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Anaplasma phagocytophilum surface protein AipA mediates invasion of mammalian host cells

David Seidman[1], **Nore Ojogun**[1], **Naomi J. Walker**[2], **Juliana Mastronunzio**[3,4], **Amandeep Kahlon**[1,5], **Kathryn S. Hebert**[1], **Sophia Karandashova**[1], **Daniel P. Miller**[1], **Brittney K. Tegels**[1], **Richard T. Marconi**[1], **Erol Fikrig**[3], **Dori L. Borjesson**[2], and **Jason A. Carlyon**[1,*]

[1]Department of Microbiology and Immunology, Virginia Commonwealth University School of Medicine, Richmond, Virginia, USA

[2]Department of Pathology, Microbiology, and Immunology, University of California School of Veterinary Medicine, Davis, California, USA

[3]Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut, USA

Summary

Anaplasma phagocytophilum, which causes granulocytic anaplasmosis in humans and animals, is a tick-transmitted obligate intracellular bacterium that mediates its own uptake into neutrophils and non-phagocytic cells. Invasins of obligate intracellular pathogens are attractive targets for protecting against or curing infection because blocking the internalization step prevents survival of these organisms. The complement of *A. phagocytophilum* invasins is incompletely defined. Here, we report the significance of a novel *A. phagocytophilum* invasion protein, AipA. *A. phagocytophilum* induced *aipA* expression during transmission feeding of infected ticks on mice. The bacterium upregulated *aipA* transcription when it transitioned from its non-infectious reticulate cell morphotype to its infectious dense-cored morphotype during infection of HL-60 cells. AipA localized to the bacterial surface and was expressed during *in vivo* infection. Of the AipA regions predicted to be surface-exposed, only residues 1 to 87 ($AipA_{1–87}$) were found to be essential for host cell invasion. Recombinant $AipA_{1–87}$ protein bound to and competitively inhibited *A. phagocytophilum* infection of mammalian cells. Antiserum specific for $AipA_{1–87}$, but not other AipA regions, antagonized infection. Additional blocking experiments using peptide-specific antisera narrowed down the AipA invasion domain to residues 9 to 21. An antisera combination targeting $AipA_{1–87}$ together with two other *A. phagocytophilum* invasins, OmpA and Asp14, nearly abolished infection of host cells. This study identifies AipA as an *A. phagocytophilum* surface protein that is critical for infection, demarcates its invasion domain, and establishes a rationale for targeting multiple invasins to protect against granulocytic anaplasmosis.

[*]Corresponding author: Jason A. Carlyon, Department of Microbiology and Immunology, Virginia Commonwealth University School of Medicine, P. O. Box 980678, Richmond, VA 23298-0678, Phone: (804)-628-3382, Fax: (804)-828-9946. jacarlyon@vcu.edu.
[4]Present address: Juliana Mastronunzio, Fairfield University, Fairfield, Connecticut, USA
[5]Present address: Amandeep Kahlon, Maxxam Analytics, Burnaby, British Columbia, Canada

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Introduction

Obligate intracellular bacteria use outer membrane proteins (OMPs) called invasins to enter eukaryotic host cells. Since these organisms are incapable of extracellular survival, infection can be prevented or cured by blocking the internalization step. Thus, it is desirable to identify and characterize invasins of obligate intracellular bacteria, specifically the functional domains that mediate entry into host cells. *Anaplasma phagocytophilum* is an obligate intracellular bacterium in the order *Rickettsiales* and family *Anaplasmataceae* that infects neutrophils to cause granulocytic anaplasmosis in humans and animals. Though primarily an *Ixodes* spp. tick-borne illness (Truchan *et al.*, 2013), human granulocytic anaplasmosis (HGA) can also be transmitted perinatally, nosocomially, and through blood transfusion (Annen *et al.*, 2012; Carlyon, 2012; Jereb *et al.*, 2012; Alhumaidan *et al.*, 2013). The disease presents as a non-specific febrile illness that can be accompanied by leukopenia, thrombocytopenia, elevated levels of serum transaminases, and increased susceptibility to potentially fatal secondary infections (Truchan *et al.*, 2013). HGA is an emerging infection in the United States, Europe, and Asia (Truchan *et al.*, 2013). The number of reported HGA cases in the United States increased over six-fold from 2003 to 2012, the latest period for which disease reporting statistics are available (Hopkins *et al.*, 2005; Centers for Disease Control and Prevention, 2013).

A. phagocytophilum undergoes a biphasic developmental cycle that begins when an infectious dense-cored (DC) organism binds to and enters its host cell, where it resides within a host cell-derived vacuole. Between 4 and 8 h, the DC develops into the non-infectious reticulate cell (RC) morphotype that subsequently divides by binary fission to yield a bacteria-filled vacuolar inclusion. From 8 to 20 h, the intravacuolar population consists exclusively of replicating RCs. Most RCs transition back into DCs between 28 and 32 h. DCs then exit host cells between 28 to 36 h and initiate the next round of infection (Troese *et al.*, 2009). *A. phagocytophilum* OMPs that are upregulated during RC-to-DC transition, bacterial exit, and reinfection are attractive candidates to evaluate for both their roles in infection and their prospect as protective antigens.

Given the potential severity of HGA, the limited choices of antibiotics for treating the disease, and the lack of a vaccine, a thorough understanding of *A. phagocytophilum* cellular invasion is critical. We recently identified OmpA (APH0338) and Asp14 (14-kDa *A. phagocytophilum* surface protein; APH0248) as being important for *A. phagocytophilum* entry into mammalian cells (Ojogun *et al.*, 2012; Kahlon *et al.*, 2013). OmpA binds to α2,3-sialic acid of the sialyl Lewis x (sLe^x) tetrasaccharide that caps P-selectin glycoprotein ligand-1 (PSGL-1) on myeloid cell surfaces. OmpA also recognizes α2,3-sialic acid residues that decorate as yet defined glycoproteins on endothelial cells (Ojogun *et al*, 2012). The Asp14 receptor is unknown. Evidence implicates involvement of one or more *A. phagocytophilum* invasins in addition to OmpA and Asp14 in mediating infection (Kahlon *et al.*, 2013). Identifying these invasins and evaluating if targeting them alone or in concert with OmpA and Asp14 can block bacterial entry may foster development of effective prophylaxes against HGA.

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A whole genome transcriptional profiling study revealed *A. phagocytophilum* genes that are upregulated during infection of mammalian versus tick cells (Nelson *et al.*, 2008). Several of these encode putative OMPs, one of which is APH0915. In this study, we show that APH0915, hereafter referred to as AipA (*A. phagocytophilum* invasion protein A), is important for bacterial entry into mammalian cells and identify its invasion domain. We further demonstrate that a combination of antisera targeting AipA, OmpA, and Asp14 synergistically blocks infection. Our findings not only advance understanding of how *A. phagocytophilum* employs multiple invasins to promote infection, but also sets the stage for development of a multi-target vaccine that protects against granulocytic anaplasmosis.

Results

A. phagocytophilum differentially expresses AipA during the infectious stage of its biphasic developmental cycle, the transmission bloodmeal of infected ticks, and infection of mammalian hosts

AipA is a 355-amino acid (36.9 kDa) protein and a putative OMP (Nelson *et al.*, 2008). In silico analysis of the AipA amino acid sequence predicted that residues 107 to 127, 136 to 155, and 220 to 243 form transmembrane domains that position residues 1 to 106 and 156 to 219 on the bacterial surface (Figure 1A). Protein BLAST searches revealed that AipA does not display high sequence identity to sequenced proteins of other organisms, including *Anaplasmataceae* and *Rickettsiales* members. Because *A. phagocytophilum* proteins encoded by genes that are upregulated late during the biphasic developmental cycle are important for infection (Huang *et al.*, 2010b; Troese *et al.*, 2011; Mastronunzio *et al.*, 2012), we examined AipA expression throughout the infection cycle in human promyelocytic HL-60 cells. *aipA* mRNA levels were approximately 10- to 20-fold higher between 30 and 36 hours – time points that correspond to RC-to-DC transition, exit, and reinfection – than between 4 and 26 hours, time points that correspond to conversion to and replication of non-infectious RC organisms (Troese *et al.*, 2009) (Figure 2A). Also, the *aipA* mRNA level of the DC inoculum was comparable to that detected at 36 h, a time point that correlates with reinfection.

