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Anaplasma phagocytophilum induces actin phosphorylation to selectively regulate gene transcription in Ixodes scapularis ticks

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Anaplasma phagocytophilum, the agent of human anaplasmosis, persists in ticks and mammals. We show that A. phagocytophilum induces the phosphorylation of actin in an Ixodes ricius tick cell line and Ixodes scapularis ticks, to alter the ratio of monomeric/filamentous (G/F) actin. A. phagocytophilum–induced actin phosphorylation was dependent on Ixodes p21–activated kinase (IPAK1)–mediated signaling. A. phagocytophilum stimulated IPAK1 activity via the G protein–coupled receptor Gβγ subunits, which mediated phosphoinositide 3-kinase (PI3K) activation. Disruption of Ixodes gβγ, pi3k, and pdk1 reduced actin phosphorylation and bacterial acquisition by ticks. A. phagocytophilum–induced actin phosphorylation resulted in increased nuclear G actin and phosphorylated actin. The latter, in association with RNA polymerase II (RNAPII), enhanced binding of TATA box–binding protein to RNAPII and selectively promoted expression of salp16, a gene crucial for A. phagocytophilum survival. These data define a mechanism that A. phagocytophilum uses to selectively alter arthropod gene expression for its benefit and suggest new strategies to interfere with the life cycle of this intracellular pathogen, and perhaps other Rickettsia–related microbes of medical importance.

Human Granulocytic Anaplasmosis is an increasingly common tick-borne illness in the United States, Europe, and Asia (Dumler et al., 2005; Bakken and Dumler, 2008). The agent of this disease, Anaplasma phagocytophilum, survives within human neutrophils using several strategies, including delaying apoptosis, inhibiting NADPH oxidase activity, and subverting phagolysosome biogenesis to reside in an inclusion that does not fuse with lysosomes (Carlyon and Fikrig, 2003). Tyrosine phosphorylation of translocated bacterial effector proteins is another key feature that enables pathogens to thwart host cell signaling (Selbach et al., 2009). Several proteins translocated by bacterial type III and IV secretion systems are involved in pedestal formation (Tir of EPEC and Citrobacter), cell scattering (CagA of Helicobacter), invasion (Tarp of Chlamydia), and cell proliferation (BepD–F of Bartonella; Covacci and Rappuoli, 2000). Bacterial protein tyrosine kinases and phosphatases also play a role in pathogenicity and enable the microbe to short circuit host defense mechanisms and thwart signaling (Covacci and Rappuoli, 2000). A. phagocytophilum AnkA protein is tyrosine phosphorylated by Abl-1 kinase to facilitate infection (Lin et al., 2007; Ijdo et al., 2007). A. phagocytophilum AnkA also binds to granulocyte DNA and nuclear proteins, leading to speculation about the functional nature of AnkA–host cell DNA interactions (Park et al., 2004). The agent of human granulocytic anaplasmosis also induces the tyrosine phosphorylation of ROCK1 in human neutrophils to aid in intracellular survival (Thomas and Fikrig, 2007). Collectively, these studies demonstrate that this unique obligate intracellular pathogen has evolved diverse mechanisms to persist within mammalian cells and that tyrosine phosphorylation of proteins

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plays an important role in the manipulation of host cellular events to promote A. phagocytophilum survival.

A. phagocytophilum is closely related to other arthropod-borne bacteria in the genera Rickettsia and Ehrlichia that infect the mammalian host (Dunler et al., 2001). Intracellular microorganisms, including Rickettsia, Shigella, Listeria, and vaccinia virus, among others, use actin polymerization to move within and spread between cells (Frischknecht et al., 1999a; Goosney et al., 1999; Gouin et al., 2004; Cossart and Toledano-Arana, 2008). These pathogens recruit host actin and cytoskeletal proteins to their surface and activate the assembly of an actin comet tail (Goldberg, 2001; Gouin et al., 2005; Cossart and Toledano-Arana, 2008). In contrast, Salmonella, Neisseria, and Bartonella intercept actin rearrangements during internalization (Dramsi and Cossart, 1998; Patel and Galán, 2005; Patel et al., 2009). Some microbes manipulate the actin cytoskeleton by directly injecting effectors or virulence factors into cells, thereby specifically targeting crucial intracellular signaling pathways (Sansonetti, 2002; Münter et al., 2006; Bhavsar et al., 2007). Yersinia activates the effector protein YpkA to phosphorylate G-actin and cause the disassembly of actin stress fibers (Navarro et al., 2007). Vaccinia virus achieves actin-based motility by mimicking the tyrosine kinase signaling pathways that control actin nucleation dynamics (Frischknecht et al., 1999b). In mammalian cells, the Rickettsia surface protein RicA activates the Arp2/3 complex to induce actin polymerization and filopodia formation (Martinez and Cossart, 2004). The essential role of actin-based motility and actin dynamics has not been examined in the arthropod vector.

Some bacteria use arthropod components and signaling events to survive in the vector or to facilitate transmission to the host. A. phagocytophilum is naturally maintained in a tick-rodent cycle. Humans are merely incidental hosts. Uninfected Ixodes scapularis larvae acquire A. phagocytophilum within 2 d of tick engorgement on A. phagocytophilum–infected mice, and once in the tick, the bacteria migrate through the gut to infect the salivary glands (Hodzic et al., 1998). The larvae molt into nymphs and later into adults, whereas the bacteria persist within the secretory acini of the salivary glands (Hodzic et al., 1998; Katavolos et al., 1998). Upon tick feeding, the bacteria replicate and migrate from the salivary glands to the mammalian host (to invade granulocytes), and the transmission of A. phagocytophilum occurs between 24 and 48 h after tick engorgement (Hodzic et al., 1998; Katavolos et al., 1998). I. scapularis, the black-legged tick, is a vector for viral and bacterial pathogens including A. phagocytophilum and Borrelia burgdorferi, the agent of Lyme disease (Schwan, 1996; Dunler et al., 2005). The extended period of association of these microbes with the vector has resulted in the development of intimate relationships between pathogen and arthropod. For example, B. burgdorferi uses Salp15, a tick salivary gland protein, to facilitate infection of the mammalian host (Ramamoorthi et al., 2005). Salp15 is selectively increased in B. burgdorferi–infected tick salivary glands during engorgement, and silencing of the salp15 gene in I. scapularis reduced the capacity of tick-borne spirochaetes to infect mice (Ramamoorthi et al., 2005). In addition, Salp15 binds B. burgdorferi, thereby protecting the spirochete from antibody-mediated killing (Ramamoorthi et al., 2005). A. phagocytophilum up-regulates Salp16, a tick salivary gland protein, to survive in its arthropod vector (Sukumaran et al., 2006). Acquisition of A. phagocytophilum from the infected mammalian host was severely inhibited and the bacterial loads were substantially lower in the salivary glands of salp16-silenced ticks, thereby suggesting that A. phagocytophilum specifically requires salp16 to infect salivary glands (Sukumaran et al., 2006). When Salp16 is not present in I. scapularis, as demonstrated in RNAi studies, A. phagocytophilum can no longer effectively persist within tick salivary glands (Sukumaran et al., 2006). The mechanisms used by A. phagocytophilum to influence its arthropod vector, including the expression of I. scapularis genes, are not known. We now explore whether A. phagocytophilum selectively modulates arthropod signaling by altering protein phosphorylation and whether these processes influence I. scapularis gene expression and survival of A. phagocytophilum within ticks.

