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Research Article

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Anaplasma phagocytophilum induces *Ixodes scapularis* ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold

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In the United States, *Ixodes scapularis* ticks overwinter in the Northeast and Upper Midwest and transmit the agent of human granulocytic anaplasmosis, *Anaplasma phagocytophilum*, among other pathogens. We now show that the presence of *A. phagocytophilum* in *I. scapularis* ticks increases their ability to survive in the cold. We identified an *I. scapularis* antifreeze glycoprotein, designated IAFGP, and demonstrated via RNAi knockdown studies the importance of IAFGP for the survival of *I. scapularis* ticks in a cold environment. Transfection studies also show that IAFGP increased the viability of yeast cells subjected to cold temperature. Remarkably, *A. phagocytophilum* induced the expression of *iafgp*, thereby increasing the cold tolerance and survival of *I. scapularis*. These data define a molecular basis for symbiosis between a human pathogenic bacterium and its arthropod vector and delineate what we believe to be a new pathway that may be targeted to alter the life cycle of this microbe and its invertebrate host.

Introduction

Diverse arthropods have intimate relationships with microbes (1–5). Several examples include pheromonal symbiosis of desert locusts (6) and nutritional symbiosis of flower thrips (7) with environmentally acquired bacteria. Aphid-*Buchnera* and tsetse-*Wigglesworthia* obligate endosymbioses and *Wolbachia*-insect facultative endosymbioses are examples of arthropods having relationships with bacteria acquired by vertical transmission (8–10). Arthropods also transmit microbes to vertebrates, sometimes resulting in human illness (11), and little is known about whether these vectors can establish beneficial interactions with medically important pathogens.

The black-legged tick, *Ixodes scapularis*, can transmit several bacteria that cause disease in man, including *Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis (12–16). *I. scapularis* are blood-sucking ectoparasites that ingest *A. phagocytophilum* while feeding on infected animals (16). Upon entering ticks, *A. phagocytophilum* establishes itself in the salivary glands of the ticks and is then transstadially maintained through different stages of the arthropod life cycle (17, 18). Persistence in the arthropod throughout the year is particularly important for a microbe, such as *A. phagocytophilum*, which cannot be transovarially transmitted from an infected adult female tick to its offspring (17, 18). *I. scapularis* larvae that feed in midsummer overwinter as nymphs, and nymphs that engorge in early summer overwinter as adults (18, 19). These ticks must survive multiple abiotic stress conditions, including freezing temperatures, desiccation, and ice encasement.

Arthropods have evolved physiological and behavioral strategies to withstand lower environmental temperatures that are collectively termed "freeze avoidance" or "freeze tolerance." Each of these processes elicits different adaptive responses, and specific antifreeze proteins are involved (20–22). Antifreeze proteins are a diverse class of compounds that bind to ice crystals and restrict their growth (23–26). Antifreeze proteins require structural complementarity with ice to adsorb to its surface; however, the molecular mechanisms remain to be elucidated (27–30). Antifreeze proteins are classified into 2 main types, antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) (24, 29). AFPs and AFGPs are divided into 4 and 8 subtypes, respectively (24, 29, 30). A characteristic feature of AFGPs is the presence of repeating tripeptide units (alanine-alanine-threonine), in which the secondary hydroxyl group of the threonine residue is glycosylated with a disaccharide (30). Higher molecular mass glycoproteins of 20 to 33 kDa are referred to as AFGPs 1 to 4, and those less than 20 kDa are referred to as AFGPs 5 to 8 (24, 29, 30). AFGPs may also have the L-threonine residue substituted with L-arginine, and L-alanine residues substituted with L-proline (31, 32). Most experiments have focused on understanding the role of type 1 to 4 AFPs. A limited number of studies have been performed on AFGPs, due to their structural complexity and the difficulty in obtaining pure AFGP.

Since their identification in the body fluids of polar fishes (23, 33), AFPs have been found in more than 55 terrestrial arthropods, including insects, spiders, mites, and centipedes (20, 34–40). AFGPs in arthropods have not been reported. In the present study, we have identified an *I. scapularis* AFGP and determined whether *I. scapularis* has enhanced cold tolerance due to an association with *A. phagocytophilum*.

Results

A. phagocytophilum–infected ticks survive better at cold temperatures. *A. phagocytophilum* survives the winter in tissues of *I. scapularis*. We therefore determined whether this bacterium influences the survival of ticks exposed to cold temperatures. Uninfected or

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A. phagocytophilum–infected ticks that were maintained at 23°C in laboratory were incubated at –20°C and analyzed for survival at various time points (Figure 1A). Survival curves showed the lethal time point$_{50}$ (LT_{50}) at –20°C for uninfected ticks to be approximately 25 minutes (Figure 1A). At the LT_{50}, *A. phagocytophilum*–infected ticks were more cold tolerant (survival rate of 80%) than uninfected (survival rate of 40%) ticks (Figure 1A). None of the ticks, regardless of infection, survived when incubated for 45 minutes at –20°C (Figure 1A). We then performed 18 independent survival assays with multiple batches of ticks (10 ticks/group/experiment) at the LT_{50}. We found increased survival rates for *A. phagocytophilum*–infected (73%, $P < 0.001$) ticks in comparison with uninfected (41%) controls (Figure 1B). After cold shock, ticks were monitored for their ability to move from a starting point after 10 minutes. The mobility (distance-traveled rate) of *A. phagocytophilum*–infected ticks after cold shock was higher ($P < 0.0001$) than controls, suggesting increased fitness of the *A. phagocytophilum*–infected ticks at cold temperatures (Figure 1C and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI42868DS1).

To determine whether sequential cooling affected the survival of ticks, we incubated uninfected and *A. phagocytophilum*–infected ticks at different temperatures (23°C, 4°C, 0°C, –10°C, and –20°C) in a sequential manner for 1 hour at each respective temperature (see Methods). The increased survival and mobility of *A. phagocytophilum*–infected ticks in comparison with uninfected ticks that was seen upon rapid cold shock was also significant ($P < 0.05$) upon sequential cooling (Figure 1, D and E). To eliminate any bias in our studies, we performed a series of scrambled assays with a mixed population of *A. phagocytophilum*–infected and uninfected ticks at the LT_{50} (see Methods). Scrambled assays showed enhanced survival and mobility for *A. phagocytophilum*–infected ($P < 0.05$) ticks in comparison with the controls (Figure 1, F and G).

To determine whether the *A. phagocytophilum* burden was associated with increased arthropod cold tolerance, we performed studies with 25 uninfected and 25 *A. phagocytophilum*–infected ticks and quantified the *A. phagocytophilum* burden in each tick. After cold treatment, quantitative real-time PCR (QRT-PCR) was performed with *p44* gene–specific primers to assess the *A. phagocytophilum* load. The results indicated a significant correlation between the *A. phagocytophilum* burden in each tick and its cold tolerance capacity (Figure 2). Collectively, the results from Figures 1 and 2 demonstrate a beneficial association between the presence of *A. phagocytophilum* within *I. scapularis* and the viability of ticks exposed to extreme cold temperatures.

Identification of an afgp gene in I. scapularis. Several insects and terrestrial arthropods have antifreeze proteins (20, 22, 35). We assessed whether *I. scapularis* has any antifreeze proteins and whether *A. phagocytophilum* influences the expression of these genes. An expressed sequence tag (EST) sequence that encodes a potential antifreeze protein (TC43064) was identified from the *I. scapularis* database. The sequence showed significant relatedness to a cod, *Boreogadus saida*, *afgp* gene rather than to insect *afp* genes. By RT-PCR, using primers designed from the 5′ and 3′ ends of the *I. scapularis* TC43064 sequence, an amplification product of approximately 900 bp was detected in cDNA generated from unfed *I. scapularis* ticks (Supplemental Figure 2). The PCR product was cloned into the pGEM-T Easy Vector and sequenced from both ends (Figure 3). The GC-rich TC43064 sequence contained a full-length open reading frame and coded predicted protein that, because of high similarity with *B. saida* AFGPs, was named *I. scapularis* AFGP (IAFGP; GenBank HM213906). Sequencing of additional cDNA clone obtained from different PCR primers (see Methods) yielded an additional 32 upstream nucleotides beyond the putative start codon of *iafgp* (Figure 3).