Next, we examined if the differential *aipA* transcription pattern observed in infected HL-60 cells correlated with differential AipA protein expression. AipA amino acids 1–87 ($AipA_{1–87}$), 165–204 ($AipA_{165–204}$) and 249–355 ($AipA_{249–355}$) contain segments that are hydrophilic and predicted to be accessible on the protein's surface (Figure 1B). $AipA_{1–87}$ and $AipA_{165–204}$ are predicted to be exposed on the bacterial surface, while $AipA_{249–355}$ is not (Figure 1B). $AipA_{1–87}$ and $AipA_{249–355}$ were expressed in *Escherichia coli* as proteins N-terminally fused to glutathione-*S*-transferase. Despite numerous attempts, we were unable to express soluble GST-tagged full-length AipA and $AipA_{165–204}$ (data not shown). Mouse antiserum raised against $AipA_{1–87}$ and $AipA_{249–355}$ fusion proteins recognized a band of the expected size in lysates of *A. phagocytophilum* infected but not uninfected HL-60 cells (Figure 2B). Anti-$AipA_{249–355}$ recognized an additional band having an apparent mobility that was slightly smaller than 75 kDa, suggesting that AipA may dimerize. Alternatively, anti-$AipA_{249–355}$ may recognize an epitope that is shared with or is similar in sequence to that of the unknown *A. phagocytophilum* protein. We used $AipA_{1–87}$ antibody to screen

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infected HL-60 cells by immunofluorescence microscopy. Consistent with our transcriptional data, approximately 70% and 96% of the *A. phagocytophilum* inclusions contained AipA-expressing bacteria at 28 and 32 h, respectively, whereas considerably fewer inclusions were AipA-positive at earlier time points (Figure 2C). These data demonstrate that AipA is both transcriptionally and translationally upregulated during periods when *A. phagocytophilum* converts to and is in its infectious DC morphotype.

A. phagocytophilum genes that are induced during the tick transmission bloodmeal, such as *ompA* and *asp14*, encode proteins that are important for establishing infection in mammalian hosts (Ojogun *et al.*, 2012; Kahlon *et al.*, 2013). Therefore, we examined *aipA* expression in the salivary glands of *A. phagocytophilum* infected *I. scapularis* nymphs over the course of transmission feeding on naïve mice. *aipA* mRNA was undetectable in unfed infected ticks (Figure 2D). However, *aipA* transcripts were significantly induced at 24 h of tick feeding and were increasingly expressed through to 72 h. To ensure that *A. phagocytophilum* transcription was not globally upregulated during tick transmission feeding, we also examined *groEL* (*aph0240*) expression. *groEL* was detected at the highest level in infected unfed nymphs and decreased in expression over the duration of the bloodmeal. Thus, *A. phagocytophilum* specifically induces select genes, including *aipA*, as it adapts during the transmission bloodmeal to colonize the mammalian host.

Consistent with a prior report that *A. phagocytophilum* preferentially expresses *aipA* during growth in HL-60 cells versus *I. scapularis* embryo-derived ISE6 cells (Nelson *et al.*, 2008), $AipA_{1–87}$ antibody failed to detect a protein of the expected size in lysates of ISE6 cells in which *A. phagocytophilum* had been continually passaged for one, six, or seven weeks (Figure 2E). APH0032, which is an *A. phagocytophilum* protein that we previously demonstrated to be expressed during infection of ISE6 cells (Huang *et al.*, 2010b), was detected in all infected samples. HGA patient serum and serum from a dog that had been naturally infected with *A. phagocytophilum* each detected GST-$AipA_{1–87}$ (Figure 2F), confirming that AipA is expressed and elicits a humoral immune response during *A. phagocytophilum* infection of humans and dogs. Two additional HGA patient sera recognized GST-$AipA_{1–87}$ (data not shown).

AipA is an *A. phagocytophilum* surface protein

To confirm whether AipA localizes to the bacterial outer membrane, we employed confocal microscopy to screen infected RF/6A endothelial cells with $AipA_{1–87}$ antibody in conjunction with antiserum targeting the *A. phagocytophilum* major surface protein, Msp2 (P44) (Carlyon, 2012). Both antibodies detected intravacuolar organisms, yielding the ring-like staining pattern on their peripheries that is characteristic for Msp2 (P44) and other confirmed *A. phagocytophilum* OMPs (Ge *et al.*, 2007; Ojogun *et al.*, 2012; Kahlon *et al.*, 2013) (Figure 3A). Msp2 (P44) signal colocalized with and extended beyond the AipA signal. Next, to determine if immunoaccessible AipA domains are exposed on the bacterial surface, we treated the surfaces of intact, host cell-free DC bacteria with trypsin, an approach that has been used to confirm surface localization of *A. phagocytophilum* and *Chlamydia trachomatis* OMPs (Wang *et al.*, 2006; Ojogun *et al.*, 2012; Kahlon *et al.*, 2013). AipA residues 1 to 87 include six lysine and three arginine residues, making this putative

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surface exposed region susceptible to tryptic digest. If $AipA_{1–87}$ or portions thereof were exposed on the *A. phagocytophilum* surface, then incubating intact bacteria with trypsin should result in proteolytic cleavage of this region of the protein, which, in turn, would result in an inability to detect AipA. Trypsin-treated DC organisms were solubilized, Western-blotted, and probed with antiserum specific for $AipA_{1–87}$ or a confirmed surface-exposed epitope of Asp55 (55-kDa *A. phagocytophilum* surface protein) (Ge *et al.*, 2007). Blots were also probed with antiserum targeting APH0032, which does not localize to the *A. phagocytophilum* outer membrane (Huang *et al.*, 2010b). After surface trypsinolysis, APH0032 was detected but AipA and Asp55 were not (Figure 3B). Thus, AipA residues 1 to 87 are exposed on the *A. phagocytophilum* surface.

GST-AipA requires amino acids 1 to 87 to bind and competitively inhibit *A. phagocytophilum* infection of mammalian host cells

Since AipA is an exposed surface protein that is induced during key infection stages in the *A. phagocytophilum* life cycle, we investigated if it facilitates interactions with mammalian host cell surfaces to promote infection. We assessed if GST-tagged $AipA_{1–87}$ and $AipA_{249–355}$ bind to RF/6A cells. GST alone served as a negative control. GST antibody detected GST-$AipA_{1–87}$ that had adhered to the host cells by both immunofluorescence microscopy and flow cytometry (Figure 4, A and B). GST-$AipA_{249–355}$ bound poorly, at best, and GST alone did not bind to host cells. Based on their differential adhesion capabilities, we rationalized that GST-$AipA_{1–87}$ but not GST-$AipA_{249–355}$ would be able to serve as a competitive agonist to inhibit *A. phagocytophilum* infection. Indeed, preincubating HL-60 and RF/6A cells with GST-$AipA_{1–87}$ significantly reduced the percentages of infected cells and the number of bacterial inclusions per cell by nearly four-fold relative to incubation with GST alone (Figure 4, C to F). In contrast, GST-$AipA_{249–355}$ did not inhibit *A. phagocytophilum* infection of HL-60 cells and reduced infection of RF/6A cells by only a small degree. These data suggest that AipA residues 1 to 87 contain a domain that contributes to *A. phagocytophilum* invasion of myeloid and endothelial cells.

