changes in mRNA levels of selected genes in response to *A. phagocytophilum* infection in ticks. Gene expression analysis in response to *A. phagocytophilum* infection in ticks showed that, as in ISE6 tick cells, all genes were downregulated in tick salivary glands (Fig. 2A). However, while CG10 and T2 mRNA levels decreased in the guts and whole females in response to infection, the mRNA levels for CG2, CG8, T1, and T3 were higher in infected guts and ticks (Fig. 2A). Subolesin mRNA levels increased in response to pathogen infection in all tick samples (Fig. 2A).

Effect of gene knockdown on female tick feeding. The knockdown of the CG8, CG10, T1, and T2 genes resulted in a 29% to 73% decrease in female tick weight after feeding compared to the weight of the controls (*P* < 0.05) (Fig. 2B; see also Table S3 in the supplemental material). As expected from previous experiments, subolesin knockdown reduced the weight of fed ticks by 88% compared with the weight of unrelated Rs86 dsRNA-injected ticks (*P* < 0.01) (Fig. 2B).

Effect of gene knockdown on *A. phagocytophilum* infection levels in ISE6 tick cells and ticks. Gene knockdown by RNAi was used for functional characterization of the CG2, CG8, CG10, T1, T2, and T3 genes during *A. phagocytophilum* infection of ISE6 tick cells and ticks (Fig. 2C and D; see also Table S4 and Table S5 in the supplemental material). Significant gene knockdown was obtained for 4 genes, the CG2, CG8, T2, and subolesin genes, in ISE6 tick cells (see Table S4 in the supplemental material). However, gene knockdown produced significant differences in *A. phagocytophilum* infection levels only for CG8, T2, and subolesin (Fig. 2C). Pathogen infection levels were lower and higher in CG8- and

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**FIG 1** Gene expression in *I. scapularis* ISE6 cells and tick developmental stages. The CG2, CG8, CG10, T1, T2, and T3 mRNA levels in ISE6 cells and ticks were determined by real-time RT-PCR. Amplification efficiencies were normalized against the amplification efficiency for tick 16S rRNA, and normalized mRNA levels (average ± SD) are expressed in arbitrary units.
T2-knockdown cells, respectively. Subolesin knockdown resulted in higher pathogen infection levels in ISE6 tick cells.

In ticks, significant gene knockdown was obtained for all genes in both guts and salivary glands (see Table S5 in the supplemental material). As expected, gene knockdown in ticks had different effects on pathogen infection levels in guts and salivary glands (Fig. 2D). Knockdown of the CG2 and CG8 genes resulted in lower infection levels in both guts and salivary glands. Pathogen infection levels were higher in the salivary glands of CG10-knockdown ticks, while lower infection levels were obtained in T1-knockdown ticks. Knockdown of the T2 and T3 genes decreased infection levels in the guts but increased them in the salivary glands of T2-knockdown ticks. In this experiment, subolesin knockdown did not affect pathogen infection levels in ticks. Only CG8 and T2 knockdown affected *A. phagocytophilum* infection levels in both ISE6 tick cells and ticks (Fig. 2C and D). No correlation between the effect of gene knockdown on tick feeding and pathogen infection levels was found. After gene knockdown, pathogen infection levels varied between guts and salivary glands, and while the greatest effect of gene knockdown on tick feeding was shown for subolesin (Fig. 2B), gene knockdown did not have an effect on pathogen infection levels (Fig. 2D).

**Sequence analysis of *I. scapularis* genes functionally relevant for *A. phagocytophilum* infection of ISE6 tick cells and ticks.** Additional sequence analysis was conducted on the *I. scapularis* CG8 and T2 genes, affecting *A. phagocytophilum* infection after gene knockdown in both ISE6 tick cells and ticks. The *I. scapularis* CG8 sequence contained 24 spectrin (cd00176) repeats. The T2 protein contained one voltage-dependent anion-selective channel (VDAC; cd07306) domain. Sequence databases were searched for CG8 and T2 orthologs. Sequences homologous to *I. scapularis* CG8 (GenBank accession no. XP_002433506) were not identified in other tick species, but *I. scapularis* T2 (GenBank accession no. XP_002408065) orthologs were found in *Rhipicephalus (Boophilus) microplus* (GenBank accession no. ADT82652) and *Amblyomma variegatum* (GenBank accession no. DAA34069). Phyloge-
A genetic analysis of CG8 (Fig. 3A) and T2 (Fig. 3B) sequences showed that these tick sequences clustered together in a clade close to the sequence of the water flea (*Daphnia pulex*; Arthropoda: Crustacea). Similar results were obtained with all methods used to infer evolutionary histories (data not shown). Alignment of T2 tick ortholog protein sequences revealed differences in sequence length that could be due to incomplete sequence information or evolution, with a 79% homology in 234 shared amino acids (Fig. 3C). The analysis of molecular pathways involving CG8 and T2 proteins in the KEGG database showed that these molecules are involved in tight junction (map04530) and apoptosis (map04210) pathways.