The *iafgp* sequence contained an open reading frame of 756 nucleotides with a 3′ untranslated region that included a polyadenylation signal sequence at position 146 from the stop codon (Figure 3). The full-length *iafgp* cDNA contained a KOZAK-compatible (41) ATG codon at the 5′ end, encoding a putative IAFGP protein with a predicted molecular mass of 23.2 kDa (Figure 3). Computer analysis (SignalP 3.0 Server; Technical University of Denmark) revealed a signal peptide cleavage site between amino acids 19 and 20 (Figure 3). The deduced IAFGP sequence showed approximately 70% identity with several other fish AFGPs (Supplemental Figure 3, A and B). Ala-Ala-Thr repeat sequences characteristic of fish AFGPs and posttranslational modification signals including O-glycosylation sites (determined at NetOGlyc 3.1 Server; Technical University of Denmark) were identified in IAFGP (Figure 3).

Expression of I. scapularis iafgp is developmentally regulated and induced at cold temperatures. We determined whether *iafgp* expression was regulated during tick development. The *iafgp* mRNA levels were assessed by QRT-PCR using β-actin as an internal control (Figure 4A). *iafgp* was expressed in larvae and nymphs and increased in adults (Figure 4A and Supplemental Figure 4). No difference in *iafgp* expression was seen between male and female adult ticks (Figure 4A and Supplemental Figure 4). To assess whether cold temperatures influenced *iafgp* mRNA levels, uninfected unfed ticks (20 ticks/temperature) were incubated at different temperatures (23°C, 10°C, 4°C, and 0°C). The expression levels of *iafgp* were increased (4-fold, $P < 0.001$) at lower (4°C and 0°C) temperatures in comparison with higher (23°C and 10°C) temperatures (Figure 4B).

Expression of iafgp is influenced by A. phagocytophilum. As *A. phagocytophilum* influences the cold tolerance of ticks, we determined whether *A. phagocytophilum* also alters *iafgp* expression. *A. phagocytophilum*–infected ticks were incubated at different temperatures (23°C, 10°C, 4°C, and 0°C), and *iafgp* mRNA levels were compared with those of uninfected ticks (20 ticks/group/temperature). The expression of *iafgp* was elevated in *A. phagocytophilum*–infected ticks in comparison with the controls at all tested temperatures (Figure 4C; 3-fold, $P < 0.05$). These results show that *iafgp* expression is influenced by the presence of *A. phagocytophilum* within ticks.

iafgp-deficient ticks have reduced ability to survive at cold temperatures. To determine whether IAFGP directly plays a role in tick survival at cold temperatures, we generated *iafgp*-deficient uninfected ticks by RNA interference (RNAi). Mock and *iafgp* dsRNAs were synthesized in vitro. Mock dsRNA was prepared from empty L4440 vector that contained restriction enzyme site sequences (see Methods). Equal amounts (4.2 nanoliters) containing equal molecules of the respective dsRNAs were microinjected into the bodies of ticks, which were allowed to rest for 3 days at 23°C. At day 3, QRT-PCR showed a significant reduction of *iafgp* mRNA in *iafgp*-dsRNA–injected ticks compared with the mock-injected (7-fold, $P < 0.01$) controls (Figure 5A). The unchanged levels of tick *beta-actin* mRNA confirmed the specificity of RNAi. We then determined survival and mobility rates of mock- and *iafgp*-dsRNA–injected ticks at the LT_{50} (25 min, –20°C). We found a significant reduction in survival ($P < 0.0001$) and mobility

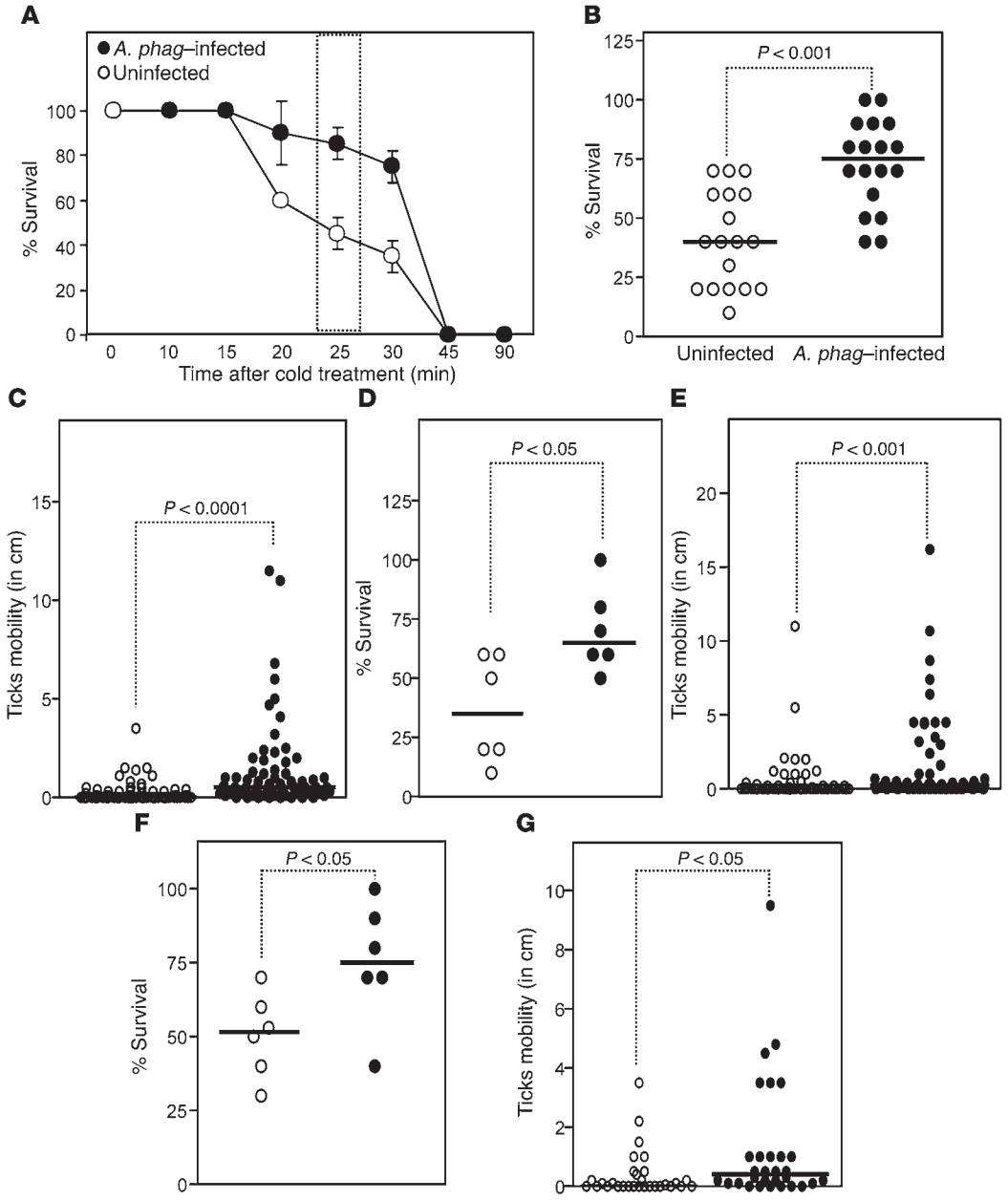

Figure 1

A. phagocytophilum–infected nymphs survive better at cold temperatures. (**A**) Survival of uninfected and *A. phagocytophilum*–infected (*A. phag*–infected) unfed nymphs at –20°C for 0, 10, 15, 20, 25, 30, 45, or 90 minutes. Data from 2 independent experiments with 10 ticks/group/time point/experiment are shown. The dashed box indicates the LT_{50} time point for ticks at –20°C. Mean ± SD error bars from 2 independent experiments are shown for comparison. (**B**) Survival of uninfected and *A. phagocytophilum*–infected unfed nymphs at the LT_{50} (–20°C, 25 minutes) from 18 independent experiments. Each circle represents 1 independent experiment (10 ticks/group/experiment). The difference in the survival between *A. phagocytophilum*–infected and uninfected nymphs is significant ($P < 0.001$). (**C**) Mobility (in cm) by uninfected or *A. phagocytophilum*–infected unfed nymphs after cold shock at LT_{50}. Each circle represents 1 individual tick. Survival (**D** and **F**) and mobility (**E** and **G**) measurements of uninfected and *A. phagocytophilum*–infected unfed nymphs at LT_{50} in a sequential (**D** and **E**) and scrambled (**F** and **G**) cold tolerance assay. Each circle in **D** and **F** represents 1 independent experiment (10 ticks/group/experiment) and in **E** and **G** represents 1 individual tick. In all panels, white circles represent uninfected ticks and black circles represent *A. phagocytophilum*–infected ticks. For all panels, statistical significance was calculated using Mann-Whitney *U* nonparametric test. $n = 70$ (**C**); $n = 60$ (**E**); $n = 30$ (**G**) for both uninfected and *A. phagocytophilum*–infected ticks. Horizontal lines in **B**–**G** indicate the median of the readings from independent experiment/ticks.