Antiserum targeting AipA residues 1 to 87 inhibits *A. phagocytophilum* infection of host cells

Given that AipA amino acids 1 to 87 are exposed on the *A. phagocytophilum* surface and contribute to infection, we assessed if treating DC organisms with heat-inactivated $AipA_{1–87}$ antiserum prior to incubating them with HL-60 cells would inhibit infection. OmpA antiserum, for which we previously validated its ability to inhibit *A. phagocytophilum* infection (Ojogun *et al.*, 2012), was a positive control. Anti-$AipA_{1–87}$ and anti-OmpA each reduced the number of infected cells and the number of bacterial inclusions per cell by approximately 40% (Figure 5, A and B). In contrast, $AipA_{249–355}$ antiserum had no effect on *A. phagocytophilum* infection. Consistent with our published studies of *A. phagocytophilum* invasins (Ojogun *et al.*, 2012; Kahlon *et al.*, 2013), neither $AipA_{1–87}$ nor OmpA antiserum inhibited bacterial adhesion to HL-60 cells (data not shown).

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AipA targets a sLe^x-capped PSGL-1-independent receptor

sLe^x-capped PSGL-1 is the only known *A. phagocytophilum* receptor on myeloid host cells (Herron *et al.*, 2000), and OmpA binds the α2,3-sialic acid determinant of sLe^x (Ojogun *et al.*, 2012). Since *A. phagocytophilum* interactions with sLe^x-capped PSGL-1 involve at least one bacterial surface protein in addition to OmpA (Carlyon *et al.*, 2003; Yago *et al.*, 2003; Reneer *et al.*, 2006; Sarkar *et al.*, 2007; Reneer *et al.*, 2008; Ojogun *et al.*, 2012), we investigated if AipA$_{1–87}$ antiserum could inhibit bacterial binding to Chinese hamster ovary (CHO) cells transfected to express sLe^x-capped PSGL-1 (PSGL-1 CHO cells). These cells are excellent models for studying *A. phagocytophilum* interactions with sLe^x-capped PSGL-1 as they, but not untransfected CHO cells that do not express the receptor, support bacterial binding (Carlyon *et al.*, 2003; Xia *et al.*, 2003; Yago *et al.*, 2003; Reneer *et al.*, 2006; Sarkar *et al.*, 2007; Reneer *et al.*, 2008; Troese *et al.*, 2009). DC organisms were incubated with AipA$_{1–87}$ or AipA$_{249–355}$ antiserum prior to being added to PSGL-1 CHO cells. Bacteria pretreated with preimmune serum were a negative control, whereas bacteria pretreated with OmpA antiserum served as a positive control. Additional positive controls for blocking *A. phagocytophilum* adhesion were PSGL-1 CHO cells that had been pretreated with KPL-1 or CSLEX1, which are monoclonal antibodies that block the bacterium's access to the PSGL-1 N-terminus or the α2,3-linked sialic acid determinant of sLe^x, respectively (Goodman *et al.*, 1999; Herron *et al.*, 2000; Troese *et al.*, 2009). Incubating DC organisms with OmpA antibody and incubating PSGL-1 CHO cells with KPL-1 or CSLEX1 significantly reduced the numbers of bound DC organisms by two- to three-fold (Figure 5C). *A. phagocytophilum* bound poorly to untransfected CHO cells. Preimmune serum, anti-AipA$_{1–87}$, and anti-AipA$_{249–355}$ failed to inhibit bacterial binding to PSGL-1 CHO cells. Therefore, AipA contributes to *A. phagocytophilum* cellular invasion by interacting with an sLe^x-capped PSGL-1-independent receptor.

A combination of antisera targeting AipA, OmpA, and Asp14 blocks *A. phagocytophilum* infection of host cells

A. phagocytophilum infection requires cooperative interactions of multiple invasins with the host cell surface (Truchan *et al.*, 2013). Incubating DC organisms with antiserum targeting full-length OmpA (Ojogun *et al.*, 2012), full-length Asp14 (Kahlon *et al.*, 2013), or AipA$_{1–87}$ significantly, but only partially, reduced *A. phagocytophilum* infection of mammalian host cells. We therefore investigated if blocking multiple bacterial-host interactions by treating DC organisms with combinations of AipA$_{1–87}$, OmpA, and/or Asp14 antisera could improve blocking efficacy. The result was synergistic: whereas antisera combinations targeting two of the three invasins together more effectively inhibited infection than an antiserum targeting an individual protein, the most effective blocking of *A. phagocytophilum* infection was achieved using antisera against all three invasins (Figure 6). These data demonstrate the potential of simultaneously targeting AipA, OmpA, and Asp14 as an effective means for preventing *A. phagocytophilum* infection.

The AipA invasion domain is contained within residues 9 to 21

We next sought to pinpoint the AipA invasion domain. Our competitive agonist and antisera blocking studies indicated that this domain lies within residues 1 to 87. Based on

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hydrophobicity and surface probability analyses (Figure 1B), we rationalized that, of the AipA region of interest, amino acids 9 to 21 and/or 61 to 84 were most likely to facilitate interactions with host cells that promote infection. Accordingly, we generated rabbit antisera against each of these peptides for use in antibody blocking experiments. The AipA region encompassed by residues 165 to 204 ($AipA_{165–204}$) is predicted to be exposed on the *A. phagocytophilum* outer membrane, hydrophilic, and accessible on the surface of AipA (Figure 1B). Yet, the contribution of $AipA_{165–204}$ to infection was unknown due to our inability to express it as a soluble recombinant protein. Therefore, we also raised antisera to peptides corresponding to AipA amino acids 165 to 182 and 183 to 201. Each AipA peptide antiserum recognized endogenous AipA in lysates of *A. phagocytophilum* infected, but not uninfected HL-60 cells and was specific for the peptide against which it was raised (Figure 7, A and B). Incubating DC organisms with $AipA_{9–21}$ antiserum reduced infection of HL-60 cells by approximately 47% relative to preimmune control serum, an inhibitory effect that was analogous to the reduction achieved using antiserum against $AipA_{1–87}$ or OmpA (Figure 7C). Antisera against each of the other three AipA peptides and $AipA_{249–355}$ had minimal or no inhibitory effect on infection. Thus, the AipA invasion domain is contained within residues 9 to 21.

Discussion

Invasins of obligate intracellular pathogens are dualistic: they are essential for bacterial entry into host cells but, as such, they are also “Achilles’ Heels” that can be blocked to prevent infection and pathogen survival. Targeting invasins of arthropod-transmitted pathogens that are induced during the arthropod blood meal would be an attractive approach because of its potential to prevent not only establishment of infection but also disease transmission. Given that *A. phagocytophilum* infection is predicated on the cooperative actions of multiple bacterial surface-associated invasins (Truchan *et al.*, 2013), most of which are induced during the tick transmission bloodmeal (Ojogun *et al.*, 2012; Kahlon *et al.*, 2013), effective prophylaxis against granulocytic anaplasmosis can potentially be achieved by identifying and targeting these invasins. AipA is an attractive target to include in a multicomponent granulocytic anaplasmosis vaccine. It is an invasin that is induced during tick transmission feeding, is preferentially expressed during the bacterium’s infectious stage, and functions synergistically with OmpA and Asp14 to mediate optimal infection of mammalian host cells.