CG8 and T2 proteins levels decrease and subolesin levels increase in *A. phagocytophilum*-infected ISE6 tick cells. The CG8, T2, and subolesin protein levels were characterized in ISE6 tick cells in response to *A. phagocytophilum* infection. The results showed that while *A. phagocytophilum* major surface protein 4 (MSP4) and subolesin levels increased in infected cells compared to uninfected control cells, CG8 and T2 levels decreased with infection (Fig. 4A and B). Immunofluorescence analysis of uninfected and *A. phagocytophilum*-infected ISE6 tick cells corroborated the flow cytometry results (Fig. 4C). Subolesin was found in both uninfected and infected cells in the cell nuclei and cytoplasm, with higher concentrations in the latter and increased levels after infection (Fig. 4C, panels c and d). T2 was found in both perinuclear and cytoplasmic spaces, with lower levels found in infected cells, particularly in the cytoplasm, where mitochondria are located (Fig. 4C, panels g and h). CG8 was mainly found in the perinuclear region, with lower levels found in infected cells (Fig. 4C, panels i and j). Taken together, these results corroborated the proteomics results and evidenced differences in protein levels between infected and uninfected cells.

*A. phagocytophilum* inhibits apoptosis and rearranges the cytoskeleton to increase pathogen infection of ISE6 tick cells. Previous results suggested that T2 is involved in mitochondrially induced apoptosis mediated by CASP9. Therefore, CASP9-mediated apoptosis was characterized in uninfected and *A. phagocytophilum*-infected ISE6 tick cells. The results showed that while apoptosis was induced or inhibited after treatment with the riddiften-B and NSCI apoptosis inducer and inhibitor, respectively, infection with *A. phagocytophilum* inhibited CASP9-mediated apoptosis.

**FIG 3** Analysis of CG8 and T2 protein sequences. (A) Phylogenetic analysis of CG8 protein sequences. (B) Phylogenetic analysis of T2 protein sequences. The evolutionary histories were inferred using the neighbor-joining method. Tick sequences are marked with dots. (C) Amino acid sequence alignment of tick T2 ortholog sequences in *I. scapularis* (GenBank accession no. XP_002408085), *R. microplus* (GenBank accession no. ADT82652), and *A. variegatum* (GenBank accession no. DAA34069). Asterisks denote amino acids conserved among all sequences analyzed.
apoptosis by more than 1.5-fold (Fig. 5A). The activity of HK, a protein interacting and partially regulating T2, was lower in *A. phagocytophilum*-infected ISE6 tick cells than uninfected cells (Fig. 5B). These results demonstrate that *A. phagocytophilum* infection downregulates T2 expression, resulting in the inhibition of mitochondrially induced apoptosis (Fig. 5C).

**DISCUSSION**

The infection of *I. scapularis* ISE6 tick cells with *A. phagocytophilum* reduces the levels of proteins involved in cell growth and transport, thus reflecting the effect of pathogen multiplication on these cell processes (unpublished results). Herein, the genes encoding tick proteins involved in cell growth and transport were functionally characterized for their role during pathogen infection.

Several studies have characterized the *A. phagocytophilum*-tick interface at the molecular level (10–21). However, this appears to be the first report of the functional analysis of tick genes confirmed to be differentially expressed at both the protein and mRNA levels in response to *A. phagocytophilum* infection. Furthermore, the predicted function of the genes confirmed to affect pathogen infection in both ISE6 tick cells and ticks, CG8 and T2, suggested that they are involved in *A. phagocytophilum* infection/multiplication and tick response to infection.