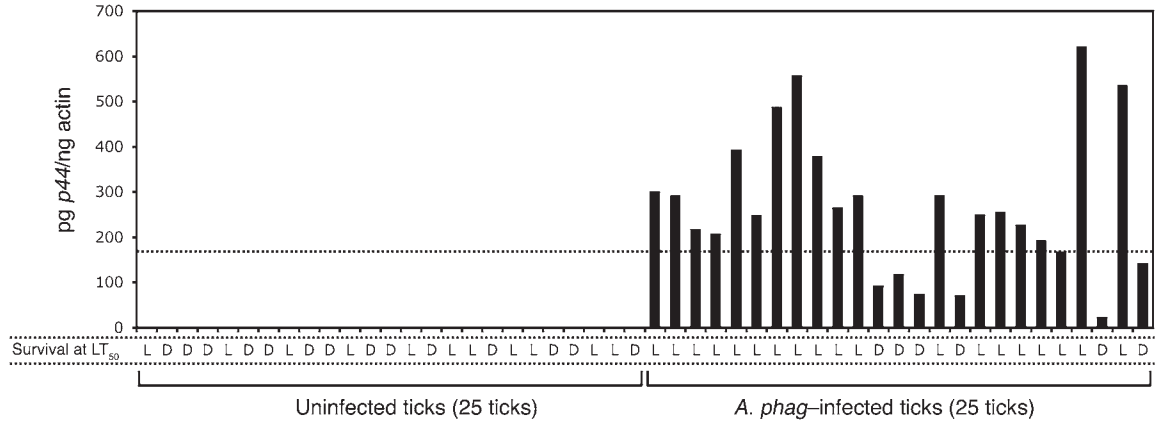

Figure 2
Increased *A. phagocytophilum* burden correlates with increased cold tolerance of ticks. *A. phagocytophilum*-burden in Live (L) or Dead (D) *A. phagocytophilum*–infected unfed ticks at LT_{50} (–20°C, 25 minutes) as determined by QRT-PCR. Uninfected ticks were used as controls. *A. phagocytophilum* burden was quantified with *p44* gene–specific primers and normalized to tick *beta-actin* levels. Histograms represent the *A. phagocytophilum* burden in each individual tick (25 ticks/group/assay). Horizontal dotted line in the graph points to the threshold level of *A. phagocytophilum* burden required to increase cold tolerance in ticks. All of the dead *A. phagocytophilum*–infected ticks showed reduced bacterial loads that fell below the threshold level. 1 complete set of data from 3 independent experiments is shown.

($P < 0.0001$) of *iafgp*-dsRNA–injected ticks in comparison with the mock-injected controls (Figure 5, B and C), demonstrating a direct role for IAFGP in tick cold tolerance. To further demonstrate that the reduced survival and mobility of ticks was due to specific silencing of *iafgp*, the survival and mobility assays were repeated by silencing another *I. scapularis* gene, *trospA* (Supplemental Figure 5). We found no significant differences in the survival and mobility of *trospA*-dsRNA–injected ticks in comparison with the mock-injected controls (Supplemental Figure 5).

To understand whether increased cold tolerance in *A. phagocytophilum*–infected ticks is solely related to *A. phagocytophilum*–induced expression of *iafgp*, we generated *iafgp*-deficient ticks using RNAi. Mock- and *iafgp*-dsRNA–injected ticks were then allowed to feed on *A. phagocytophilum*–infected mice for 48 hours. Both groups of ticks were assessed for the *A. phagocytophilum* burden and cold tolerance (see Methods). The LT_{50} time point at –20°C for 48-hour–fed ticks was between 45 and 50 minutes. For our experiments, we considered incubation at –20°C, 50 minutes as cold treatment condition for fed ticks. QRT-PCR showed a reduction of *iafgp* mRNA levels in *iafgp*-dsRNA–injected ticks when compared with the mock-injected (3-fold, $P < 0.05$) ticks (Figure 5D). We found a reduction in the *A. phagocytophilum* burden ($P < 0.05$) and survival ($P < 0.01$) of *iafgp*-dsRNA–injected ticks in comparison with the controls (Figure 5, E and F). The survival rates of ticks were the same in the mock- and *trospA*-dsRNA–injected ticks (Supplemental Figure 5). These results show that the increased cold tolerance in *A. phagocytophilum*–infected ticks is directly related to the *A. phagocytophilum*–induced expression of *iafgp*.

A. phagocytophilum infection enhances the cold tolerance of tick cells. *A. phagocytophilum* can infect and persist in *I. ricinus* tick cell line (IRE/CTVM19) for an extended period of time. By RT-PCR, using primers designed from the 5′ and 3′ ends of *iafgp* gene followed by sequencing, we were able to identify *I. ricinus iafgp* gene from these cells (Supplemental Figure 6, A and B). We designated *I. ricinus iafgp* as *iafgp-Iric* (GenBank HM213905). The deduced amino acid sequence of IAFGP-IRIC showed 80% identity with IAFGP (Supplemental Figure 6C). Ala-Ala-Thr repeat sequences and O-glycosylation sites were also evident in IAFGP-IRIC (Supplemental Figure 6B). We then determined whether *A. phagocytophilum* also induces *iafgp-Iric* in vitro. These tick cells,

Figure 3
Nucleotide and predicted amino acid sequence of IAFGP. The nucleotide sequence of the *iafgp* gene was obtained by sequencing PCR product cloned in the pGEMT-Easy Vector. The deduced amino acid sequence is shown as a single letter below the nucleotide sequence. The in-frame putative IAFGP translational start and stop codons are indicated in bold. The amino acid sequence corresponding to N-terminal signal peptide is boxed (solid line). Ala-Ala-Thr repeats are underlined, and several O-glycosylation sites are indicated by asterisks. Upstream nucleotide sequence obtained from sequencing additional cDNA clone is shown in lower case. The Kozak sequence at 5′ end and polyadenylation signal at 3′ end are indicated in bold letters and enclosed in dashed boxes.