RESULTS

A. phagocytophilum induces phosphorylation of tick actin

The tyrosine phosphorylation of Ixodes proteins upon A. phagocytophilum infection was first examined using an Ixodes ricinus tick cell line (Bell-Sakty et al., 2007). Immunofluorescence showed increased phosphorylation of proteins in A. phagocytophilum–infected cells in comparison with the uninfected controls (Fig. 1 A). Although the phosphotyrosine signal was more localized to the periphery and filamentous filopodial structures in uninfected cells, A. phagocytophilum–infected cells showed an irregular distribution of the phosphorylated proteins as large clusters (Fig. 1 A). Immunoblotting demonstrated that A. phagocytophilum markedly induced phosphorylation of a major Ixodes protein (Fig. 1 B and Table S1) that was identified as actin by mass spectrometry analysis. Actin was shown to be heavily phosphorylated at residue Y53 (Jungbluth et al., 1995). The tyrosine residue (Y53) is also conserved in I. scapularis actin (National Center for Biotechnology Information [NCBI] protein accession no. XP_002408110). However, the liquid chromatography–tandem mass spectrometry (LC–MS/MS) phosphopeptide identification results revealed that the peptide LCYVALIDFQEMATAASSSSLEK contained the phosphosite and that tyrosine residue corresponds to Y178 of I. scapularis actin (NCBI protein accession no. XP_002408110). Immunoprecipitation of a tick cell extract with phosphotyrosine antibody, followed by immunoblotting with actin antibody, confirmed that A. phagocytophilum induces phosphorylation of actin (Fig. 1 C and Table S1). We also found that A. phagocytophilum induces the threonine but not serine phosphorylation of actin (Fig. S1, A and B; and Table S1).

To determine the temporal development of A. phagocytophilum–induced phosphorylation of actin, tick cells were analyzed over 10 d. Phosphorylation was evident at 24 h and persisted through day 10 (Fig. 1 D and Table S1). A. phagocytophilum infection was apparent at all time points during the course of infection (Fig. 1 E). The infection rate of A. phagocytophilum in
A. phagocytophilum induces phosphorylation of actin in unfed ticks and during acquisition by ticks.

To assess whether actin phosphorylation occurs in vivo, the phosphorylation pattern in A. phagocytophilum–infected ticks was examined. Actin phosphorylation was induced in A. phagocytophilum–infected unfed nymphs in comparison with the uninfected controls, suggesting an extended and stable modification of actin (Fig. 1 F and Table S1). Immunofluorescence of unfed tick salivary glands also demonstrated elevated tick cells was found to be 70 ± 9% (Fig. S1, C and D). To determine whether inhibition of protein tyrosine phosphatases altered A. phagocytophilum–induced extended actin phosphorylation, tick cells were treated with pervanadate, a protein tyrosine phosphatase inhibitor. Actin phosphorylation was comparable in pervanadate–treated and untreated A. phagocytophilum–infected cells (Fig. 1 D), suggesting that inhibition of tick tyrosine phosphatase activity did not influence A. phagocytophilum–induced actin phosphorylation.

Figure 1. A. phagocytophilum induces actin phosphorylation in tick cells and ticks. (A) Immunofluorescence images of uninfected and A. phagocytophilum–infected tick cells at 48 h after infection, stained for phosphorylated tyrosine (pTyr). Bar, 10 µm. Representative images are shown from three independent experiments. (B) Coomassie-stained SDS-PAGE image of anti-pTyr immunoprecipitated proteins from uninfected (UI) and A. phagocytophilum–infected (I) lysates at 48 h after infection. The arrow denotes the dominant phosphorylated band identified as actin in A. phagocytophilum–infected cells. Phosphorylation of a protein with a higher molecular mass was also noted. (C) Lysates with (I) or without (UI) A. phagocytophilum were immunoprecipitated with antibodies against pTyr and probed with antibodies against actin. The level of actin before immunoprecipitation (total actin) served as the loading control. (D) Lysates from cells infected (I) or not (UI) with A. phagocytophilum were isolated at different time points (8, 24, and 48 h and 7 and 10 d) and assessed for actin phosphorylation in the presence (+) or absence (–) of pervanadate, a protein tyrosine phosphatase inhibitor. (E) The presence of A. phagocytophilum in infected tick cells was assessed by immunoblotting with antisera specific for the A. phagocytophilum P44 antigen (P44). Actin served as loading control. (F) Lysates were prepared from unfed ticks infected (I) or not (UI) with A. phagocytophilum. 20 µg of total lysates were probed with pTyr–specific antibody. pY-actin is denoted by an arrow. Total actin served as loading control. Whole tick (G), salivary gland (H), and gut tissue (I) lysates were prepared from I. scapularis fed for 48 h on uninfected or A. phagocytophilum–infected mice and were analyzed as described in F. Representative data are shown from three independent experiments in all panels.

A. phagocytophilum induces phosphorylation of actin in unfed ticks and during acquisition by ticks.

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levels of phosphorylated actin upon *A. phagocytophilum* infection (Fig. S1 E). To examine whether acquisition of *A. phagocytophilum* by ticks induces actin phosphorylation, uninfected nymphs were fed on either *A. phagocytophilum*–infected or uninfected mice. Ticks were collected at 24, 48, and 72 h during engorgement and at 48 and 72 h after feeding. Actin phosphorylation was evident at all time points examined (data at 48 h during feeding are shown; Fig. 1 G and Table S1). An elevated level of a phosphorylated protein with a lower molecular mass was also seen in *A. phagocytophilum*–infected ticks compared with the uninfected controls. *A. phagocytophilum*–induced actin phosphorylation was then specifically assessed in the tick gut, the site of microbial entry, and the salivary glands, the site of pathogen persistence. After 48 h of engorgement, *A. phagocytophilum*–induced actin phosphorylation was significantly increased (2.6–4.0 fold) in the tick salivary glands and gut (Fig. 1, H and I; and Table S1), suggesting that *A. phagocytophilum*–induced actin phosphorylation occurs in both these tissues.

*A. phagocytophilum*–induced actin phosphorylation is mediated by phosphoinositide 3-kinase (PI3K)–p21–activated kinase (PAK1) signaling

Studies in *Drosophila melanogaster* have shown that PAK1 plays an important role in linking to tyrosine kinase signaling pathways through Dock (Nck homologue), thereby leading to changes in the reorganization of the actin cytoskeleton (Galisteo et al., 1996). The adaptor protein Nck has also been shown to directly couple PAK1 signaling to receptor tyrosine kinases in several mammalian tissue culture systems (McCarty, 1998). Furthermore, the association of PI3K and PAK1 phosphorylates actin and reorganizes the actin cytoskeleton in an opossum kidney epithelial cell line (Papakonstanti and Stournaras, 2002). These studies suggest PI3K–PAK1 signaling to be a central component in actin phosphorylation. To characterize whether these kinases are regulating *A. phagocytophilum*–induced phosphorylation of actin, we searched the *I. scapularis* genome database and identified partial coding sequences for both the Gβ subunits (accession no. iPip3k and iPak1) as *Ixodes pacificus* (iip3k) and PK1 as *Ixodes pacificus* (ipak1). The GenBank accession nos. for *i3k* and *ipak1* are HM165193 and HM165194, respectively. The *I. scapularis* PI3K and PAK1 homologues (Fig. S2, A and B) showed high similarity (80–85%) and identity (65–70%) to the PI3K and PAK1 proteins from *Drosophila melanogaster*, *Aedes aegypti*, mice, and man (Fig. S3, A and B). G protein–coupled receptor Gβγ subunits stimulate PAK1 through activation of PI3K but act independently of Rac1/Cdc42 GTPases (Menard and Mattingly, 2004). We found the annotated coding sequences for both the Gβ (accession no. XP_002401352) and Gγ subunits (accession no. EEC12500) from the NCBI Protein database. In the current study, we designated the tick G protein–coupled receptor Gβ subunit as *igβ* and the γ subunit as *igy*. The *I. scapularis* Gβ and Gγ homologues showed 55 and 44% amino acid identity, respectively, to Gβ1 and Gγ2 in humans (Fig. S4, A and B).

We found that the expression of *ipak1*, *i3k*, and both *igβ* and *igy* were significantly elevated in *A. phagocytophilum*–infected unfed ticks and in uninfected ticks that acquired *A. phagocytophilum* from infected mice (Fig. S5, A–H). These data suggest that *A. phagocytophilum* elevates the expression of the G protein–coupled receptor Gβγ subunits, which leads to activation of the PI3K–PAK1 signaling pathways.