**Spectrin alpha chain or alpha-fodrin (CG8)**. The spectrin re-

**FIG 4** T2 and CG8 protein levels decrease in *A. phagocytophilum*-infected ISE6 tick cells. (A) Flow cytometry profile histogram showing in cells from early and late infection (infected cells [IC]) and uninfected cells (UC) the isotype control (IgG IC, green; IgG UC, yellow), T2 (T2 IC, purple; T2 UC, orange), and CG8 (CG8 IC, red; CG8 UC, blue) MFI peaks visualized by use of an FITC-conjugated secondary antibody and CellQuest Pro software. (B) Ratio of MFI for infected cells to MFI for uninfected cells for MSP4, subolesin (SUB), T2, and CG8 in cells from early and late infection and uninfected cells. Positive and negative values denote higher and lower protein levels in infected cells, respectively, with respect to the levels in the uninfected controls. MFI was calculated as the MFI of the test labeled sample minus the MFI of the isotype control. (C) Representative images of immunofluorescence analysis of uninfected and *A. phagocytophilum*-infected ISE6 tick cells. Tick cells were stained with rabbit anti-tick proteins antibodies (green, FITC). (a and b) Bright-field image of infected cells (a) and preimmune control serum-treated infected cells (b), which gave results to similar those for uninfected cells; (c and d) uninfected (c) and infected (d) cells stained with subolesin antibodies; (e and f) uninfected (e) and infected (f) cells stained with anti-MSP4 antibodies; (g and h) uninfected (g) and infected (h) cells stained with anti-T2 antibodies; (i and j) uninfected (i) and infected (j) cells stained with anti(CG8) antibodies. Bars, 10 μm (a, b) and 20 μm (c to j).
peats in the I. scapularis CG8 sequence are found in several proteins involved in the cytoskeletal structure (39, 40). CG8 is an actin-cross-linking and molecular scaffold protein that links the plasma membrane to the actin cytoskeleton and functions in the determination of cell shape, arrangement of transmembrane proteins, and organelle organization, all necessary for cell growth and/or maintenance. In other systems, fodrin-mediated actin rearrangements occur during pathogen infection of host cells (41), but fodrin activation could result in cell apoptosis (42). These results suggest a dual role for CG8, in which it induces cytoskeletal rearrangements necessary for pathogen infection while at the same time it acts as a host cell defense mechanism to control pathogen infection through induction of cell apoptosis.

This dual function of CG8 was also suggested in tick cells and ticks infected with A. phagocytophilum. CG8 was downregulated in infected tick cells and tick salivary glands, likely manipulated by the pathogen, because the gene is involved in the host cell response to infection. CG8 mRNA levels were upregulated in the guts of infected ticks. However, CG8 knockdown resulted in lower A. phagocytophilum infection levels in ISE6 tick cells and tick guts and salivary glands because CG8 is also required for pathogen infection. In fact, downregulation of CG8 was more pronounced in ISE6 tick cells and tick salivary glands late in A. phagocytophilum infection, when inhibition of cell apoptosis is crucial to increase infection, but rearrangement of the actin filaments that are required for pathogen infection may be less relevant at this infection stage. When RNAi produced gene knockdown before infection, the effect on cytoskeleton rearrangement occurred at early infection stages and thus resulted in lower pathogen infection levels both in ISE6 tick cells and in tick guts and salivary glands. Another example of how A. phagocytophilum alters the cytoskeleton is through actin phosphorylation to selectively regulate gene transcription in association with RNA polymerase II (RNAPII) and the TATA-binding protein in ticks (18). Interestingly, certain spectrin mutations or polymorphisms have been shown to constitute new factors of innate resistance to malaria in vitro (43).

The reduction in tick weight obtained after CG8 gene knockdown suggested that, although mRNA levels decreased with blood feeding in female ticks, the protein plays a role during tick feeding and/or digestion of a blood meal. The increase in CG8 mRNA levels that occurred from tick larvae to unfed males suggested a role for this gene during development, at least in male ticks. These results are probably associated with the rearrangements of the cytoskeleton that occur during blood feeding and development in ticks (44).