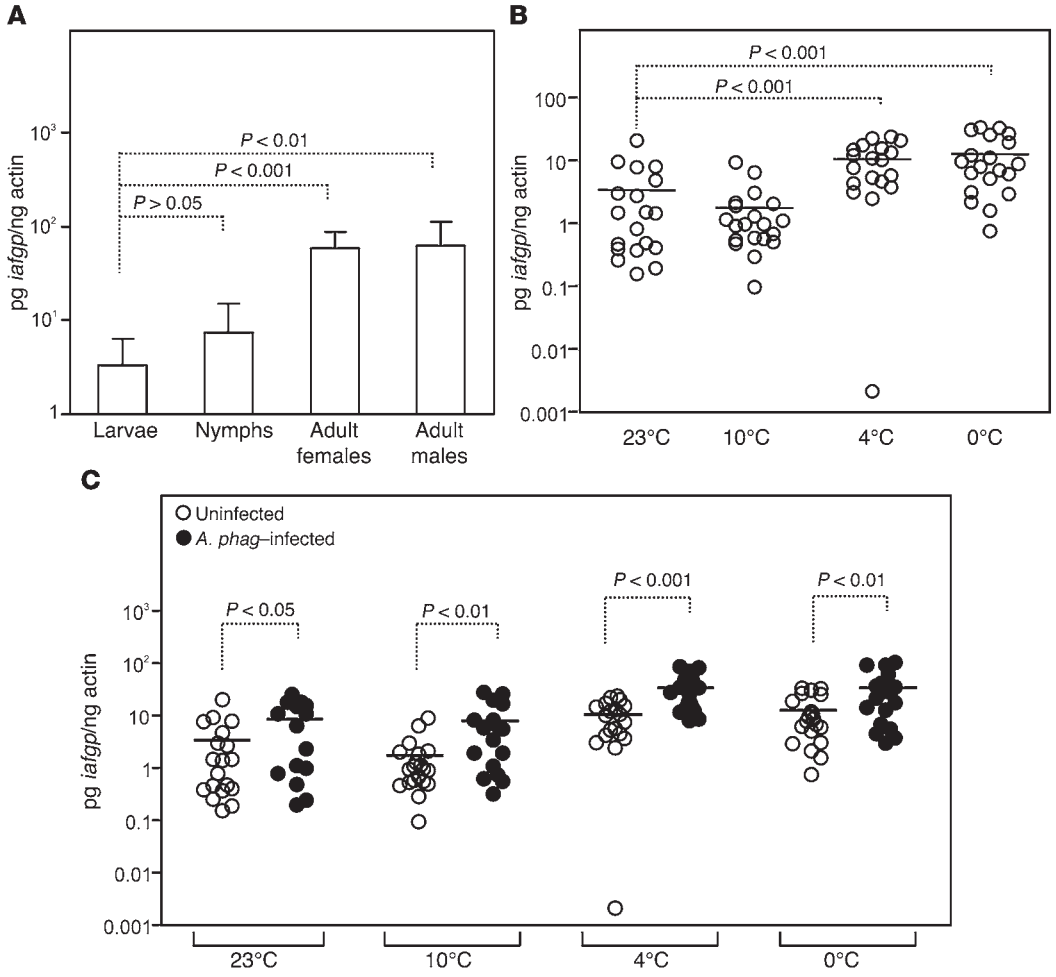

Figure 4

Expression of *iafgp* is developmentally regulated and induced by cold and *A. phagocytophilum*. (**A**) The expression of *iafgp* is developmentally regulated in *I. scapularis* ticks. Total RNA from unfed larvae (n = 10), unfed nymphs (n = 10), unfed adult males (n = 10 ticks), and unfed adult females (n = 10) was prepared in triplicate and the amount of *iafgp* transcripts was quantified by QRT-PCR and normalized to tick *beta-actin*. Error bars indicate + SD from the mean. (**B**) The expression of *iafgp* in nymphs is induced by cold temperatures. Total RNA from nymphs incubated at different temperatures (23°C, 10°C, 4°C, and 0°C) was prepared, and the *iafgp* transcript levels were quantified by QRT-PCR. Each circle represents 1 individual tick. The differences in *iafgp* levels at 4°C and 0°C in comparison with 23°C is significant ($P < 0.05$, Student's t test). (**C**) Total RNA from uninfected nymphs (white circles) and *A. phagocytophilum*–infected nymphs (black circles) incubated at different temperatures (23°C, 10°C, 4°C, and 0°C) was prepared, and transcript levels of *iafgp* were quantified by QRT-PCR. Levels of *iafgp* were normalized to tick *beta-actin*. Each circle represents 1 individual tick. The elevated levels of *iafgp* transcripts in *A. phagocytophilum*–infected nymphs in comparison with the uninfected controls is significant at all tested temperatures ($P < 0.05$, Student's t test). Horizontal lines in panels **B** and **C** indicate average of the readings from independent ticks.

when incubated at –5°C for 2 hours, demonstrated a significant induction of *iafgp-Iric* mRNA (3-fold, $P < 0.01$) in comparison with 28°C temperature (Figure 6A). *A. phagocytophilum* infection also induced *iafgp-Iric* expression in these cells (4-fold, $P < 0.01$; Figure 6B). To determine whether *A. phagocytophilum* infection increases cold tolerance in tick cells, we incubated the cells at –20°C for 10 minutes and stained with FM4-64 dye to analyze membrane integrity and internalization of the dye upon cold treatment (Figure 6C). We found less membrane disruption and internal membrane staining in *A. phagocytophilum*–infected cells (3-fold, $P < 0.001$) compared with controls (Figure 6, C and D). Uninfected and *A. phagocytophilum*–infected cells incubated at 28°C showed no phenotypic differences (Figure 6C). These results further show that *A. phagocytophilum* infection and an increase in *iafgp-Iric* expression are associated with the induction of cold tolerance in tick cells.

In vitro antifreeze activity of IAFGP mediates yeast survival at cold temperatures. To characterize the functional role of IAFGP, we developed a cold tolerance assay in yeast (see Methods). IAFGP was expressed in *Saccharomyces cerevisiae* EBY100 cells fused to the Aga2p mating agglutinin protein under the control of galactose-inducible promoter (Figure 7A). The expression of *iafgp* was induced by growing yeast transformants in SDGAA medium. 10^2 cells from an overnight culture were plated on SDGAA, and the number of viable cells before cold shock was determined by their colony-forming ability (Figure 7, B and C). From the same culture vial, cells were shifted to –20°C, and at 4 and 24 hours samples were thawed and 10^2 cells were plated on SDGAA plates to deter-