The exposure of AipA on the infectious DC form surface makes it accessible to blocking antibodies. Indeed, preincubating DC organisms with AipA antiserum significantly reduced infection of HL-60 cells. Pretreatment of DC bacteria with a combination of antibodies targeting AipA, OmpA, and Asp14 nearly abolished infection, whereas pretreatment with antibodies against one or two of the three invasins was less effective. Thus, AipA, OmpA, and Asp14 are collectively critical for infection and targeting all three together blocks infection *in vitro*. Spotted fever group *Rickettsia* species, which are in the Order *Rickettsiales* with *A. phagocytophilum*, also use multiple surface proteins to promote entry into host cells (Martinez et al., 2004; Cardwell *et al.*, 2009; Chan *et al.*, 2009; Chan *et al.*, 2010; Riley *et al.*, 2010). Moreover, this pathogenic strategy is common among numerous other Gram negative bacterial pathogens, including *Chlamydia pneumonia* (Molleken *et al.*,

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2010; Molleken *et al.*, 2013), *Legionella pneumophila* (Garduno *et al.*, 1998; Stone *et al.*, 1998; Cirillo *et al.*, 2001; Chang *et al.*, 2005; Vandersmissen *et al.*, 2010; Duncan *et al.*, 2011), *Bordetella pertussis* (Brennan *et al.*, 1996), *Haemophilus influenzae* (Jurcisek *et al.*, 2007; Giufre *et al.*, 2008; Chang *et al.*, 2011; Dicko *et al.*, 2011; Jalalvand *et al.*, 2013; Singh et al., 2013) and *Leptospira* species (Barbosa *et al.*, 2006; Pinne *et al.*, 2010; Verma *et al.*, 2010; Zhang *et al.*, 2012).

GST-AipA is capable of binding to mammalian cells, which suggests that it functions not only as an invasin but also as an adhesin. Yet, AipA antibodies and GST-AipA$_{1–87}$ each failed to inhibit *A. phagocytophilum* binding to mammalian cells. In these experiments, the role of AipA as an adhesin was presumably masked by the presence of other adhesins/invasins, such as OmpA and Asp14, on the bacterial surface (Ojogun *et al.*, 2012; Kahlon *et al.*, 2013). The AipA receptor is unknown. However, because AipA antibody failed to inhibit *A. phagocytophilum* binding to PSGL-1 CHO cells, it can be concluded that AipA recognizes a sLe^x-capped PSGL-1 independent receptor. AipA and Asp14, which also engages a sLe^x-capped PSGL-1 independent receptor (Kahlon *et al.*, 2013), complement the sLe^x-targeting activity of OmpA (Ojogun *et al.*, 2012).

Bacterial genes that are upregulated during transmission feeding of arthropod vectors are critical for various vector-transmitted bacteria to establish infection in their mammalian hosts (Hinnebusch *et al.*, 1996; Perry *et al.*, 1997; Grimm *et al.*, 2004; Tilly *et al.*, 2006). Consistent with these phenomena, *aipA* is not expressed by *A. phagocytophilum* during its residence in ISE6 cells or *I. scapularis* nymphs, but is induced when the bacterium is cultivated in mammalian tissue culture cells and during tick transmission feeding. Furthermore, *A. phagocytophilum* expresses AipA during infection of humans and dogs. Thus, AipA is dispensable for bacterial colonization of the tick vector, but is important for infecting mammalian hosts. Similar expression profiles have been observed for the other identified invasins OmpA, Asp14, and APH1235 (Mastronunzio *et al.*, 2012; Ojogun *et al.*, 2012; Kahlon *et al.*, 2013). Also, like APH1235 (Troese *et al.*, 2011; Mastronunzio *et al.*, 2012), AipA is pronouncedly upregulated when the bacterium is in the DC stage. In agreement with the invasive role of the DC morphotype, both proteins are important for establishing and/or maintaining infection in mammalian host cells.

How AipA is transported to and associates with the bacterial outer membrane is unclear, as it lacks a canonical signal peptide that would target it for Sec-dependent or twin-arginine secretion. This conundrum is further complicated as AipA is unique to *A. phagocytophilum* and bears no homology to any known crystal structure. Perhaps AipA is an atypical transmembrane protein or a peripheral membrane protein that is anchored to the bacterial outer membrane via a posttranslational modification. AipA colocalizes with the confirmed outer membrane protein, Msp2 (P44) and functions in concert with OmpA and Asp14, both of which have also been shown to colocalize with Msp2 (P44) (Kahlon *et al.*, 2013; Ojogun *et al.*, 2012). Msp2 (P44) has been proposed to form heteromeric complexes that mediate interactions with host cells (Park *et al.*, 2003). Given that AipA, Asp14, and OmpA synergistically promote *A. phagocytophilum* infection of host cells, it will be important to determine if they do so as a multimeric invasin complex that includes Msp2 (P44).

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The AipA invasion domain lies within residues 9 to 21, which is a hydrophilic region of the protein that is exposed on the bacterial surface. Antiserum targeting this span reduces *A. phagocytophilum* infection of host cells by a level comparable to that achieved by antiserum targeting the entire surface-exposed N-terminal domain of AipA. The observed inhibition is specific to anti-$AipA_{9–21}$, as antisera targeting peptides corresponding to all other predicted hydrophilic regions of AipA exhibited no inhibitory effect. Previously, we discovered that the N-terminal ectodomain of OmpA is required for recognition of α2,3-linked sialic acid of sLe^x (Ojogun *et al.*, 2012) and the Asp14 invasion domain lies within residues 101–124 (Kahlon *et al.*, 2013). Currently, we are further delineating the OmpA and Asp14 invasion domains and confirming whether targeting them in concert with $AipA_{9–21}$ effectively blocks *A. phagocytophilum* infection.

Granulocytic anaplasmosis can be debilitating or fatal, and there is no vaccine that protects against the disease. Understanding the *A. phagocytophilum* invasion mechanism would greatly augment development of novel preventative or therapeutic measures. Here, we have demonstrated that AipA is a promising target because it is an invasin that is markedly expressed at key stages in the *A. phagocytophilum* infection cycle and is accessible to blocking antibody. Furthermore, we show for the first time that simultaneously targeting multiple *A. phagocytophilum* invasins effectively blocks infection *in vitro*. Moving forward, it will be important to surmise the efficacy of AipA, OmpA, and Asp14 as vaccinogens for eliciting protection against *A. phagocytophilum* infection *in vivo*.

Experimental Procedures

Cultivation of uninfected and *A. phagocytophilum*-infected host cell lines

ISE6 cells were kindly provided by Ulrike Munderloh of the University of Minnesota. Human promyelocytic HL-60 cells (CCL-240; American Type Culture Collections (ATCC), Manassas, VA), RF/6A (rhesus monkey choroidal endothelial cells, ATCC CRL-1780), ISE6 cells and *A. phagocytophilum* (NCH-1 strain) infected HL-60, RF/6A, or ISE6 cells were cultured as previously described (Huang *et al.*, 2012). PSGL-1 CHO cells and untransfected CHO cells, both of which were provided by Rodger McEver of The Oklahoma Medical Research Foundation, were maintained as previously described (Troese *et al.*, 2009).

In silico analyses of AipA

The AipA sequence was assessed for transmembrane domains using the TMpred and TMHMM algorithms (Hofmann *et al.*, 1993; Krogh *et al.*, 2001), each of which yielded highly similar predictions. Results obtained using TMpred are presented in Figure 1. Protean, which is part of the Lasergene software package (version 8.02; DNASTAR, Madison, WI), was used to assess AipA for regions of hydrophobicity and probability of being surface-exposed using the Kyte-Doolittle (Kyte *et al.*, 1982) and Emini (Emini *et al.*, 1985) algorithms, respectively.

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Recombinant protein and antiserum production

A PCR amplicon of *aipA* (*aph0915*) nucleotides 1 to 261, encoding AipA amino acids 1 to 87, was generated using primers 5′-**CACC**TTGAGTTTTACAATGTCGAAGTTATCGC-3′ (nucleotides in bold text correspond to a Gateway entry vector-compatible sequence) and 5′-CTATCCTAGCATCCTTCTAGAAGCGGAAG-3′ (underlined nucleotides denote an added stop codon). A PCR product corresponding to *aipA* nucleotides 745 to 1068, encoding AipA amino acids 249 to 355, was generated using primers 5′-**CACC**ATCTATCAAGGAAATTACGAAGATCGCAAC-3′ and 5′-GAGCAGCATGCTTTA-3′. The amplicons were cloned into the pDest-15 vector (Life Technologies, Carlsbad, CA) downstream of and in frame with the gene encoding GST as described previously (Ojogun *et al.*, 2012). Expression and purification GST-tagged AipA residues 1 to 87 (GST-AipA$_{1–87}$), 249 to 355 (GST-AipA$_{249–355}$), and GST alone were performed as previously described (Troese *et al.*, 2011). GST-tagged full-length AipA and AipA amino acids 165 to 204 remained insoluble over a wide range of conditions and thus could not be purified. Generation of murine polyclonal antisera against each GST fusion protein was performed as described previously (Troese *et al.*, 2011). Rabbit polyclonal antisera were raised against synthetic keyhole limpet hemocyanin (KLH)-conjugated peptides corresponding to AipA amino acid residues 9 to 21, 61 to 84, 165 to 182, and 185 to 201 (New England Peptide, Gardner, MA). Specificity of each AipA peptide antiserum for its target peptides was determined by the enzyme-linked immunosorbent assay using the TMB substrate kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions.