Voltage-dependent anion-selective channel or mitochondrial porin (T2). The VDAC domain present on I. scapularis T2 suggested that this molecule is the channel known to guide the metabolic flux across the mitochondrial outer membrane that plays a key role in mitochondrially induced apoptosis (45, 46). T2 is the most abundant protein in the mitochondrial outer membrane and regulates the flux of mostly anionic metabolites through the outer mitochondrial membrane, which is highly permeable to small molecules (45, 46). As suggested by immunofluorescence assays conducted here, T2 is also localized extramitochondrially (47). T2 binds to and is regulated in part by HK, an interaction that renders mitochondria less susceptible to proapoptotic signals, most likely by interfering with T2’s capability to

![FIG 5 Role of T2 in mitochondrially induced apoptosis.](image-url)
Subolesin knockdown increased in pathogen infection levels, probably due to the role of subolesin in intracellular innate immunity as a mechanism to subvert host cell defense against pathogen infection. Upon infection, cells undergo apoptosis as a defense against pathogen replication, which was characterized by reduced HK levels in A. phagocytophilum-infected tick cells. Pathogens have evolved mechanisms to subvert the apoptotic processes through downregulation of T2 (A. phagocytophilum in tick cells) and VDAC1 and VDAC2 (group A Streptococcus pyogenes in epithelial cells [48]) and the expression of proteins that interact with Bcl-2 and VDAC1 (herpesvirus in murine fibroblasts [49]). A. phagocytophilum uses different mechanisms to inhibit apoptosis in both mammalian and tick cells (4, 5, 50). The Anaplasmata xenolocated substate 1 (Ats-1) is secreted by A. phagocytophilum and translates inside the mammalian cell mitochondria to inhibit apoptosis (51). The activity of this A. phagocytophilum antiapoptotic effector in tick cells is unknown but could be related to the findings reported herein. Recently, I. scapularis X-linked inhibitor of apoptosis (XIAP) E3 ubiquitin ligase was shown to be required for A. phagocytophilum infection in ticks (52), again showing how inhibition of tick cell apoptosis through different mechanisms is required for pathogen infection.

As previously discussed for CG8, gene expression and RNAi experiments suggested that T2 might be involved in tick feeding and/or digestion of a blood meal and during development of male ticks. In ticks, like in other organisms, mitochondrial function is likely essential for many physiological processes, such as feeding and development (53, 54).

Subolesin. Subolesin was used as a control in these studies, but new data on protein levels were obtained. Subolesin is a candidate tick protective antigen discovered in I. scapularis and subsequently found to be conserved in many tick species and other eukaryotes, where it is the ortholog of insect and mammalian akirins (14, 55–58). Subolesin expression is induced in response to pathogen infection in ticks and plays an important role in the tick immune response to pathogen infection through the regulation of genes involved in innate immunity (14, 16, 56, 59). As in previous experiments, subolesin mRNA levels did not change in response to A. phagocytophilum infection of ISE6 tick cells but increased in tick guts and salivary glands (12, 60). However, subolesin protein levels increased in A. phagocytophilum-infected ISE6 tick cells, suggesting that posttranscriptional mechanisms affect subolesin levels in response to infection (21, 61). Subolesin knockdown resulted in higher A. phagocytophilum infection levels in ISE6 tick cells, supporting its role in the tick immune response. However, in tick guts and salivary glands, subolesin knockdown did not affect pathogen infection levels, probably due to the role of subolesin in tissue structure and development, which are required for pathogen infection (16, 25, 62), pointing to differences between in vitro and in vivo studies (12, 21, 60).

Although subolesin and akirin function as transcription regulatory factors and should therefore be located in cell nuclei (14, 56–58, 63), the results shown herein suggest, as did previous experiments (25), that at least for tick subolesin, the protein is also present in the cell cytoplasm. This result agrees with the hypothesized role for tick subolesin in different pathways that help to provide an understanding of the effect of gene knockdown by RNAi and vaccination (13, 14). However, further experiments are needed to characterize the subcellular localization for this protein.

Conclusions. The experiments described here characterized new genes involved in A. phagocytophilum infection/multiplication and the tick cell response to infection, thus advancing our understanding of the molecular events at the tick-pathogen interface. The pathogen manipulated CG8 and T2 tick gene expression to inhibit apoptosis as a mechanism to subvert host cell defenses and increase infection. In the gut, the tick response to infection through CG8 upregulation was used by the pathogen to increase infection due to cytoskeleton rearrangement, which is required for pathogen infection. These results also evidenced the different roles that guts and salivary glands play during pathogen infection (64). Remarkably, both mechanisms have also been reported in mammalian host neutrophils infected with A. phagocytophilum (4–8), demonstrating that the pathogen uses similar strategies to establish infection in both vertebrate and invertebrate hosts.

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