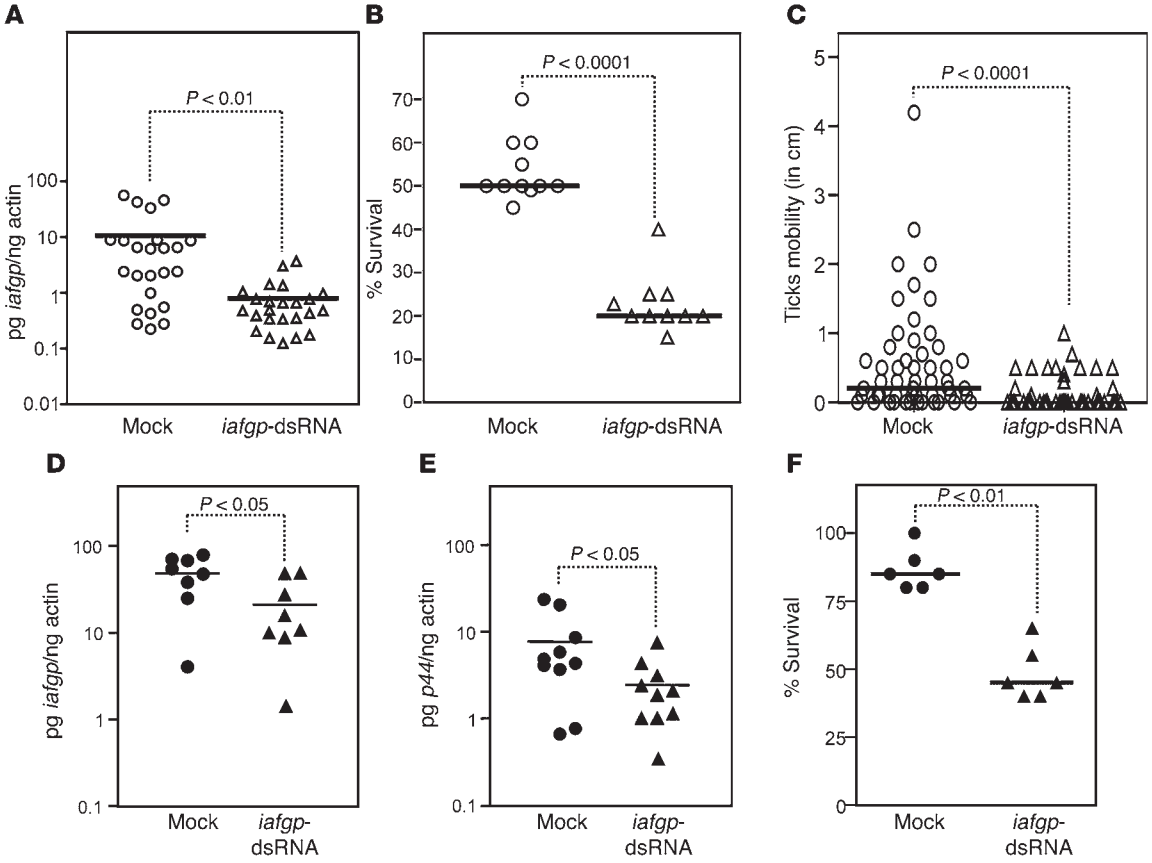

Figure 5

Silencing of *iafgp* by RNAi reduces survival of ticks at cold temperatures. (**A**) QRT-PCR showing reduced *iafgp* mRNA levels in *iafgp*-dsRNA–injected uninfected ticks compared with the mock-dsRNA control. (**B**) Survival of mock-dsRNA–injected and *iafgp*-dsRNA–injected ticks at the LT_{50} time point. (**C**) Mobility (in cm) by mock-dsRNA–injected and *iafgp*-dsRNA–injected ticks at LT_{50} time point (–20°C, 25 min). (**A**–**C**) White circles represent mock-dsRNA–injected ticks, and white triangles represent *iafgp*-dsRNA–injected ticks. QRT-PCR showing reduced *iafgp* mRNA levels (**D**) and *A. phagocytophilum* burden (**E**) in mock-injected (black circles) and *iafgp*-dsRNA–injected (black triangles) ticks partially fed (48 hours) on *A. phagocytophilum*–infected mice. (**F**) Survival percentage at –20°C, 50-minute time point of mock-dsRNA–injected (black circles) and *iafgp*-dsRNA–injected (black triangles) ticks partially fed (48 hours) on *A. phagocytophilum*–infected mice. Each circle in **A**, **C**, **D**, and **E** represents 1 individual tick, and each circle in **B** and **F** represents 1 independent experiment with 10 ticks/group/experiment. Statistics in **A**, **D**, and **E** were performed using 2-tailed Student's *t* test and in **B**, **C**, and **F** using Mann-Whitney *U* test. $n = 60$ (**C**) for both mock and *iafgp*-dsRNA ticks. Horizontal lines in **A**, **D**, and **E** represent average and in **B**, **C**, and **F** represent median of the readings from independent experiments/ticks.

mine cell viability after cold shock. Yeast cells transformed with the empty plasmid pYD1 were used as experimental controls. Recovery of the plasmids (isolated from EBY100 transformants and transformed in *E. coli*) confirmed the presence of both *iafgp* and empty plasmids in respective yeast strains used in the cold-tolerance assay (Supplemental Figure 7). Cells containing empty vector showed a significant loss of viability after 24 hours incubation at –20°C in comparison with the cells expressing IAFGP ($P < 0.01$; Figure 7, B and C). These results demonstrate that expression of IAFGP increases cold tolerance in yeast.

Discussion

Arthropods and microbes have intimate interactions (4). Many microbes are parasitic or commensal, but some are advantageous to the host (1, 5, 9, 42–47). Studies have elucidated the role of microbial factors involved in beneficial relationships with arthropods (4, 42, 43); however, the host molecules that contribute to these beneficial relationships remain unknown. We have now identified an arthropod antifreeze glycoprotein, IAFGP, that is actively involved in a mutualistic interaction between *A. phagocytophilum* and *I. scapularis*, facilitating survival of both species at cold temperatures.

Several studies have examined the cold-hardiness of *Ixodes* ticks, but these studies have not assessed whether a microbe can influence its vector during cold exposure (48–50). As *A. phagocytophilum* overwinter in ticks (18), we determined whether this microbe altered *I. scapularis* gene expression and whether this was related to survival in the cold. We observed a significant increase in the expression of an antifreeze glycoprotein gene, *iafgp*, at cold temperatures when *I. scapularis* were infected with *A. phagocytophilum* compared with ticks that did not harbor this pathogen. We found no significant differences in *iafgp* expression between uninfected and *Babesia microti*–infected ticks, suggesting that *iafgp* expression is specifically influenced by the presence of *A. phagocytophilum* (Supplemental Figure 8). The increased survival, as determined by a series of rapid, sequential, and scrambled cold-tolerance assays, demonstrated

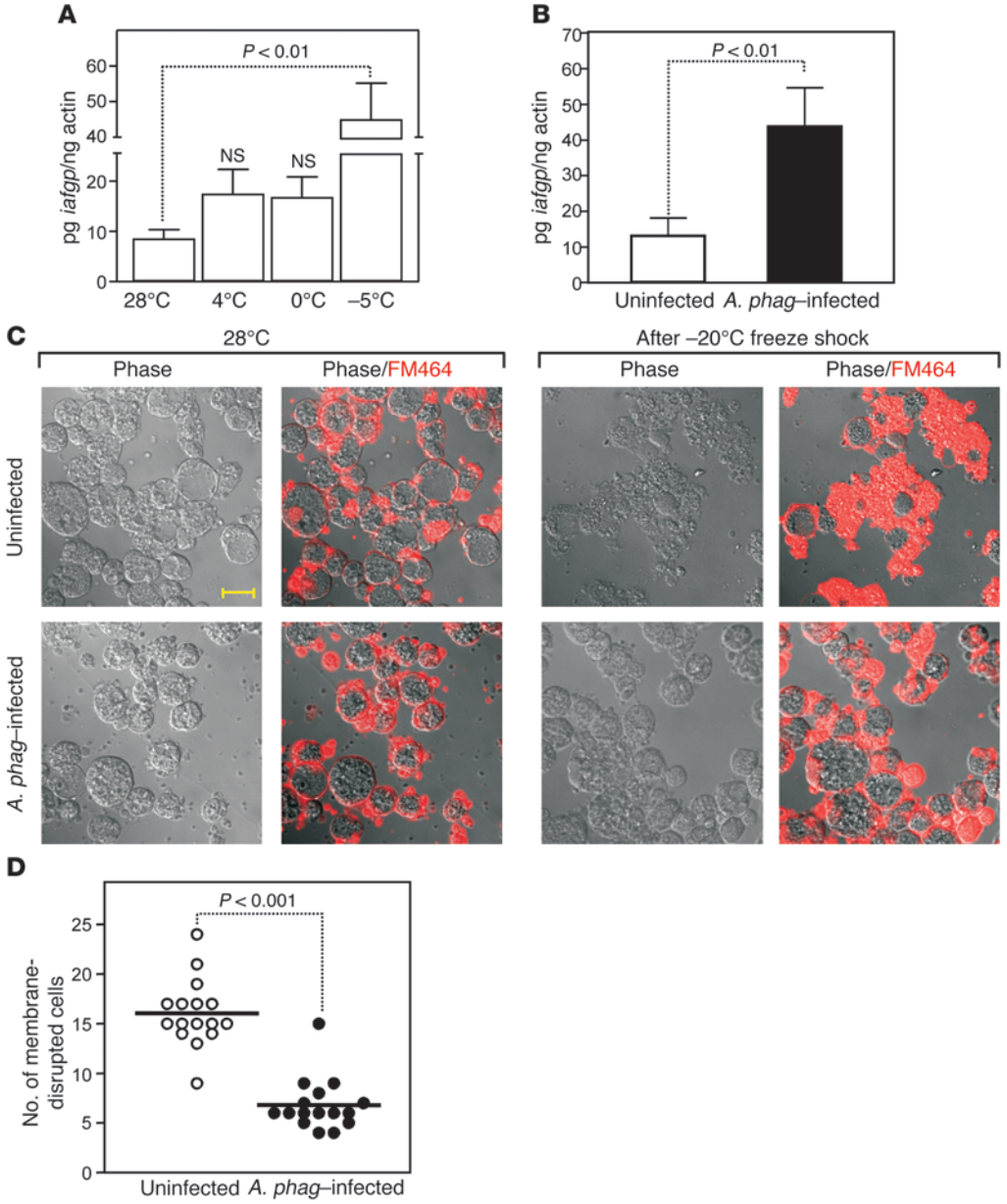

Figure 6

A. phagocytophilum infection and induction of *iafgp* expression enhances cold tolerance in tick cells. (**A**) QRT-PCR showing the levels of *iafgp* transcripts in tick cells incubated at different temperatures (28°C, 4°C, 0°C, –5°C). (**B**) QRT-PCR showing the level of *iafgp* transcripts in uninfected (white bar) and *A. phagocytophilum*–infected (black bar) tick cells at 48 hours after infection. Results from 3 independent experiments are shown. Error bars indicate + SD from the mean. The level of *iafgp* transcripts was normalized to tick *beta-actin*. (**C**) Immunofluorescence images of tick cells stained with FM4-64 dye showing increased membrane disruption in uninfected cells in comparison with *A. phagocytophilum*–infected cells after cold shock at –20°C for 10 minutes. Uninfected and *A. phagocytophilum*–infected tick cells incubated at 28°C served as experimental controls. Representative images from 3 independent experiments are shown. Original magnification, ×65. Scale bar: 20 μM. (**D**) Quantitative assessment of the number of membrane-disrupted cells in uninfected (white circles) and *A. phagocytophilum*–infected (black circles) tick cells after cold shock at –20°C for 10 minutes is shown. Each circle represents 1 microscopic field. Statistics were performed using Student's *t* test and the *P* values are shown. Horizontal lines in the graph indicate average of the readings from independent microscopic observations.