To study whether inhibition of *i3k*, *ipak1*, or tyrosine kinases affects *A. phagocytophilum*–induced actin phosphorylation, we infected tick cells with *A. phagocytophilum* and simultaneously treated them with LY294002 (an inhibitor of PI3K), PK–18 (a potent PAK1 inhibitor), or Genistein (a protein tyrosine kinase inhibitor). At 48 and 72 h, cells treated with each of the three inhibitors showed a considerable reduction in actin phosphorylation (Fig. 2 A and Table S1) and a significant decrease in the *A. phagocytophilum* burden (Fig. 2 B) in comparison with the controls. To study whether these inhibitors reduced actin phosphorylation and the bacterial load in vivo, we performed microinjections of these inhibitors into *A. phagocytophilum*–infected nymphs. At 24 h, we found a considerable reduction in actin phosphorylation in *A. phagocytophilum*–infected unfed ticks when compared with the mock controls (Fig. S3 I and Table S1). During acquisition of *A. phagocytophilum* by ticks, actin phosphorylation and bacterial loads were also significantly reduced in PK–18 or Genistein inhibitor–treated ticks at 48 h after engorgement (Fig. S5, J and K; and Table S1).

**Silencing of ipak1 reduces actin phosphorylation and *A. phagocytophilum* acquisition by ticks**

To establish the interaction of tick IPAK1 with actin, we performed immunoprecipitation studies of control and *A. phagocytophilum*–infected tick cells. Immunoblots performed with mammalian PAK1 antibody demonstrated specific cross-reactivity with IPAK1 (Fig. S5L, Table S1). When performed immunoprecipitation studies of control and *A. phagocytophilum*–infected ticks (Fig. 2, D and E; and Table S1). The association of *A. phagocytophilum* with actin phosphorylation and bacterial loads was also significantly reduced in PK–18 or Genistein inhibitor–treated ticks at 48 h after engorgement (Fig. S5, J and K; and Table S1). During acquisition of *A. phagocytophilum* by ticks, actin phosphorylation and bacterial loads were also significantly reduced in PK–18 or Genistein inhibitor–treated ticks at 48 h after engorgement (Fig. S5, J and K; and Table S1). The *A. phagocytophilum* burden in the *ipak1*–dsRNA–injected ticks was also markedly diminished when compared with the controls (Fig. 2 F). These data further suggest that IPAK1 signaling and actin phosphorylation are required for *A. phagocytophilum* survival in ticks.
**A. phagocytophilum** induces IPAK1 activity through IPI3K activation

To determine whether *A. phagocytophilum*-induced actin phosphorylation was dependent on IPAK1 activation, total lysates from the 48 h during tick feeding were immunoprecipitated with PAK1 antibody. IPAK1 activation was examined by determining the phosphorylation of myelin basic protein (MBP), a known exogenous substrate for PAK1 activity. We found that *A. phagocytophilum* induces strong activation of IPAK1 (Fig. 3 A and Table S1). Total lysates used for immunoprecipitation of IPAK1 (input) were probed with actin antibody as the loading control (Fig. 3 A). The association of PI3K with PAK1 regulates PAK1 activity (Papakonstanti and Stournaras, 2002). We found that *A. phagocytophilum* also induces IPI3K activity, as determined by the generation of phosphatidylinositol-3,4,5-triphosphate PI(3)P from phosphatidylinositol (Fig. 3 B). We therefore generated *ipi3k*-deficient *A. phagocytophilum*–infected ticks. The level of *ipi3k* mRNA and the *A. phagocytophilum* burden were significantly reduced in the *ipi3k*-dsRNA–injected groups compared with the mock controls (Fig. S6, B and C). Furthermore, *A. phagocytophilum*–induced tyrosine phosphorylation of actin was markedly diminished in *ipi3k*-dsRNA–injected ticks (Fig. S6 D and Table S1), suggesting an important role for the association of PI3K with PAK1 in actin phosphorylation. We then examined the IPAK1 activity in mock or *ipi3k*-dsRNA–treated ticks by immunoprecipitation of IPAK1. IPAK1 kinase activity was dramatically reduced in *ipi3k*-deficient ticks when compared with the mock controls (Fig. 3 C and Table S1). *A. phagocytophilum*–induced IPAK1 activity is dependent on Gβγ stimulation but independent of Rac1/Cdc42 activation

Heterotrimeric G proteins have been implicated in PAK1 and PI3K activation (Menard and Mattingly, 2004). The *A. phagocytophilum*–induced expression of *igβ* and *igγ* (Fig. S5, E–H) prompted us to investigate the upstream mechanisms. Silencing of *igβ* and *igγ* (Fig. S6, E and F) decreased the *A. phagocytophilum* burden (Fig. S6, G and H) and reduced the tyrosine phosphorylation of actin (Fig. S6 I and Table S1). IPAK1 and IPI3K activities were dramatically reduced in both
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Figure 3. *A. phagocytophilum* infection induces IPAK1 and IPI3K activity through Gβγ stimulation but independent of Rac1/Cdc42 activation. (A) Lysates from ticks infected (I) or not (UI) with *A. phagocytophilum* were immunoprecipitated (IP) with anti-PAK1, and PAK1-mediated phosphorylation of the substrate MBP was analyzed by in vitro kinase assay. Total lysates used for the kinase assay were probed with actin antibody as the loading control (input). (B) IP13K activity read out by ELISA detecting PI conversion to PI(3)P, IP-3 immunoprecipitates from lysates of *A. phagocytophilum*-infected or uninfected ticks. (C and D) IPAK1 activity in *ipi3k*-silenced ticks (C) or *igβ* or *igy*-silenced ticks (D) in comparison with their respective mock controls was measured as in A. Total lysates used for the kinase assays were probed with actin antibody as the loading control. In A, C, and D, IPAK immunoprecipitates were used at three different dilutions indicated by wedges (10, 15, and 25 µl IP beads). (E) IP3K activity in *igβ*- or *igy*-silenced ticks in comparison with the mock control was determined as in B. (F) Rac1/Cdc42 activation upon binding to PAK-PBD (Rac1/CDC42 binding domain of PAK1) upon *A. phagocytophilum* infection. Total tick lysates were used for affinity precipitation of Rac1/Cdc42 GTPases with PAK-PBD beads. Total lysates before precipitation were probed with actin as the loading control. Statistics were performed using the Student’s *t* test, and the *p*-value is shown in B and E. Error bars show mean ± SD. Representative data from two independent experiments is shown in all panels.

*iigβ*- and *igy*-deficient ticks when compared with the controls (Fig. 3, D and E; and Table S1). PAK1 has been implicated in the rearrangement of the actin cytoskeleton by acting downstream of the small GTPases Rac1 and Cdc42. Upon binding to Rac1/Cdc42, PAK1 autophosphorylates, thereby increasing its kinase activity toward exogenous substrates (Papakonstanti and Stournaras, 2002). We assessed whether *A. phagocytophilum* activates these small GTPases by performing a Rac1/Cdc42 activation assay using the p21-binding domain of PAK1 (PBD) that specifically binds to and precipitates the active (GTP) form of Rac1 and Cdc42. No differences were observed between GTP-Rac1 and GTP-Cdc42 precipitates obtained from *A. phagocytophilum*-infected or uninfected lysates bound to PAK1-PBD agarose beads (Fig. 3 F and Table S1). Each of the GTPases was assessed with specific antibodies, and an input loading control was provided by tick lysates (before precipitation) probed with actin antibody (Fig. 3 F). Our results suggest that *A. phagocytophilum* induces the Ixodes G protein–coupled receptor Gβγ subunits to stimulate IPAK1 and IPI3K activation and that the Rac1/Cdc42 GTPases do not induce IPAK1 activity.