Differential aipA expression studies

HL-60 cells were synchronously infected with *A. phagocytophilum* DC organisms (Troese *et al.*, 2009). The infection time course was allowed to proceed for 36 h, a time period that enabled the bacteria to complete their biphasic developmental cycle and initiate a second round of infection (Troese *et al.*, 2009). RNA isolated from aliquots taken every 4 hours was subjected to reverse transcriptase-quantitative PCR (RT-qPCR) as described previously (Troese *et al.*, 2011) using AipA specific primers 5′-CCTCAACTAAAGAAGCGTCATCAAA-3′ and 5′-GTACGGTGTACAAAACGAGGAACA-3′, which targeted nucleotides 179 to 388. Relative *aipA* transcript levels were normalized to the transcript levels of the *A. phagocytophilum* 16s rRNA gene (*aph1000*) using the $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001; Kahlon *et al.*, 2013). To determine if *aipA* was transcriptionally upregulated in the DC versus RC morphotype, normalized *aipA* transcript levels were calculated as fold changes in expression relative to expression at 16 h, a time point at which the entire *A. phagocytophilum* population existed in the RC form (Troese *et al.*, 2009; Mastronunzio *et al.*, 2012). *aipA* expression during blood meal acquisition by *A. phagocytophilum* infected nymphs from mice was monitored as described (Mastronunzio *et al.*, 2012) using AipA primers targeting nucleotides 179 to 388. As a control, expression of the *A. phagocytophilum groEL* gene (*aph0240*) during tick transmission feeding was monitored using gene-specific primers (Kahlon *et al.*, 2013).

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Western blotting and confocal microscopy

Antisera generated in this study and prior studies targeted AipA, APH0032 (Huang *et al.*, 2010b), Asp55 (Ge *et al.*, 2007), and Msp2 (P44) (Troese *et al.*, 2011). Sera from an HGA patient and a dog that had been naturally infected with *A. phagocytophilum* were previously described (Ojogun *et al.*, 2012) and provided by Dr. Janet Foley of The University of California-Davis, respectively. Western blot analyses (Troese *et al.*, 2011) were performed on uninfected or *A. phagocytophilum* infected host cells or *A. phagocytophilum* DC organisms that had been subjected to surface trypsinolysis as described previously (Kahlon *et al.*, 2013). *A. phagocytophilum* infected host cells were analyzed by spinning disk confocal microscopy as described (Huang *et al.*, 2010a; Huang *et al.*, 2012).

AipA antiserum inhibition of *A. phagocytophilum* infection

Inhibition of host cell infection by preincubating DC organisms with heat-inactivated polyclonal antiserum targeting GST-AipA$_{1–87}$, GST-AipA$_{249–355}$, GST-OmpA (Ojogun, 2012), GST-Asp14 (Kahlon *et al.*, 2013), AipA$_{9–21}$, AipA$_{61–84}$, AipA$_{165–182}$, or AipA$_{183–201}$ (2 mg mL^{-1}) was assessed as previously described (Ojogun *et al.*, 2012). Serum against GST alone and preimmune serum served as negative controls. In instances where DC bacteria were incubated with combinations of antisera targeting AipA, Asp14, and/or OmpA, each respective antiserum was at a concentration of 2 mg mL^{-1}, and control antiserum was matched accordingly. Following antibody treatment, bacterial adhesion to and infection of HL-60 cells were monitored using spinning disk confocal microscopy (Ojogun *et al.*, 2012).

Binding of GST-AipA to mammalian host cells and competitive inhibition of *A. phagocytophilum* infection

Mammalian host cells cells were incubated with 4 μM GST, GST-AipA$_{1–87}$, or GST-AipA$_{249–355}$ for 1 h at 37°C. Spinning disk confocal microscopy and flow cytometry were used to assess the binding of recombinant proteins to host cells and competitive inhibition of *A. phagocytophilum* infection as previously described (Ojogun *et al.*, 2012; Kahlon *et al.*, 2013).

Assessment of the relevance of AipA to *A. phagocytophilum* adherence to PSGL-1 CHO cells

To determine if AipA was important for *A. phagocytophilum* recognition of sLe^x-capped PSGL-1, DC organisms were incubated with antiserum targeting AipA$_{1–87}$, AipA$_{249–355}$, OmpA (Ojogun *et al.*, 2012), or preimmune control serum as described above. Next, the treated bacteria were incubated with PSGL-1 CHO cells or untransfected CHO cells for 1 h, followed by two rounds of washing with PBS to remove unbound bacteria, and enumeration of bound organisms using spinning disk confocal microscopy as described (Troese *et al.*, 2009). As positive controls for inhibition of bacterial adherence to sLe^x-capped PSGL-1, PSGL-1 CHO cells were incubated with the PSGL-1 N-terminus-specific antibody, KPL-1 (BD Biosciences, San Jose, CA), or the sLe^x-specific antibody, CSLEX1 (BD Biosciences) for 30 min prior to the addition of bacteria. Mouse IgG and mouse IgM served as isotype controls for KPL-1 and CSLEX1, respectively.

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Statistical analyses

One-way analysis of variance (ANOVA) was performed using the Prism 5.0 software package (Graphpad; San Diego, CA) to assess statistical significance as described (Ojogun *et al.*, 2012). Statistical significance was set at $P < 0.05$.