enhanced cold tolerance of *A. phagocytophilum*–infected ticks. The mobility of ticks further confirmed increased fitness of *A. phagocytophilum*–infected ticks at cold temperatures. Finally, a higher *A. phagocytophilum* burden correlated with increased viability of ticks at cold temperatures. A direct correlation of AFGP concentration to antifreeze activity has been shown in other studies (24, 51, 30). Our data suggest that *A. phagocytophilum* induces the expression of *iafgp*, leading to elevated levels of IAFGP synthesis that enhance the cold tolerance of ticks — thereby facilitating survival of both *I. scapularis* and *A. phagocytophilum* at extreme temperatures.

The small genome size, low rate of horizontal gene transfer events, and absence of mobile genetic elements that are charac-

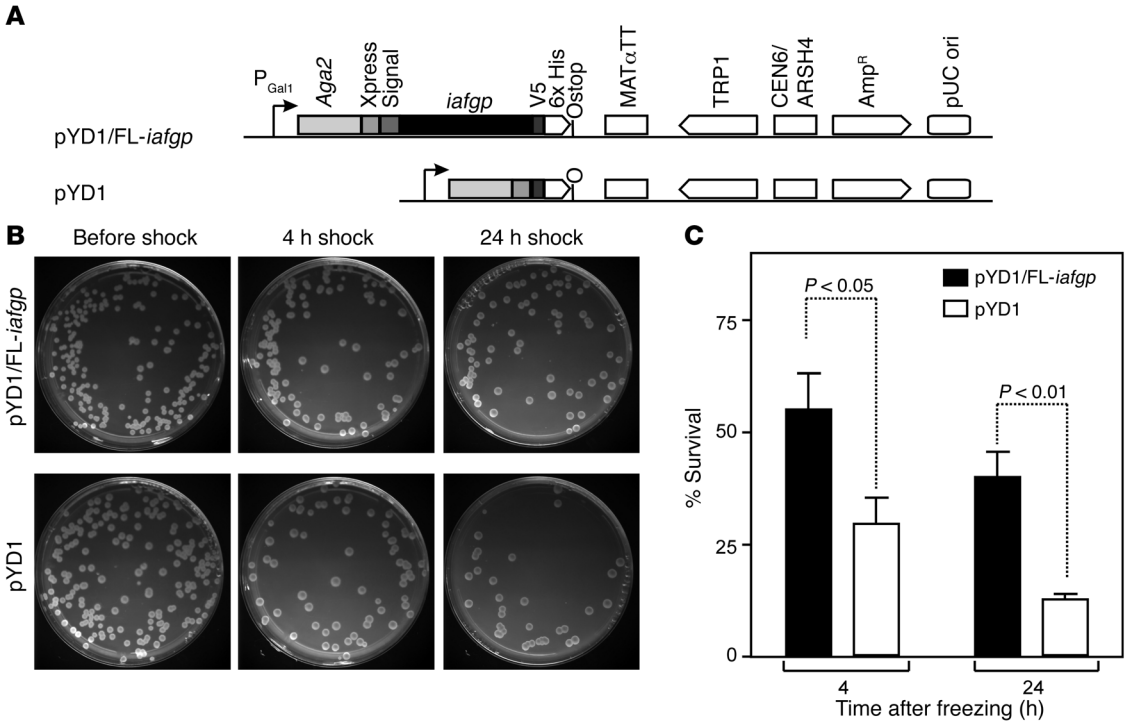

Figure 7
IAFGP increases cold tolerance in yeast. (**A**) Schematic representation of the constructs used for testing IAFGP expression in yeast cells. Full-length *iafgp* (FL-*iafgp*) was cloned in frame with AGA2 in pYD1 vector and transformed into EBY100 yeast cells. (**B**) Representative images from 3 independent experiments of SDGAA plates containing pYD1/FL-*iafgp* and the pYD1 empty vector transformed yeast cells before and after 4 hours or 24 hours at –20°C. (**C**) Quantitative survival assessment of pYD1/FL-*iafgp* (black bars) and pYD1 empty vector (white bars) transformed yeast cells after 4 hours or 24 hours at –20°C. Data from 3 independent experiments with triplicate clones are shown. Error bars indicate + SD from the mean value. Statistics were performed using Student's *t* test, and the *P* values are shown.

teristic of many beneficial microbes (52) are also evident with *A. phagocytophilum* (53). Many arthropods contain specialized cells called bacteriocytes that are filled with "primary symbionts" that are mostly beneficial to the hosts (4, 43). The primary symbionts are vertically transmitted from parent to their offspring. Arthropods also harbor "secondary" symbionts that usually do not reside exclusively in these specialized organs and are found extracellularly or intracellularly in different tissues (4, 43). These symbionts are acquired by horizontal transmission such as direct intimate contact or indirect contamination via a vector or the environment. Unlike primary symbionts in which beneficial associations with the arthropod are maintained from generation to generation, secondary symbionts exhibit favorable associations with the host that are typically facultative (4). Our finding that, in the absence of vertical transmission, *A. phagocytophilum* that are acquired from mammals and maintained in ticks significantly contributed to the arthropod fitness during cold reflects a facultative beneficial relationship between this microbe and *I. scapularis*.

The frequency of *A. phagocytophilum* infection in *I. scapularis* varies among ticks in different parts of United States (12–15, 54). Factors that influence the efficient vector acquisition of the bacteria while feeding on vertebrates as well as the ecological and landscape changes, such as yearly temperature fluctuations, associated with tick activity, can potentially influence *A. phagocytophilum* infection rates (19, 55). If *A. phagocytophilum*–infected ticks persist better during winter, then illnesses that these ticks transmit may also increase with alterations in the climate. Our laboratory-based survival and mobility studies may provide insight in understanding the epidemiological distribution of *A. phagocytophilum*–infected ticks in the field.

The extended winter questing activity of *Ixodes* ticks has been systematically examined both in Europe and in the United States (56, 57). The fact that *Ixodes* ticks are active during the winter, when arthropods are usually quiescent, supports our data for a critical role of AFGPs during this season. Studies from Lee and Baust show that *Ixodes uriae* has the greatest overall range of thermal tolerance, from –30°C to +40°C, reported for any Antarctic terrestrial arthropod (48). The authors proposed that the regulation of the cold tolerance in *I. uriae* could be due to the presence of antifreeze proteins. Arthropods in nature seek refuge in areas (microenvironment) where they are protected from hard freezing. Chill injury is the most important condition they come across. In addition to the role as inhibitors of ice crystal growth, AFGPs protect membrane stability during low temperature stress and chill injury (58, 59). Hays et al. found that AFGPs prevent leakage from liposomes, suggesting that AFGPs interact with lipids to stabilize membranes at low temperature (60). Collectively, these studies suggest a role for AFGP during chill injury.