*A. phagocytophilum* infection alters the ratio of G/F-actin in ticks

PAK1 directly phosphorylates actin, resulting in the disassembly of stress fibers, cortical actin organization, and formation...
of filopodia (Papakonstanti and Stournaras, 2002). Moreover, phosphorylation of actin inhibits actin filament nucleation and elongation, leading to a reduction in actin polymerization (Liu et al., 2006). We determined the ratio of globular G (monomeric) to filamentous F (G/F) actin in A. phagocytophilum–infected tick cells to examine whether A. phagocytophilum–induced actin phosphorylation inhibits actin nucleation and thereby causes alterations in the G/F-actin ratio. A. phagocytophilum infection in tick cells increased G-actin and markedly reduced F-actin (threefold; Table S1) in comparison with the uninfected controls (Fig. 4A and Table S1). In addition, infection with A. phagocytophilum also altered the ratio of G/F-actin in unfed nymphal ticks in vivo (unpublished data). Detailed imaging of tick cells using confocal microscopy revealed that filamentous actin-enriched stress fibers/actin bundles were dramatically reduced in the cytosol of A. phagocytophilum–infected cells compared with the uninfected controls (Fig. 4B). In addition, filopodial structures that protrude outside the cell periphery were diminished, with a concomitant increase in G actin staining in cell nuclei of A. phagocytophilum–infected cells (Fig. 4B). When these changes in morphology were quantified, the number of filamentous cells per field, the number of filaments per cell, and the percentage of cells positive for filaments per field were significantly decreased in A. phagocytophilum–infected cells in comparison with the uninfected controls (Fig. 4C–E). Decreased F-actin suggested inhibition of actin polymerization, and increased G-actin in infected cells correlated with the induction of actin phosphorylation by A. phagocytophilum.

**A. phagocytophilum**–induced phosphorylated actin accumulates in cell nuclei and selectively alters *I. scapularis* gene transcription

We assessed whether G-actin was increased in nuclear extracts of A. phagocytophilum–infected cells and found increased levels of nuclear actin in comparison with the uninfected controls (Fig. 5A and Table S1). Confocal microscopy also confirmed higher levels of G-actin in the nuclei of A. phagocytophilum–infected cells (Fig. 5B). Immunoprecipitation with phosphotyrosine antibody showed increased levels of phosphorylated actin in A. phagocytophilum–infected nuclear extracts when compared with the uninfected controls (Fig. 5A and Table S1). To identify a mechanism for A. phagocytophilum infection–mediated transcriptional effects, we quantified levels of RNA polymerase II (RNAPII) and the transcription factor TATA box–binding protein (TBP) in uninfected and A. phagocytophilum–infected tick cells. Although the total levels of the proteins were not significantly different as a result of infection, immunoprecipitation with RNAPII antibody showed enhanced RNAPII association with TBP, suggesting that a stable interaction of TBP and RNAPII is induced upon infection with A. phagocytophilum (Fig. 5A and Table S1).

Actin has been shown to associate with eukaryotic RNA polymerases and is directly involved in gene transcription (Bettinger, et al., 2004; Hofmann, 2009). We noted an increased association of G-actin or phosphorylated actin with RNAPII in A. phagocytophilum–infected tick cell nuclei in comparison with the uninfected controls (Fig. 5C and D). These data suggest a role for A. phagocytophilum–induced actin phosphorylation in transcriptional effects.
Figure 5. *A. phagocytophilum*-induced phosphorylated/G–actin accumulates in tick cell nuclei. (A) Nuclear extracts were prepared from uninfected (UI) and *A. phagocytophilum*-infected (I) cells and subjected to immunoprecipitation (IP) and immunoblot (IB) with the indicated antibodies. Immunoblots for RNAPII and TBP served as loading control. Three independent experiments yielded similar results. (B) Confocal microscopy showing G–actin (red) and F–actin (green) in *A. phagocytophilum*-infected (I) or uninfected (UI) tick cells. Bar, 10 µM. (C and D) Uninfected and infected tick cells were stained with DNase I for G–actin (red; C) or anti-phosphotyrosine (red; D) and anti RNAPII (green) and TOPRO-3 (blue). Representative images from three independent experiments are shown. Bars, 20 µm.
phosphorylated actin in altering gene transcription. To address the role of phosphorylated actin in modulating *Exodes* gene transcription, we examined an *A. phagocytophilum*–modulated tick gene, *salp16*, which we have shown to be essential for *A. phagocytophilum* survival in ticks (Sukumaran et al., 2006). During *A. phagocytophilum* acquisition by the tick, *salp16* expression levels were elevated in mock-injected ticks, as expected, but were significantly reduced in both *ipak1*-silenced and PAK1 inhibitor–injected ticks (Fig. 6, A and B). Silenced *ipak1* levels and reduced actin phosphorylation correlated with significantly decreased *salp16* levels in *A. phagocytophilum*-infected ticks. To assess whether the reduction in *salp16* gene expression was specific in *ipak1*-silenced or PAK1 inhibitor–treated *A. phagocytophilum*-infected ticks, we analyzed the expression levels of *salp20* gene from *I. scapularis*. We found no significant alterations in the expression levels of *salp20* (Fig. 6, C and D).

To establish a possible mechanism for the specific enhancement of expression of *salp16* in *A. phagocytophilum*-infected ticks, we identified a putative RNAPII–dependent promoter TATA motif in the genomic locus of *salp16* and *salp20* (as control), as indicated in the experimental procedures. Electrophoretic mobility shift assays (EMSAs) showed enhanced binding of crude nuclear extracts from *A. phagocytophilum*–infected ticks to the putative *salp16* promoter TATA binding motif in comparison with the nuclear extract proteins from uninfected controls (Fig. 6 E). EMSAs with the *salp20* probe showed no differences in binding for nuclear extract proteins from *A. phagocytophilum*-infected or uninfected nuclear extract proteins (Fig. 6 F). These data were consistent with the quantitative (Q) RT-PCR showing no alterations in *salp20* gene expression in *ipak1*-silenced or inhibitor-treated *A. phagocytophilum*–infected ticks (Fig. 6, C and D). EMSA performed with tick nuclear extract proteins from *A. phagocytophilum*–infected ticks and antibodies against RNAPII, TBP, phosphotyrosine, or actin blocked the band shift with *salp16* probe (Fig. 7 A), suggesting an important role for these nuclear proteins in regulating *salp16* gene transcription. EMSAs with actin antibody (increasing concentrations) and nuclear extracts from *A. phagocytophilum*-infected ticks showed a band shift with the *salp20* probe. However, the band shift was reduced or blocked with the *salp16* probe, suggesting a specific role for phosphorylated nuclear actin in selective regulation of *salp16* gene transcription (Fig. 7 B).

In addition to *salp16* and *salp20* genes, we analyzed several other *salp* genes such as *salp15* (Fig. 7, A and B), *salp17* (Fig. 7, C and D), and *salp25D* (Fig. 7, E and F). Upon *ipak1*-silencing or treatment with inhibitors (PK-18 or Genistein), we found no significant difference in the expression levels of these genes. Furthermore, no alterations were seen in β-tubulin or *gapdh* expression levels upon either *ipak1*-silencing or treatment with inhibitors (PK-18 or Genistein; Fig. 7, G–J). Although not exhaustive, the unchanged transcription levels of this panel of genes suggest that IPAK1–mediated actin phosphorylation is not a global regulator but rather has a selective effect on *salp16* after *A. phagocytophilum* infection. Collectively, our data suggest a novel role for *A. phagocytophilum*–induced phosphorylated actin
in association with RNAPII and TBP as important components of the transcriptional activation required to selectively regulate *I. scapularis* *salp16* gene transcription.