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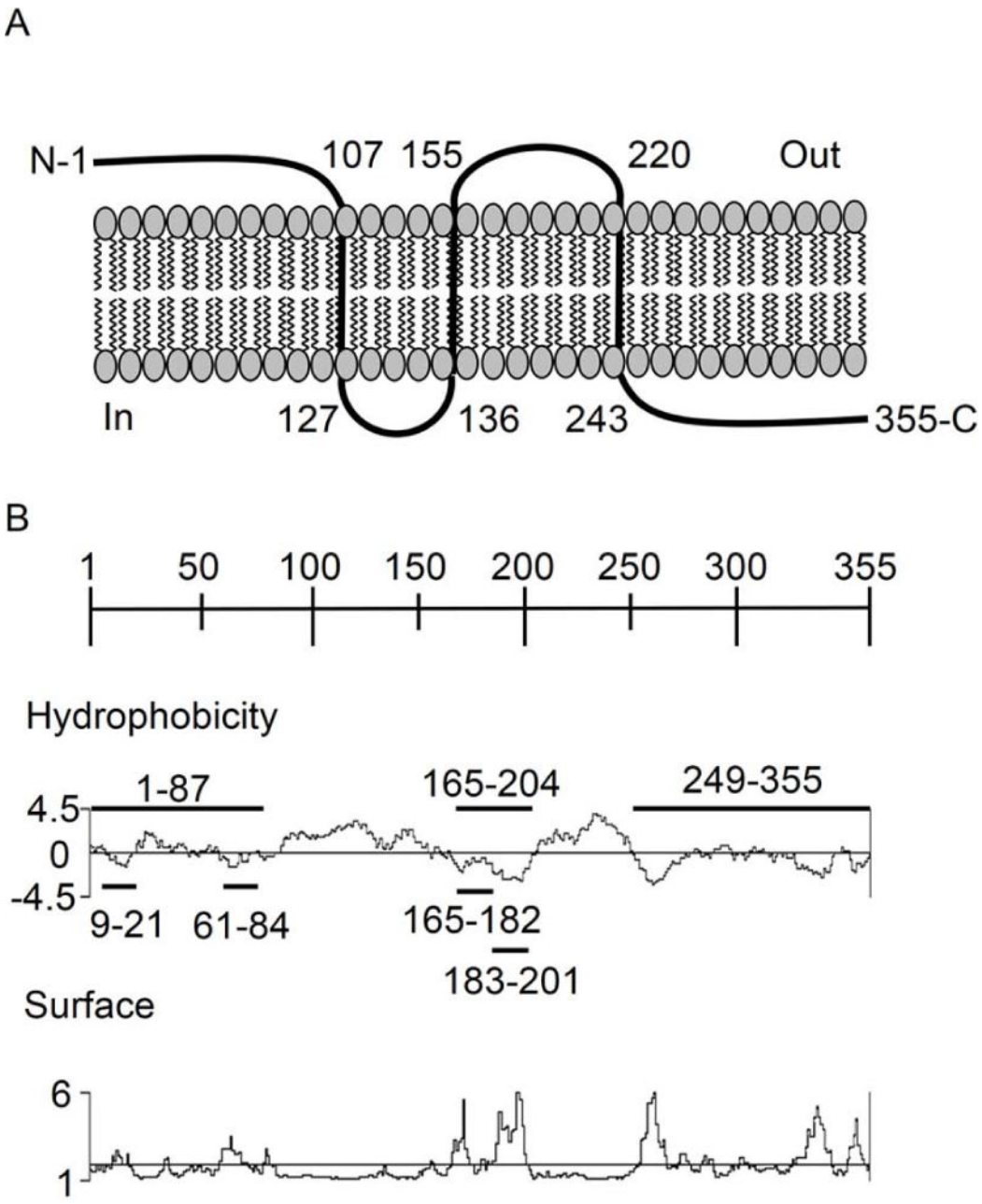

Figure 1. Schematic diagrams of *A. phagocytophilum* AipA membrane topology and sequence

(A) Diagram of the predicted topology of AipA in the *A. phagocytophilum* outer membrane. N, AipA amino terminus. C, AipA carboxy terminus. Numerical values indicate amino acid coordinates for predicted transmembrane spanning regions. (B) Diagrams of the AipA sequence. The scale indicates 50-amino acid intervals. For the Hydrophobicity diagram, the Kyte-Doolitle algorithm was used to determine hydrophobic (histogram above the axis) and hydrophilic (histogram below axis) regions. For the Surface diagram, the Emini algorithm was used to determine regions that are likely accessible on the surface of the AipA (histogram above the axis) or not (histogram below the axis). The AipA amino acid

segments against which antisera were raised are indicated on the Hydrophobicity plot by horizontal lines.

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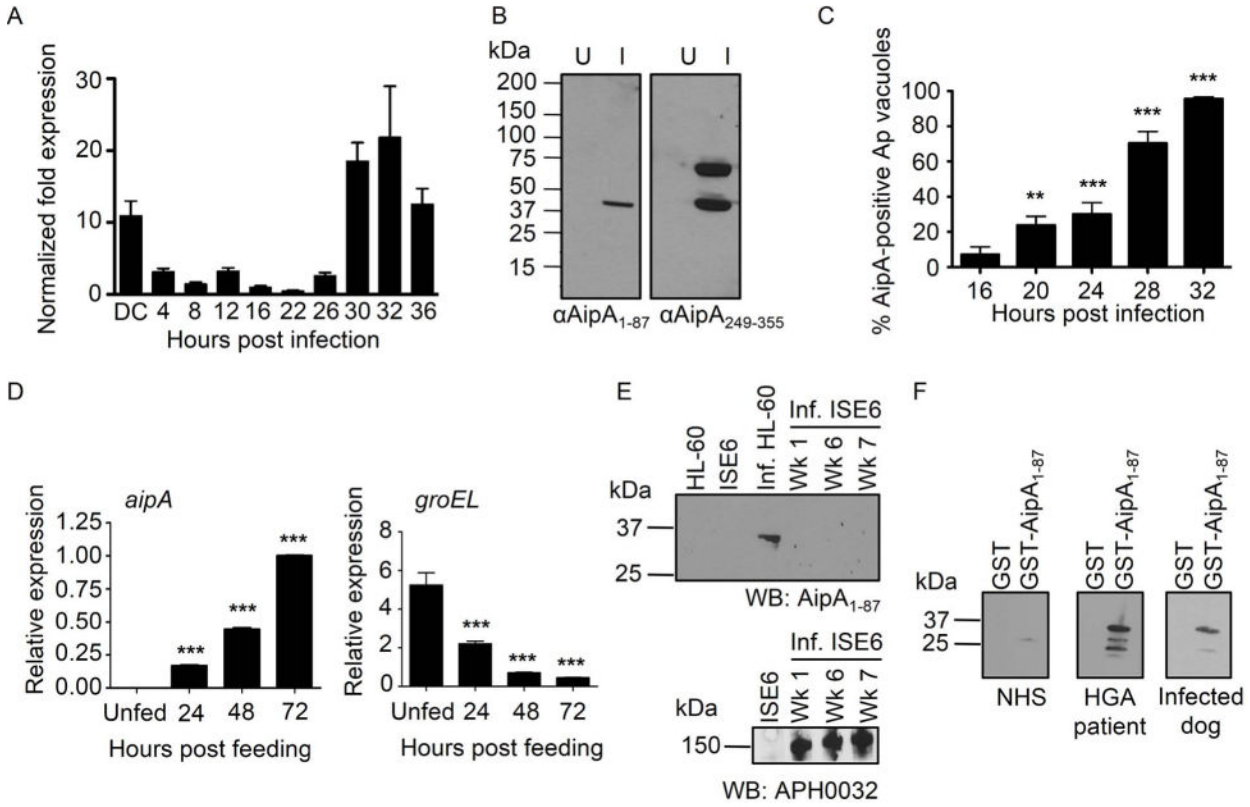

Figure 2. Differential expression profiling of AipA throughout the *A. phagocytophilum* life cycle

(A) aipA transcriptional profile during *A. phagocytophilum* infection of HL-60 cells. DC bacteria were incubated with HL-60 cells to establish a synchronous infection. Total RNA isolated from the DC inoculum and infected host cells at several postinfection time points was subjected to reverse transcriptase-quantitative PCR (RT-qPCR). Relative *aipA* transcript levels were normalized to *A. phagocytophilum* 16s rRNA gene transcript levels. To determine relative *aipA* transcription between RC and DC organisms, normalized *aipA* transcript levels per time point were calculated as the fold change in expression relative to expression at 16 h, a time point at which the entire bacterial population is in the DC morphotype. Data are the means ± standard deviations (SD) for triplicate samples and are representative of two experiments having similar results. (B) Western blot screening of whole-cell lysates of uninfected (U) and *A. phagocytophilum* infected HL-60 cells (I) using mouse antiserum raised against GST-AipA$_{1–87}$ (αAipA$_{1–87}$) and GST-AipA$_{249–355}$ (αAipA$_{249–355}$). (C) AipA expression over the course of infection of mammalian host cells. RF/6A cells that had been synchronously infected with *A. phagocytophilum* (Ap) were screened with antibodies targeting Msp2 (P44) (to denote all *A. phagocytophilum* inclusions) and AipA viewed by confocal microscopy. Data presented are the mean percentages ± SD of Msp2 (P44)-positive A. *phagocytophilum* inclusions that were also AipA-positive. At least 100 bacterial inclusions were scored per time point. (D) *aipA* and *groEL* expression during transmission feeding of *A. phagocytophilum* infected ticks on naïve mice. *A. phagocytophilum*-infected I. *scapularis* nymphs were allowed to feed on mice for 72 h. Total RNA recovered from unfed and transmission-fed ticks that had been removed at 24, 48, and 72 h postattachment was subjected to RT-qPCR. Relative *aipA* and *groEL* transcript

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levels were normalized to A. *phagocytophilum* 16S rRNA gene levels. (E) AipA is not expressed during *A. phagocytophilum* infection of a tick cell line. Western blot analysis of uninfected and *A. phagocytophilum* infected (Inf.) HL-60 and ISE6 cells using antiserum specific for $AipA_{1–87}$ or APH0032. The number of weeks (Wk.) during which A. *phagocytophilum* was maintained in ISE6 cells are indicated. (F) AipA is expressed *in vivo* and elicits a humoral immune response. Western blot analysis of GST and GST-$AipA_{1–87}$ screened with sera from an HGA patient and from an *A. phagocytophilum* infected dog. Results presented in panels B to F are each representative of at two to three independent experiments with similar results. Statistically significant (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$) values are indicated.