The RNAi studies, in which the selective knockdown of *iafgp* directly interferes with the survival and mobility of *I. scapularis* ticks, confirms the role of IAFGP in tick fitness at extreme cold temperatures. We noted no changes in survival or mobility with

iafgp-dsRNA–treated ticks at warmer (28°C) temperatures (data not shown), suggesting that the effects of the dsRNA on tick survival after cold shock were not due to indirect effects on any aspect of tick biology. Three scenarios may be envisioned that may elucidate the involvement of IAFGP in cold tolerance. First, IAFGP may inhibit ice crystal growth inside ticks, thereby protecting them from lethal stress and damage caused by freezing. Second, IAFGP may prevent (a) denaturation of macromolecules, (b) rupture of cell membrane by blocking ion fluxes across membranes or by inhibiting the leakage of liposomes, and (c) osmotic shrinkage with water loss in cells during freezing. Third, IAFGP may work in concert with other cold tolerance strategies of arthropods such as accumulation of polyhydroxyl alcohols and glycerol to increase the overall cold-tolerance capacity. With any of these models, our finding that *iafgp*-deficient ticks were reduced in their ability to survive after cold shock suggests a critical role for IAFGP in *I. scapularis* during the winter.

The mechanism by which IAFGP binds ice water molecules during freezing in ticks is currently not understood. We hypothesize that IAFGP may either directly bind to the ice by hydrogen bonding or by the entropic and enthalpic interactions from hydrophobic residues. The demonstration of reduced membrane disruption of *A. phagocytophilum*–infected tick cells that express more *iafgp* and increased cold tolerance upon direct expression of IAFGP in yeast supports this role. These data also suggest a mechanism of IAFGP function at the ice water molecule interface and corroborate the observations seen with other antifreeze proteins (61, 62).

In summary, these data show how an arthropod and a microbe interact to adapt to cold temperature. This beneficial interaction may enhance the long-term coexistence and fitness of both species. This study is not only important in developing an understanding of the molecular basis of mutualism between bacteria and arthropods but also may potentially form the basis for future strategies to interfere with the life cycle of vector-borne human diseases.

Methods

Bacterial isolates and ticks. *A. phagocytophilum* isolate NCH-1 (63) was used throughout this study and will be referred to herein as the *A. phagocytophilum*. This isolate infects humans and persists in ticks during its different developmental stages. *E. coli* strain DH5α (Invitrogen) was used as a cloning host for generating different plasmids. Where necessary, the antibiotic ampicillin was used at a final concentration of 50 μg/ml. *I. scapularis* ticks comprising uninfected, *A. phagocytophilum*–infected nymphs, uninfected larvae, and uninfected male and female adults and *Babesia*–infected ticks were obtained from a continuously maintained tick colony at the Department of Epidemiology and Public Health, Yale University. Larvae were fed on either uninfected or *A. phagocytophilum*–infected mice and allowed to molt to generate uninfected or *A. phagocytophilum*–infected unfed nymphs. To generate 48-hour–fed *A. phagocytophilum*–infected nymphs, mock and *iafgp*-dsRNA–injected ticks were allowed to feed on *A. phagocytophilum*–infected mice and collected at 48 hours after engorgement. Mice were obtained from Jackson Laboratories and housed at Yale Animal Resources Center. We used female C3H/HeJ mice (Jackson laboratories), 4 to 6 weeks of age, for all our experiments. All tick-feeding experimental protocols followed Yale University institutional guidelines for care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University. Tick rearing was conducted in an incubator at 23°C with 85% relative humidity and a 14-hour light/10-hour dark photoperiod regiment.

Infection rate assessment in ticks. Genomic DNA from *A. phagocytophilum*–infected unfed nymphs was extracted using DNeasy kit (QIAGEN) and processed for PCR with primers specific for the *A. phagocytophilum p44* gene (64). 10% of the unfed nymphs from each infection group were individually tested by PCR to confirm *A. phagocytophilum* infection. *A. phagocytophilum* infection rates ranged from 80% to 95% from each group. PCR reactions were performed with Taq DNA polymerase (Roche) with conditions as follows: 94°C for 3 minutes initial denaturation followed by 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute for 28 cycles. PCR products were analyzed on 1.5% agarose gels.

Identification of antifreeze glycoprotein gene in I. scapularis. The *iafgp* (TC43064) full-length nucleotide sequence was identified with BLAST search performed at the Gene Index Project (http://compbio.dfci.harvard.edu/tgi/) in the *I. scapularis* EST database using *B. saida* (NCBI DQ427107) nucleotide sequence as query. EST sequences were further analyzed using DNASTAR software. Total RNA from unfed, uninfected *I. scapularis* ticks was isolated using QIAGEN RNeasy Kit (QIAGEN), processed for cDNA synthesis using iScript cDNA synthesis (Bio-Rad), and used as a template for RT-PCR. RT-PCR was performed using Master Amp PCR Optimization Kit (Epicentre Technologies). The *iafgp* gene fragment was amplified using oligonucleotides 5′-ATGACGACTCTGCTTCGTCTGACTATCCT-3′ and 5′-CGTTATTTATTCCCRCGTTTTCAT-3′. The generated PCR products were cloned into pGEM-T easy vector (Promega) and sequenced using primers 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′, 5′-TAATACGACTCACTATA-3′, and 5′-CACACAGGAAACAGCTATGAC-3′ from both ends at KECK DNA Sequencing Facility, Yale University. To obtain additional 32-bp upstream nucleotide sequence (KOZAK sequence) beyond the putative start codon of *iafgp* gene, PCR was performed using 5′-GAGACTCCAAAGGGACCAACCTA-3′ and 5′-CGTTATTTATTCCCRCGTTTTCAT-3′ and sequenced from the 5′ end with the pGEMT sequencing primer (mentioned above). Figure 3 in the text shows sequence of both 32 bp upstream sequence and *iafgp* coding sequence. Amino acid sequence similarity and identity of IAFGP were determined by aligning deduced IAFGP amino acid sequences with fish AFGPs from *B. saida*, *Chaenodraco wilsoni*, *Pogonophryne scotti*, *Dissostichus mawsoni*, *Chaenocephalus aceratus*, *Trematomus hansoni*, *Notothenia neglecta*, *Notothenia microlepidota*, *Harpagifer antarcticus*, and *Eleginus gracilis* using DNASTAR software. Accession numbers for these protein sequences are shown in the legend of Supplemental Figure 3.

Cold treatment for ticks to quantify iafgp mRNA levels. *A. phagocytophilum*–infected and uninfected control unfed nymphs that were maintained at 23°C in an incubator were split into 4 groups with 20 ticks/group in individual 0.5-ml tubes. These tubes were incubated at 23°C for 2 hours and immediately shifted to different temperatures (10°C, 4°C, and 0°C). One group of ticks was continued with incubation at 23°C as controls. 100 μl of RLT buffer (QIAGEN RNeasy kit) was calibrated in 20 individual tubes/temperature. Ticks and buffer solution were incubated for 4 hours at respective temperatures. After cold treatment, ticks were immediately processed for homogenization in individual tubes with RLT buffer incubated at respective temperatures. For 4°C and 0°C incubations, homogenization was performed in 4°C cold room. All ticks were immediately processed for RNA extraction followed by cDNA synthesis. The mRNA level of *iafgp* was measured by QRT-PCR.

Survival rate measurement by rapid cold shock. Survival rate of ticks was determined with modifications from Vandyk et al. (65). For generating *I. scapularis* survival curve, unfed *A. phagocytophilum*–infected or uninfected nymphs that were maintained at 23°C in an incubator were split into 8 groups with 10 ticks/group in individual glass tubes with ventilated covers that allow aeration. Tubes containing ticks were incubated at 23°C for an additional 2 hours and directly shifted to –20°C on a flat metal

surface of the freezer (Hotpoint). A range of time in minutes was chosen at –20°C to determine LT_{50}, at which point 50% tick mortality was observed. After cold treatment at –20°C for 0, 10, 15, 20, 25, 30, 45, and 90 minutes, glass vials containing ticks were immediately shifted to 4°C for 1 hour and room temperature for 1 hour to reacclimatize ticks. After reacclimatization at room temperature, a breath test was performed by blowing air into the glass tubes and immediately ticks were placed side-by-side (1 cm apart) in a line on Whatman filter paper. Any tick moving out of the line within 10 minutes was considered to have survived the cold temperature. Percentage of tick survival at –20°C was determined for each time point, and survival curve was generated from 2 independent experiments with 10 ticks/group/time point using Microsoft Excel. Eighteen independent experiments (10 ticks/group/experiment) with different batches of *A. phagocytophilum*–infected or uninfected unfed nymphs were then performed to determine whether the presence of *A. phagocytophilum* influences tick survival at the LT_{50} time point (25 minutes, –20°C). Rapid cold treatment for mock and *iafgp*-dsRNA–injected *A. phagocytophilum*–infected nymphs (48-hour fed ticks) was performed for 50 minutes at –20°C followed by incubation at 4°C for 1 hour and room temperature for 1 hour to reacclimatize ticks. After reacclimatization ticks were analyzed for survival as described before.