*A. phagocytophilum*–induced phosphorylated actin mediates promoter specificity for selective regulation of *salp16* gene transcription

To confirm that IPAK1–mediated phosphorylated actin is responsible for the selective regulation of *salp16* transcription upon *A. phagocytophilum* infection, we analyzed the levels of phosphorylated actin, TBP, and RNAPII in mock and *ipak1*-silenced nuclear extracts from ticks. The phosphorylated actin levels were dramatically reduced in nuclear extracts from *ipak1*-silenced ticks as compared with the mock (buffer alone) control (Fig. 8 A and Table S1). The levels of TBP and RNAPII were unaffected in nuclear extracts from both mock and *ipak1*-silenced samples (Fig. 8 A and Table S1). Total actin levels from the same lysates served as loading control (Fig. 8 A). EMSAs with a *salp16* probe showed reduced binding with nuclear extract proteins from *ipak1*-silenced ticks, further demonstrating the importance of IPAK1–mediated actin phosphorylation in selective regulation of *salp16* gene transcription (Fig. 8 B). No differences were observed in the band shifts with the *salp20* probe (Fig. 8 B). The combined data from Fig. 8 (A and B) suggest that lower levels of phosphorylated actin in the nuclear extracts from *ipak1*-silenced ticks specifically affects *salp16* but not *salp20* gene transcription. To directly show that IPAK1–mediated actin phosphorylation plays a novel role in selective regulation of *salp16* gene transcription, we performed a DNA affinity precipitation (DNAP) assay using nuclear extracts from *A. phagocytophilum*–infected ticks and *salp16* or *salp20* promoter regions. The DNAP assay showed that phosphorylated actin binding was enhanced and specific to the *salp16* probe (Fig. 8 C and Table S1). No differences were observed for either TBP or RNAPII binding to the *salp16* or *salp20* probes (Fig. 8 C and Table S1), further strengthening the conclusion that a potential novel role for *A. phagocytophilum*–induced phosphorylated actin in the nucleus is selective regulation of *salp16* promoter. The nuclear lysates from *A. phagocytophilum*–infected ticks used
for the DNAP assay were probed with actin antibody as the loading control (Fig. 8 C). Collectively, the results from mock/ipak1-silenced tick nuclear extracts (Fig. 8, A and B), EMSAs performed with actin antibody blocking (Fig. S7 B), and the DNAP assay (Fig. 8 C) show that salp16 promoter specificity is mediated by phosphorylated actin to selectively regulate Ixodes gene transcription.

**DISCUSSION**

Obligate intracellular bacteria have evolved a variety of mechanisms to persist in their hosts, including modulating host signaling and the actin cytoskeleton (Bhavsar et al., 2007). We addressed the survival strategies that *A. phagocytophilum* uses to persist in its arthropod vector *I. scapularis*. We provide in vitro and in vivo evidence that *A. phagocytophilum* induces the phosphorylation of actin and alters the ratio of monomeric/filamentous (G/F) actin leading to translocation of phosphorylated/G-actin into the cell nucleus. *A. phagocytophilum*-induced actin phosphorylation was dependent on Gβγ stimulation involving the activation of *Ixodes* PI3K and PAK1 but was independent of Rac1/Cdc42 small GTPases. The ability of the bacteria to enhance levels of phosphorylated actin in the nucleus selectively regulates *I. scapularis* salp16 gene transcription in association with RNAPII and TBP. This is the first study demonstrating that an intracellular bacterium can exploit phosphorylated actin to specifically control gene transcription in its arthropod host.

Generally, a small percentage of the total pool of a certain protein is phosphorylated in cells and this transient change is sufficient to activate signaling (Pawson and Scott, 2005). *A. phagocytophilum*-induced actin phosphorylation was maintained stably for several days (in vitro) to months (in vivo) suggesting that it is an extended modification. Phosphorylation and dephosphorylation of proteins is executed by protein kinases and phosphatases (Pawson and Scott, 2005). Studies with pervanadate indicated that *A. phagocytophilum* does not inhibit the host phosphatase activity to induce actin phosphorylation. The stable modification of actin also occurred in uninfected *A. phagocytophilum*-infected ticks and when clean ticks were fed on *A. phagocytophilum*-infected mice. Recently, our group has shown that *A. phagocytophilum* influences cell signaling, specifically the tyrosine phosphorylation of ROCK1, to facilitate infection in human neutrophils (Thomas and Fikrig, 2007). Actin phosphorylation was not induced in *A. phagocytophilum*-infected primary cultures of human neutrophils (Fig. S7 C), suggesting that this stable modification occurs specifically in the arthropod vector.

Heterotrimeric G proteins have been implicated in PAK1 and PI3K activation (Menard and Mattingly, 2004). Previous studies have also shown that the association of PI3K with PAK1 phosphorylates actin and reorganizes the actin cytoskeleton (Papakonstanti and Stournaras, 2002). PAK1 also directly phosphorylates actin, resulting in the disassembly of stress fibers, cortical actin organization, and cytoskeletal remodeling (Sells et al., 1997, 1999; Papakonstanti and Stournaras, 2002). It was noteworthy that *A. phagocytophilum*-induced actin phosphorylation was associated with the increase in the amount of ROCK1, to facilitate infection in human neutrophils (Papakonstanti and Stournaras, 2002). PAK1 also directly phosphorylates actin, resulting in the disassembly of stress fibers, cortical actin organization, and cytoskeletal remodeling (Sells et al., 1997, 1999; Papakonstanti and Stournaras, 2002). It was noteworthy that *A. phagocytophilum*-induced actin phosphorylation was associated with the increase in the amount of actin that interacts with IPIAK1. Furthermore, the increased levels of *Ixodes* Gβγ, gγ, ipi3k, and ipak1 in *A. phagocytophilum*-infected ticks and clean ticks acquiring *A. phagocytophilum* from mice, as well as the enhanced association of IPIAK1 and actin in tick cells, suggest that actin phosphorylation in *I. scapularis* results from PI3K- and PAK1-mediated signaling. *A. phagocytophilum*-induced IPIAK1 activation and increased actin phosphorylation was dependent on Gβγ stimulation involving PI3K activation but was independent of Rac1/Cdc42 small GTPases. The ability of PI3K and IPIAK1 to markedly inhibit both *A. phagocytophilum*-induced actin phosphorylation...
and bacterial survival in tick cells, infected ticks, and ticks that acquire bacteria from the mouse host demonstrates that PI3K-PAK1 signaling plays an important role in these events. The *Ixodes g*β*- , γ*-, *pi3k*- , and *pak1*-deficient ticks also showed a marked reduction in the levels of phosphorylated actin and the *A. phagocytophilum* burden. Collectively, these data show that *Ixodes PI3K-PAK1*-mediated signaling and IPAK1-actin association facilitates *A. phagocytophilum* survival in the vector.

In eukaryotic cells, actin exists in two forms: globular (G) monomeric actin and filamentous (F) polymerized actin. G-actin with bound ATP can polymerize to form F-actin, which hydrolyzes bound ATP releasing ADP and P, to form G-actin–ADP monomers (Gerisch et al., 1991; Pollard and Borisy, 2003). Actin is a common target of many bacterial proteins, and the cellular responses induced by a variety of stimuli and pathogens involve changes in cell morphology and the polymerization state of actin (Cameron et al., 2000; Gouin et al., 2005; Stevens et al., 2006). Our study demonstrated that *A. phagocytophilum* is unique among intracellular bacterial pathogens in altering G/F-actin levels to inhibit the actin polymerization machinery in the arthropod vector. The increased G-actin and reduced F-actin levels in *A. phagocytophilum*–infected tick cells suggest that *A. phagocytophilum* may inhibit actin filament nucleation and elongation by inducing actin phosphorylation. Our data complement the previous finding that phosphorylation of actin substantially inhibits nucleation and rate of polymerization of actin filaments in *Dictyostelium* (Jungbluth et al., 1994; Liu et al., 2006). Phosphorylation of actin was also associated with rearrangements of the actin cytoskeleton where filamentous actin–enriched stress fibers/actin bundles in the cytosol and filopodial structures protruding outside the cell periphery were dramatically reduced in *A. phagocytophilum*–infected cells. PAK1-mediated phosphorylation of actin and their direct association also results in the disassembly of actin stress fibers and cortical actin organization (Papakonstanti and Stournaras, 2002), further supporting the correlation between *A. phagocytophilum*–induced PAK1-mediated actin phosphorylation and the remodeling of the actin cytoskeleton in the arthropod vector. Overall, these findings detail a mechanism of host cytoskeletal subversion by *A. phagocytophilum* to alter actin nucleation in its arthropod vector.