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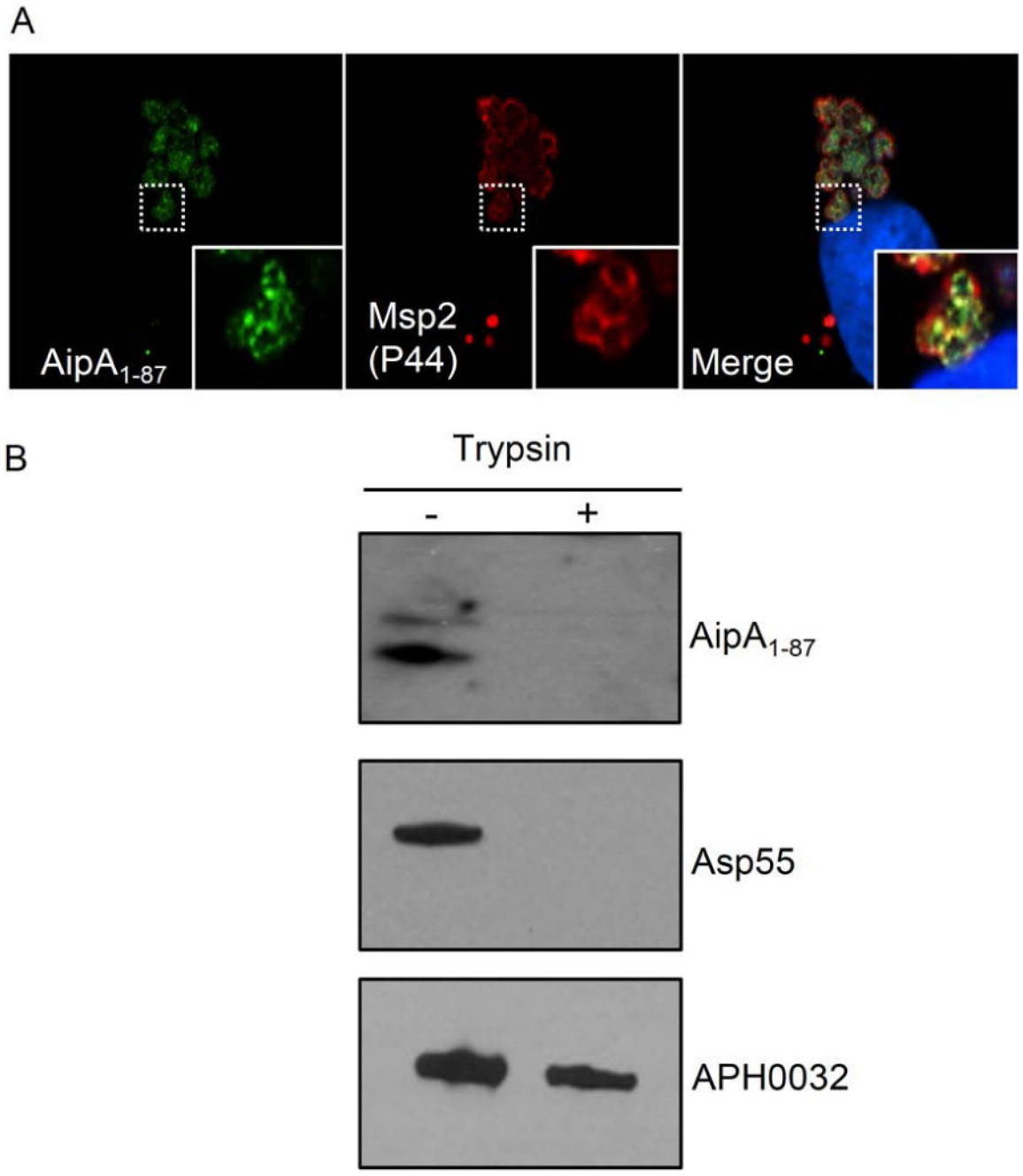

Figure 3. AipA is an *A. phagocytophilum* OMP

(A) AipA colocalizes with the confirmed OMP, Msp2 (P44). *A. phagocytophilum*-infected RF/6A cells were fixed and viewed by confocal microscopy to assess immunoreactivity with AipA antiserum (green) in conjunction with Msp2 (P44) antiserum (red). Host cell nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole; blue). The insets demarcated by solid boxes in the lower right corners of each panel are magnified versions of the representative *A. phagocytophilum*-occupied vacuole that is denoted by the hatched box in each panel. (B) AipA is exposed on the bacterial surface. Intact *A. phagocytophilum* DC organisms were incubated with trypsin or vehicle control, solubilized, and Western-blotted.

Immunoblots were screened with antiserum targeting $AipA_{1–87}$, Asp55, or APH0032. Data are representative of two experiments with similar results.

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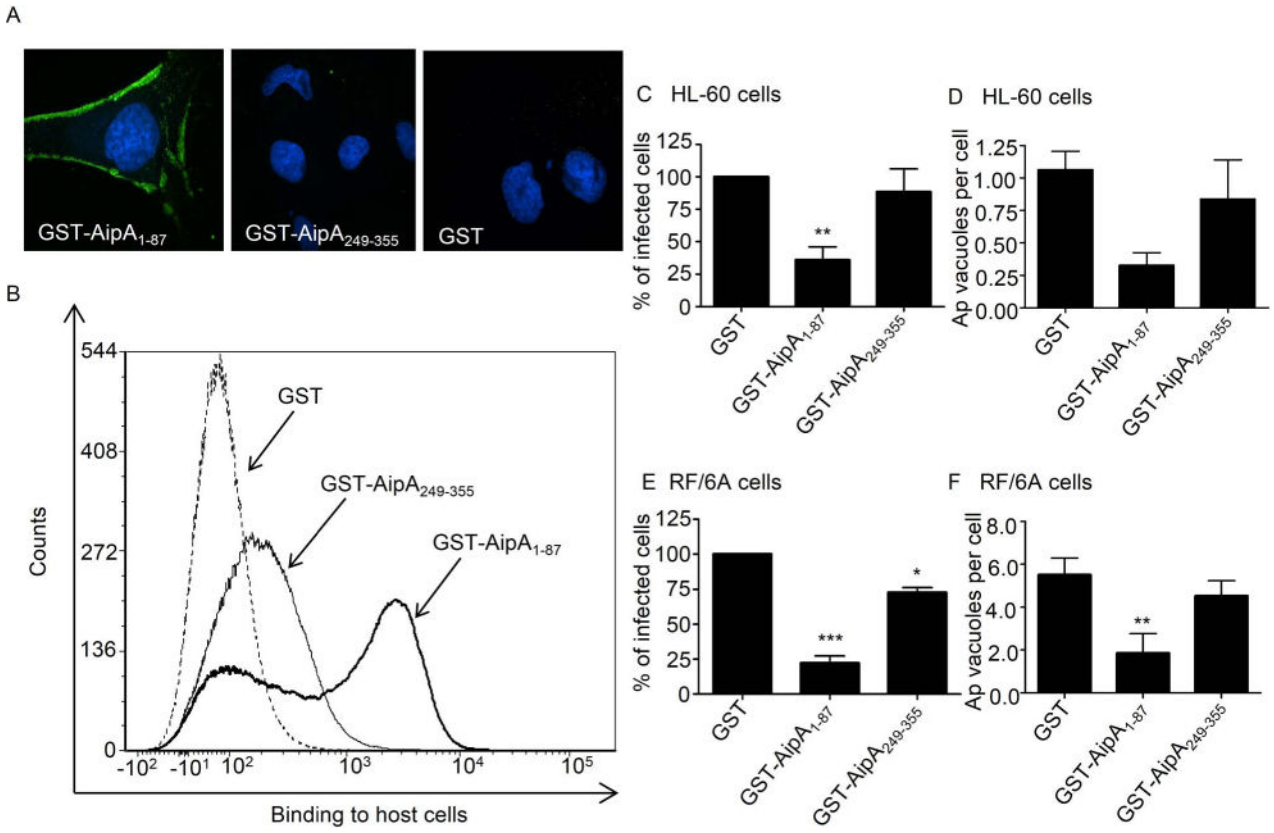