Survival rate measurement by sequential cold shock. To determine the survival rate of ticks after sequential cooling, *A. phagocytophilum*–infected or uninfected unfed nymphs (10 ticks/group/experiment) that were maintained at 23°C were sequentially incubated at 4°C for 1 hour, 0°C for 1 hour, and –10°C for 1 hour and assayed for survival rate at LT_{50}. Further details are provided in Supplemental Methods.

Survival rate measurement by scrambled assay. To eliminate any bias in the measurement of survival rate of *A. phagocytophilum*–infected unfed nymphs in comparison with the unfed uninfected controls, scrambled assays were performed. *A. phagocytophilum*–infected and uninfected unfed nymphs (10 ticks/group/experiment) were mixed in equal numbers. Glass tubes containing mixed groups of ticks were treated the same way as in rapid cold shock experiments. After cold treatment at LT_{50} (–20°C, 25 minutes), ticks were placed in a line and then numbered from 1 to 20. Survival was determined and documented for each numbered tick. After survival assay, all ticks were processed for DNA extraction (QIAGEN) and PCR to classify them as uninfected (no PCR product with *p44* gene primers) or *A. phagocytophilum*–infected ticks (PCR positive with *p44* gene primers). Six independent experiments were performed, and statistical significance ($P < 0.05$) was calculated using Mann-Whitney *U* test.

Tick mobility measurements. Along with the survival assays (rapid cold shock experiments, sequential cold shock experiments, and scrambled assays), unfed nymphs were also monitored for mobility on Whatman filter paper. After cold shock and further processing as mentioned in rapid cold shock experiments, ticks were immediately placed in a line 1 centimeter apart and monitored for movement from starting point (point where ticks were placed at the start of the assay) to the end point (point where ticks have moved after 10 minutes of observation). Distance from the start point and end point was measured in centimeters for each individual tick. Ticks that did not move after 10 minutes were scored as zero. Mobility (distance traveled) measurement for both categories of ticks (uninfected and *A. phagocytophilum*–infected unfed nymphs) from 5 independent experiments was then analyzed using GraphPad Prism software, and statistical significance ($P < 0.05$) was calculated using the Mann-Whitney *U* test.

QRT-PCR analysis. Total RNA from unfed nymphs, unfed larvae, unfed adult male and female ticks, or tick cell line was converted to cDNA. The generated cDNA was used as a template for the amplification of *iafgp* mRNA. QRT-PCR was performed using iQ-SYBR Green Supermix (Bio-Rad). The levels of *iafgp* were quantified using oligonucleotides 5′-ATGAAGACTCTGCTTCCTCTGACTATCCT-3′ and 5′-GGTGTAGCCGCCCTTTTGG-3′. Standard curve was prepared using 10-fold serial dilutions of known quantities of *iafgp* gene cloned in pGEMT-Easy Vector. As an internal control and to normalize the amount of template, tick β-actin amplicons were quantified with published primers (66). Standard curve for tick actin was prepared using 10-fold serial dilutions of known quantities from 0.5 ng to 0.000005 ng.

dsRNA synthesis and tick microinjections. The pGEMT-*iafgp* plasmid was used as a template to amplify DNA encoding a fragment of *iafgp*. The *iafgp* gene-specific primers containing BglII (5′-GAAGATCTGTTGCCGGCGTGCTCG-3′) and KpnI (5′-GGGGTACCCTTCCGTATCCGAGCCCGTA-3′) restriction enzyme sites were used in the PCR. The fragment containing *iafgp* sequence was purified and cloned into BglII-KpnI sites of the L4440 double T7 Script II vector (67). dsRNA complementary to *iafgp* sequence was synthesized using the MEGAscript RNAi Kit (Ambion Inc.) following manufacturer's instructions. Mock dsRNA was prepared from empty L4440 vector that contained restriction enzyme site sequences. Microinjections of mock and *iafgp* dsRNAs were performed as described (67). Briefly, 1 μg of L4440 empty vector or *iafgp*-containing vector was used as a template to prepare dsRNA. The prepared dsRNA was eluted in 50 μl of injection buffer (Ambion Inc.). Microinjections were made (4.2 nl/tick, 1×10^{12} molecules/μl) into the bodies of unfed nymphs as mentioned previously (67). Microinjected ticks were incubated either at room temperature for 3 days in a desiccator or fed on *A. phagocytophilum*–infected mice for 48 hours and analyzed for *iafgp* silencing by QRT-PCR. Silencing was confirmed using primers that were used for QRT-PCR and with 5′-CGGCTACGGGCTCGGA-3′ and 5′-GTTATTTATTCCCACGTTTTCATTTG-3′. Silencing of *trospA* gene was performed as described (68).

Tick cell line experiments. *Ixodes ricinus* tick cell line (IRE/CTVM19, maintained at 28°C without CO_2) was used throughout our experiments (69). *A. phagocytophilum* infection was maintained in human promyelocytic cell line (HL-60; ATCC) maintained at 37°C, 5% CO_2 in IMDM (Gibco; Invitrogen) with 20% FCS. For infecting tick cell line with *A. phagocytophilum*, bacteria were collected from *A. phagocytophilum*–infected HL-60 cells by centrifugation for 10 minutes at 3889 *g*. Cell pellets were resuspended in IMDM, lysed by 6 passages through a 25-gauge needle followed by 6 more passages through a 27-gauge needle, and the lysates were centrifuged at 350 *g* for 3 minutes to obtain cell-free bacteria in the supernatants that were used to infect the tick cell line. To quantify *iafgp* expression in tick cells, uninfected or *A. phagocytophilum*–infected tick cells were incubated at 28°C, 4°C, 0°C, and –5°C for 2 hours and immediately processed for RNA extraction, cDNA synthesis, and QRT-PCR. Cold shock experiments are described in the Supplemental Methods.

Yeast survival assays. *S. cerevisiae* strain EBY100 (Invitrogen) was used in all our analyses. The full-length *iafgp* gene was PCR amplified with oligonucleotides 5′-CGGAATTCATGACGACTCTGCTTCGTCTGACT-3′ and 5′-CCGCTCGAGCGCAGCCGCCGTAGCT-3′ containing EcoRI and XhoI sites, respectively, from pGEMT-*iafgp* plasmid DNA as a template. The obtained PCR product was digested with EcoRI-XhoI and cloned into pYD1 vector (Invitrogen) digested with EcoRI-XhoI generating pYD1/FL-*iafgp*. The plasmids pYD1/FL-*iafgp* or pYD1 (without insert) were transformed into EBY100 competent cells, and cold tolerance assays were performed as mentioned in the Supplemental Methods.

GenBank accession numbers. The GenBank accession numbers for *I. scapularis afgp* (*iafgp*) and *I. ricinus afgp* (*iafgp-Iric*) are HM213906 and HM213905, respectively.

Statistics. The statistical significance of differences observed in data sets was analyzed using Microsoft Excel and GraphPad Prism Software. For data with small variations and normal distribution, such as levels of *iafgp*

expression in ticks or *p44* levels in *A. phagocytophilum*–infected nymphs, a 2-tailed Student's *t* test was performed to compare 2 means. For data with large variations and/or a nonparametric distribution, such as survival and mobility measurements, a 2-tailed Mann-Whitney *U* test was performed to compare 2 medians. Supplemental Table 1 shows sum of ranks in all experimental samples compared using Mann-Whitney *U* test. $P < 0.05$ was considered significant in all statistical analyses.

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