Several studies have demonstrated the presence of actin in the nucleus and that nuclear actin is essential for transcription by RNA polymerases, transcription regulation, RNA processing, and export, chromatin remodeling, intranuclear movement, and structure maintenance (Pederson and Aebl, 2002; Bettinger et al., 2004; Hofmann et al., 2004; Percipalle and Visa, 2006; Hofmann, 2009). Actin may exist in monomeric form (Hofmann and de Lanerolle, 2006; Hofmann, 2009) or as short polymers of less than seven monomers in the nucleus that differ from cytosolic filamentous actin (Hofmann and de Lanerolle, 2006; Hofmann, 2009). Our results show that *A. phagocytophilum*–induced phosphorylation of actin leads to increased monomeric (G) actin levels that predominantly localize to the cell nucleus (Figs. 4 and 5). G-actin staining and phosphorylation signals were also dramatically enhanced in the nucleus of *A. phagocytophilum*–infected cells and showed a strong association with RNA PolII. Actin binds RNA polymerase I, II, and III and is required for the formation of preinitiation complexes (Pederson and Aebl, 2002; Bettinger et al., 2004; Hofmann et al., 2004; Percipalle and Visa, 2006; Hofmann, 2009). The enhanced G-actin levels in *A. phagocytophilum*–infected cell nuclei and its association with RNA PolII suggested a role for actin phosphorylation in mediating host gene transcription. It has previously been shown that *A. phagocytophilum* induces tick *salp16* gene expression and that Salp16 is essential for the survival of this microbe in ticks (Sukumaran et al., 2006). The reduction in actin phosphorylation in ipak1-deficient ticks correlated with a decrease in *salp16* gene expression. Using the *salp20* gene (which is not differentially expressed upon *A. phagocytophilum* infection) promoter fragment as control, we have analyzed the reason for the selective regulation of *salp16* gene. Our results with ipak1-silenced tick nuclear extracts (which contain less phosphorylated actin), EMSAs with actin antibody, and DNAP showed that specificity for the *salp16* promoter is mediated by the enhanced accumulation of phosphorylated actin that leads to the greater stability of RNA PolII and TBP complex formation. These results lead us to propose a model wherein *A. phagocytophilum*–induced actin phosphorylation is followed by the inhibition of actin nucleation and rate of actin polymerization, therefore resulting in decreased F-actin and increased G-actin levels in the cell (Fig. S8). The increased G-actin (phosphorylated actin) levels are recruited to the nucleus to enable the formation of stable preinitiation complexes and selective regulation of *Ixodes* gene transcription. The stable preinitiation complexes formed with phosphorylated actin may enable tight binding and the enhanced interaction of RNA PolII to TBP, which may eventually lead to selectively increased RNA PolII dependent *salp16* gene expression during *A. phagocytophilum* infection.

The fundamental mechanism by which arthropod gene regulation is manipulated by pathogens is not understood. Our results provide valuable insight into understanding how a microbe can exploit actin to selectively control arthropod gene expression to beneficially survive in the vector. This knowledge will be useful in the development of methods to interrupt the *A. phagocytophilum*–tick cycle, which may ultimately lead to the prevention of this *I. scapularis*–borne illness. We are also hopeful that these findings will be useful in developing new strategies, based on targeting pathogen–vector interactions, to combat many other arthropod–borne diseases of medical importance.

**MATERIALS AND METHODS**

*A. phagocytophilum* infection, cell lines, and culture conditions. Human promyelocytic cell line (HL-60) was acquired from American Type Culture Collection and maintained at 37°C in 5% CO2 in IMDM (Invitrogen) with 20% FCS. *A. phagocytophilum* infection was analyzed by immunofluorescence, and cell-free bacteria were collected from ~95% *A. phagocytophilum*–infected HL-60 cells by centrifugation for 10 min at 4,000 rpm. Cell pellets were resuspended in IMDM, lysed by six passages through a 25-gauge, followed by six more passages through a 27-gauge needles, and the lysates were centrifuged at 1,200 rpm for 3 min to obtain cell-free bacteria in the supernatants. Bacteria were used to infect *I. ricinus* tick cell line IRE/CTVM19 (Bell-Sakyo et al., 2007), maintained at 28°C without CO2 as previously described (Pedra et al., 2010).
A. *phagocytophilum* acquisition studies. The *A. phagocytophilum* isolate NCH-1 (which also infects humans) was maintained through serial passages of infected blood in C3H/SCID mice (The Jackson Laboratory). For the acquisition experiments, C3H/HeN immunocompetent mice (Charles River Laboratories) were injected intraperitoneally with 100 µl of *A. phagocytophilum*-infected or uninfected (controls) anticoagulated blood pooled from C3H/SCID mice. Q-PCR was performed on an aliquot of the pooled blood collected from the SCID mice to determine *A. phagocytophilum* infection. For acquisition experiments, uninfected ticks were fed on either *A. phagocytophilum*-infected or naive C3H/HeN mice (3 mice per group and 20 ticks per mouse cell). Ticks were collected from both the ends (KECK Sequencing Facility, Yale University).

**Immunoprecipitation.** Tick cell lysates were prepared from uninfected and *A. phagocytophilum*-infected cells (48 h after infection). Cell pellets (10^6 cells/ml) were washed twice with PBS and resuspended in cold modified RIPA buffer containing protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors. The lysates were precleared with protein A/G agarose beads (Thermo Fisher Scientific) at a 1:10 volume of 50% bead slurry and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatants were collected and protein concentrations were determined using the Bradford assay (BCA kit; Thermo Fisher Scientific). For analyzing the phosphorylation of proteins upon *A. phagocytophilum* infection in tick cells, antibodies specific to phosphoryrosine (Cell Signaling Technologies) were added to 500 µg/ml of the lysate and the mixture was incubated overnight at 4°C. Immunoprecipitates were also processed for Western blotting with actin antibody (Millipore) to further confirm actin phosphorylation. For determining the interaction between F-actin and phosphorylated actin,**immunolabeling was detected by anti–mouse secondary antibodies conjugated with Alexa Fluor 488 or 594, respectively. *A. phagocytophilum* was detected using polyclonal antiserum as previously described (Pedra et al., 2010). Cells were incubated with either 1 µg/ml phalloidin–Alexa Fluor 488 or with DNase I–Alexa Fluor 594 conjugates to stain for F- or G-actin, respectively. To quantify number of filamentous cell per field, a minimum of 25 microscopic fields were considered and the number of filamentous cells was counted from each field. To determine the number of filaments per cell, the total number of F-actin-positive filaments on the cell periphery that strongly stained with phalloloid was counted from a total of 30 cells in each group. The percentage of cells positive for filaments was determined by counting the number of filamentous cells/field to the total number of cells in that field. Three independent experiments were performed to determine statistical significance. Confocal images of F/G-actin were captured simultaneously at similar intensities and relevant excitation and emissions, respectively. Cells were counterstained for nuclei with TOPRO3–Alexa Fluor 647 conjugate. For tick salivary glands, immunofluorescence was performed as previously described (Sukumaran et al., 2006; Neelakanta et al., 2007). Salivary glands were dissected from unfed ticks, fixed with ice-cold acetone, and washed in PBS. Microscope slides containing fixed permeabilized tissue sections were blocked with PBS containing 0.05% Tween 20 and 5% goat serum for 1 h at 37°C and were sequentially incubated with actin and phosphoryrosyne antibodies, followed by anti–mouse secondary antibodies conjugated with Alexa Fluor 488 or 594, respectively. All Alexa conjugates were obtained from Invitrogen. Images were acquired using a laser-scanning confocal microscope (LSM 510; Carl Zeiss, Inc.).

**Identification of PI3K and PAK1 from the *Ixodes scapularis* genome.** The *I. scapularis* genome database VectorBase (http://scapularis.vectorbase.org/index.php) includes all genomic data (e.g., EST sequences, trace files, scaffolds, assemblies, and automated annotations) and the database at The Gene Index Project database includes EST sequences. The *ipak1* and *ipi3k* partial nucleotide sequences were identified with the BLAST search performed at The Gene Index Project in the *I. scapularis* EST database. EST sequences corresponding to *I. scapularis* *ipak1* (Gene Index Project accession no. TC39943) and *ipi3k* (Gene Index Project accession no. TC38262) were identified with a BLAST search using *Drosophila melanogaster* *pak1* (NCBI nucleotide accession no. DMU05680) and *pi3k* (accession no. NM_057785) nucleotide sequences as queries, respectively. EST sequences were further analyzed using DNASTAR software. Total RNA from unfed infected *I. scapularis* ticks was processed for cDNA synthesis and used as template for RT-PCR. The *ipak1* fragment was amplified using oligonucleotides 5'-ATGGCTCAGCTACGGGCAGC-3' and 5'-TTAGACCTTGTCGACACCTGTC-3' and *ipi3k* fragment was amplified using 5'-GCTCTTAAGCTCCGAGCCCTA-3' and 5'-GGAACACCCGTGTTGCCTGT-3'. The generated PCR products were cloned into pGEM-T easy vector (Promega) and sequenced using primers 5'-CCGCGGTTTCCAGCTACGAC-3' and 5'-CACACAGGACACGGTATGAC-3' from both the ends (KECK Sequencing Facility, Yale University). Amino acid sequence similarity and identity of *Ixodes* *PAK1* and PDK was determined by aligning deduced amino acid sequences with human, mouse, mosquito, and *Drosophila* PAK1 and PI3K using DNASTAR software.

**Measurement of G/F-actin ratios.** The amount of F-actin compared with free G-actin content was determined by G/F-actin in vivo assay kit (Cytokeleton, Inc.) according to the manufacturer’s instructions. In brief, unfed uninfected ticks, *A. phagocytophilum*-infected ticks or tick cells at 48 h after infection, and infected tick cells were homogenized in lysis and F-actin stabilization buffer followed by centrifugation for 1 h at 100,000 g at 30°C to separate the F-actin from G-actin pool. Supernatants of the protein extracts were collected after centrifugation and stored on ice. Pellets were resuspended in ice-cold buffer followed by centrifugation for 10 min at room temperature, and blocked with 3% bovine serum albumin (30 min at room temperature) in PBS, respectively. Cells were immunostained with antibodies directed against phosphoryrosyne, actin, or RNAIP, and labeling was detected by anti–mouse secondary antibodies conjugated with Alexa Fluor 488 or 594, respectively. *A. phagocytophilum* was detected using polyclonal antiserum as previously described (Pedra et al., 2010). Cells were incubated with either 1 µg/ml phalloidin–Alexa Fluor 488 or with DNase I–Alexa Fluor 594 conjugates to stain for F- or G-actin, respectively. To quantify number of filamentous cell per field, a minimum of 25 microscopic fields were considered and the number of filamentous cells was counted from each field. To determine the number of filaments per cell, the total number of F-actin-positive filaments on the cell periphery that strongly stained with phalloloid was counted from a total of 30 cells in each group. The percentage of cells positive for filaments was determined by counting the number of filamentous cells/field to the total number of cells in that field. Three independent experiments were performed to determine statistical significance. Confocal images of F/G-actin were captured simultaneously at similar intensities and relevant excitation and emissions, respectively. Cells were counterstained for nuclei with TOPRO3–Alexa Fluor 647 conjugate. For tick salivary glands, immunofluorescence was performed as previously described (Sukumaran et al., 2006; Neelakanta et al., 2007). Salivary glands were dissected from unfed ticks, fixed with ice-cold acetone, and washed in PBS. Microscope slides containing fixed permeabilized tissue sections were blocked with PBS containing 0.05% Tween 20 and 5% goat serum for 1 h at 37°C and were sequentially incubated with actin and phosphoryrosyne antibodies, followed by anti–mouse secondary antibodies conjugated with Alexa Fluor 488 or 594, respectively. All *A. phagocytophilum* conjugates were obtained from Invitrogen. Images were acquired using a laser-scanning confocal microscope (LSM 510; Carl Zeiss, Inc.).
distilled H$_2$O plus 1 μmol/liter cytochalasin D and incubated for 1 h on ice to disassociate F-actin by gently mixing for every 15 min. Equal amounts of both the supernatant (G-actin) and the resuspended pellet (F-actin) were subjected to analysis of immunoblot with the actin antibody (Cytoskeleton, Inc.). Total lysates (input) probed for total actin served as the loading control.

Nuclear extraction and EMSA. Nuclear extracts were prepared from uninfected and A. phagocytophilum–infected ticks or mock and ipak1–dsRNA–injected ticks (48 h during feeding) using NE-PER Nuclear and Cytoplasmic extraction kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Gel shift assays were performed with the LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific). Complementary biotinylated oligonucleotides consisting of the salp16 putative promoter TATA binding region 5′-GCCACGCCCTAGT-GATCCTGGATATATAGAAAAAGGAACTCCCTGGA-3′ and 5′-TCCAGGAGGCTTCTTCTTTCTTATATACGGGAAATGCGAT-TAGGCGTGGCC-3′ or salp20 putative promoter TATA binding region 5′-TTGTTGCTTGACAGTCGTGTTATATATATATATC-GGCGAAAGATTAGCAT-3′ and 5′-ATGTCATAATCCTTCGCC-GATATATATATACCCAGACAGCGGCAAA-3′ were annealed and biotin labeled according to the Biotin 3′ End DNA labeling kit (Thermo Fisher Scientific). The respective labeled oligonucleotides were added to a 20-μl reaction mix consisting of 1–3 μg of the nuclear extracts, DNA binding buffer, Polv (dl-dc), 1% NP-40, and MgCl2 in concentrations based on the manufacturer’s recommendations. For competition assays, unlabeled oligonucleotides were allowed to bind the nuclear extracts (30 min at room temperature) before the addition of labeled probes. EMSAs were performed with antibodies by incubating 1–3 μg of the respective antibodies with nuclear extract proteins (2 μg for 30 min at room temperature). The reactions were incubated for additional 20 min with respective labeled probes, followed by loading onto a 6% native DNA polyacrylamide gel. The gel was prerun and run with 0.5% Tris-Borate-EDTA and processed according to the manufacturer’s instructions.

Neutrophil isolation. PBMCs were isolated from a healthy donor with no acute illness, taking no antibiotics or NSAIDs, and previously screened for no exposure to other pathogen infections. Blood cells were sedimented using 6% dextran, lysed with 0.6 M KCl, and centrifuged. The neutrophil pellets were resuspended in HBSS and infected with cell-free A. phagocytophilum salivary gland extracts (Babcm), or P44 (Thomas and Fikrig, 2007) to detect A. phagocytophilum, the membrane was incubated with either 5% BSA or milk (according to the manufacturer’s instructions) in Tween-20–Tris-buffered saline to bind nonspecific sites. After the primary antibody incubations, immunoblots were treated with either anti-mouse or anti-rabbit HRP-conjugated IgG secondary antibodies based on the manufacturer’s instructions (Sigma–Aldrich). Enhanced chemiluminescence detection of antibody binding was performed with the ECL Western blotting detection system (GE Healthcare). Immunoblot films were scanned into jpeg format using a scanner (CanoScan LiDE70; Canon) and images were analyzed and quantified in Photoshop (Adobe) according to the previously described method (Miller et al., 2009; Luhtala and Parker, 2009). The relative intensity of each band on the immunoblots was calculated with respect to the loading controls (total actin or RNAPII) used in the respective experiments. Quantification of all the immunoblots from this study is shown in Table S1.

Genomic DNA Isolation and PCR to detect A. phagocytophilum infection. Total genomic DNA was isolated from the mouse peripheral blood or ticks using the DNeasy tissue kit (QIAGEN) according to the manufacturer’s protocol. A. phagocytophilum burden was determined by analyzing the levels of P44 gene using Q-PCR, as described previously (Sukumaran et al., 2006; Thomas and Fikrig, 2007).

RNA extraction, cDNA synthesis, and Q-RT-PCR analysis. To determine A. phagocytophilum burden and tick gene expression, total RNA was extracted with RNeasy extraction kit (QIAGEN) from uninfected or A. phagocytophilum–infected tick cells, whole ticks (unfed nymphs/48 h during feeding ticks), mock, ipak1–dsRNA–, ipak3–dsRNA–, igb–dsRNA–, igy–dsRNA–, or inhibitor–injected tick groups. RNA was treated on a column containing RNase-free DNase set (QIAGEN) during isolation to remove contaminating DNA. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Q-RT-PCR was performed using primers for A. phagocytophilum P44, tick genes salp16, salp20, salp15, salp25D (Ramamoorthi et al., 2005; Sukumaran et al., 2006; Narasimhan et al., 2007; Thomas and Fikrig, 2007), ipak1 (5′-CTGATCTGACAGA-TTGGAGTGA-3′ and 5′-CATGGTACACTGTTGATCCTCCTG-3′), ipak3 (5′-GCTTCTCAAGTCCGCCCCCTA-3′ and 5′-GGACAACCC-GTGCTTCTG-3′), igb (5′-GTGCCGTGTGTTGCCTTCTCCT-3′ and 5′-CAGGGATCAATGGTCTTCAAA-3′), igy (5′-ATGCACTCTGATACTGGCAAC-3′ and 5′-ATGCACTCTGGCAAC-3′), β tubulin (5′-CTACGACATCTGCTTCCTGGCAAC-3′ and 5′-GGGCCGCA-TAGTGGTCTG-3′), and gapdh (5′-CAGAAGGGGTAGGTGCTGTG-3′ and 5′-GCGGCTCAATGTTGCT-3′). Primers for β-actin cDNA were used in parallel for normalization (Neelakanta et al., 2007). Equal amounts of tick cDNA samples were used in parallel for β-actin and A. phagocytophilum P44 gene Q-RT-PCR analysis.

dsRNA synthesis and tick microinjections. Tick cDNA was prepared and used as template to amplify DNA encoding a fragment of Ixodes ghi, gyy, piki, and...
To determine whether inhibition of protein tyrosine phosphatases does further induce actin phosphorylation, uninfected or A. phagocytophilum–infected tick cells were treated with 1 mM pervanadate, a protein tyrosine phosphatase inhibitor, for respective times. For pervanadate treatment, we made a stock solution of 100 mM sodium vanadate plus 3% hydrogen peroxide in deionized water. We used inhibitor at 1:100 dilutions when stimulating cells. For PK3K, PAK1, or tyrosine kinase inhibition studies, we infected tick cells with A. phagocytophilum and simultaneously treated them with 100 μM of either LY294002 (inhibitor of PI3K) or Genistein (a protein tyrosine kinase inhibitor) or 10 μM of PK-18 (a potent PAK1 inhibitor peptide). Infected cells were treated with similar amounts of DMSO as mock controls. The inhibitors were obtained from EMD and the concentrations of these inhibitors used in this study showed no effects on cell viability as determined by IFA. For tick microinjection studies, 100 μM PK3K or Genistein or 10 μM PAK1 inhibitors were diluted 100× in 10% DMSO and microinjected into the tick body as two luts. 

Inhibitors study. To determine whether inhibition of protein tyrosine phosphatases does further induce actin phosphorylation, uninfected or A. phagocytophilum–infected tick cells were treated with 1 mM pervanadate, a protein tyrosine phosphatase inhibitor, for respective times. For pervanadate treatment, we made a stock solution of 100 mM sodium vanadate plus 3% hydrogen peroxide in deionized water. We used inhibitor at 1:100 dilutions when stimulating cells. For PK3K, PAK1, or tyrosine kinase inhibition studies, we infected tick cells with A. phagocytophilum and simultaneously treated them with 100 μM of either LY294002 (inhibitor of PI3K) or Genistein (a protein tyrosine kinase inhibitor) or 10 μM of PK-18 (a potent PAK1 inhibitor peptide). Infected cells were treated with similar amounts of DMSO as mock controls. The inhibitors were obtained from EMD and the concentrations of these inhibitors used in this study showed no effects on cell viability as determined by IFA. For tick microinjection studies, 100 μM PK3K or Genistein or 10 μM PAK1 inhibitors were diluted 100× in 10% DMSO and microinjected into the tick body as two luts. 

**Mapping of TATA motif in the putative salp16 and salp20 promoter regions.** The genomic loci corresponding to salp16 and salp20 genes were downloaded from http://www.vectorbase.org/ and contigs DS950927 and DS702893 for salp16 and DS647593, DS772527, and DS916524 for salp20 were analyzed using SeqMan and DNASTAR software. The genomic region corresponding to −1,000 bp to +100 bp from the start of salp16 or salp20 coding sequences was analyzed for putative TATA motif using three softwares: HCtata, Hamming-Clustering Method for TATA Signal Prediction in Eukaryotic Genes (http://www.itb.cnr.it/sun/webgene/); Neural network promoter prediction (http://www.fruitfly.org/seq_tools/promoter.html); and TSSW recognition of human polymerase II promoter region and start site (http://www.softberry.com). The results from all the three searches were combined and analyzed for the TATA motif and putative transcription start site. The region that showed TATA motif mapped by all three softwares with high scores was considered for EMSA assays. The region containing TATA motif in putative salp16 promoter region was PCR amplified using unique Id. scapularis genomic DNA as template with oligonucleotides 5’-CTGGCTGGGATATCCTTG-3’ and 5’-GACAATGACATATTTCTAACCGAAAAC-3’, and TATA motif fragment in the salp20 promoter region was amplified using 5’-CACAACAATACCGGCTCAGTATTCTTACTAACG-3’ and sequenced from both ends.

**Biotinylated DNAP assay.** Nuclear extracts were prepared from A. phagocytophilum–infected ticks (during 48-h engorgement) using the NE-PER Nuclear and Cytoplasmic extraction kit, and extracts were precleared with 50% slurry of streptavidin-coated Sepharose beads (GE Healthcare). Protein amounts were estimated with Bradford assay. 100–200 μg of nuclear extracts was incubated for 30 min at room temperature with 10 μg Poly (dl-IdC) for nonspecific binding and 5 μg of site-specific double-stranded biotinylated DNA probes (salp16 or salp20) in gel shift binding buffer (Thermo Fisher Scientific) supplemented with 0.1% NP-40 and 5 mM MgCl2. Biotin-labeled salp16 or salp20 probes previously used in EMSA were used in this assay. Streptavidin-coated sepharose beads were incubated for an additional 2 h at 4°C to capture the biotinylated DNA bound to the nuclear extract proteins. Beads were centrifuged and washed twice with ice-cold gel shift binding buffer and twice with PBS and then re suspended in 2× reducing sample buffer,
boiled for 5 min, and loaded on 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and probed with phosphotyrosine or TBP or RNAPII antibodies followed by incubation with anti-mouse IgG HRP-conjugated secondary antibody and enhanced chemiluminescence detection was performed as described before. Total lysates used for immunoprecipitation were probed with actin antibody to serve as the loading control.

Statistical analysis. Statistical significance between the mean values was determined using a nonpaired Student’s t test. Calculated p-values of <0.05 were considered significant.

Online supplemental material. Fig. S1 shows that A. phagocytophilum induces both phosphothreonine and phosphophosphoysine phosphorylation of actin in I. scapularis. Fig. S2 shows Ixodes PAK1 and PK3 sequences. Fig. S3 shows alignments of Ixodes Gβy subunit sequences. Fig. S4 shows alignments of Ixodes Gβy subunit alignment. Fig. S5 shows expression and inhibition of I. scapularis PAK1, PK3, Gβy, or tyrosine kinases in ticks. Fig. S6 shows that silencing of ipak, ip3ike, gβy, and gγy reduces actin phosphorylation in I. scapularis. Fig. S7 shows that antibodies against phosphotyrosine, actin, RNAPII, and TBP blocked the band shift with salp16 probe. Fig. S8 shows a model depicting A. phagocytophilum-induced actin phosphorylation in the context of Ixodes PK3-PAK1 signaling, which selectively regulates salp16 gene transcription. Table S1 shows quantification of Western blots shown in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100276/DC1.

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