Figure 4. GST-AipA requires amino acids 1 to 87 to bind and competitively inhibit *A. phagocytophilum* infection of mammalian host cells

(A and B) GST-AipA$_{1–87}$ binds to mammalian host cells. RF/6A cells were incubated with GST-AipA$_{1–87}$, GST-AipA$_{249–355}$, or GST alone. (A) The host cells were fixed, screened with GST antibody (green), and examined using confocal microscopy. Host cell nuclei were stained with DAPI. Representative merged fluorescent images from three experiments with similar results are shown. (B) Flow cytometric analysis of GST fusion protein binding to RF/6A cells. (C to F) GST-AipA$_{1–87}$ competitively inhibits *A. phagocytophilum* infection. HL-60 (C and D) and RF/6A cells (E and F) were incubated with DC bacteria in the presence of GST, GST-AipA$_{1–87}$, or GST-AipA$_{249–355}$ for 1 h. Following removal of unbound bacteria, host cells were incubated for 24 h (C and D) or 48 h (E and F) and subsequently examined using confocal microscopy to assess the percentage of infected cells (C and E) or the mean number (± SD) of pathogen-occupied vacuoles per cell (D and F). Results shown are relative to GST-treated host cells and are the means ± SD for three experiments. Statistically significant (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$) values are indicated.

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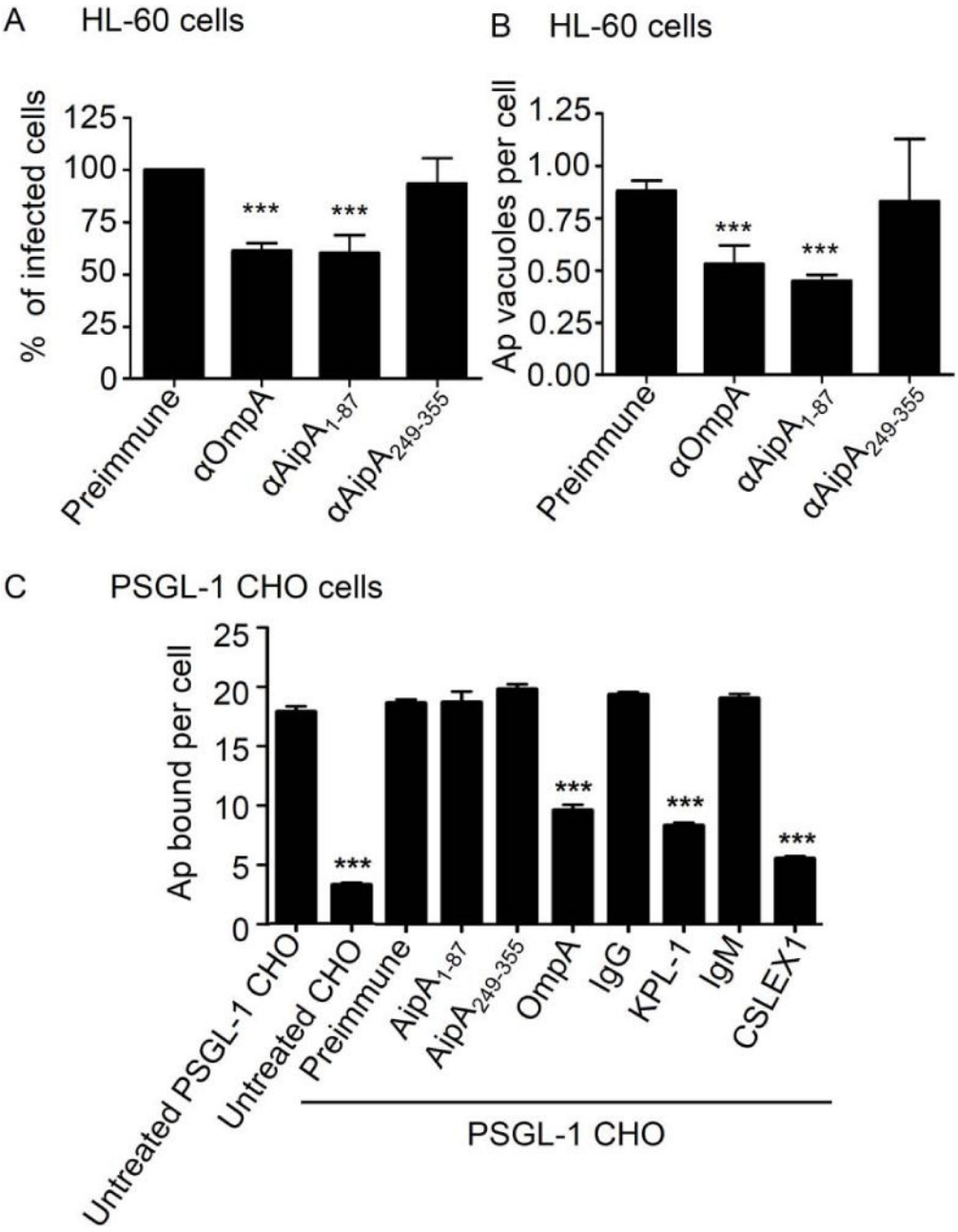

Figure 5. Pretreatment of *A. phagocytophilum* with $AipA_{1–87}$ antiserum inhibits infection of HL-60 cells but does not alter binding to sLe^x-capped PSGL-1

A. phagocytophilum DC organisms were exposed to antiserum targeting $AipA_{1–87}$, $AipA_{249–355}$, OmpA, or preimmune serum and then incubated with HL-60 (A and B), PSGL-1 CHO cells, or untransfected CHO cells (C). The infection of HL-60 cells was allowed to proceed for 24 h prior to being assessed, while bacterial binding to PSGL-1 CHO cells was assessed immediately. The mean ± standard deviations of percentages of infected HL-60 cells (A), *A. phagocytophilum* (Ap) vacuolar inclusions per HL-60 cell (B), and bound DC organisms per PSGL-1 CHO cell or untransfected CHO cell (C) were determined using immunofluorescence microscopy. Additional positive controls for blocking *A.*

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phagocytophilum to PSGL-1 CHO cells, besides incubating bacteria with OmpA antiserum, were PSGL-1 CHO cells that had been incubated with PSGL-1 N-terminus blocking antibody KPL-1 or sLe^x-blocking antibody CSLEX1 prior to the addition of bacteria. Negative controls were PGSL-1 CHO cells that had been incubated with isotype control antibodies prior to the addition of bacteria. Results shown are relative to GST-treated host cells and are the means ± SD for three experiments. Statistically significant (***, $P < 0.001$) values are indicated.

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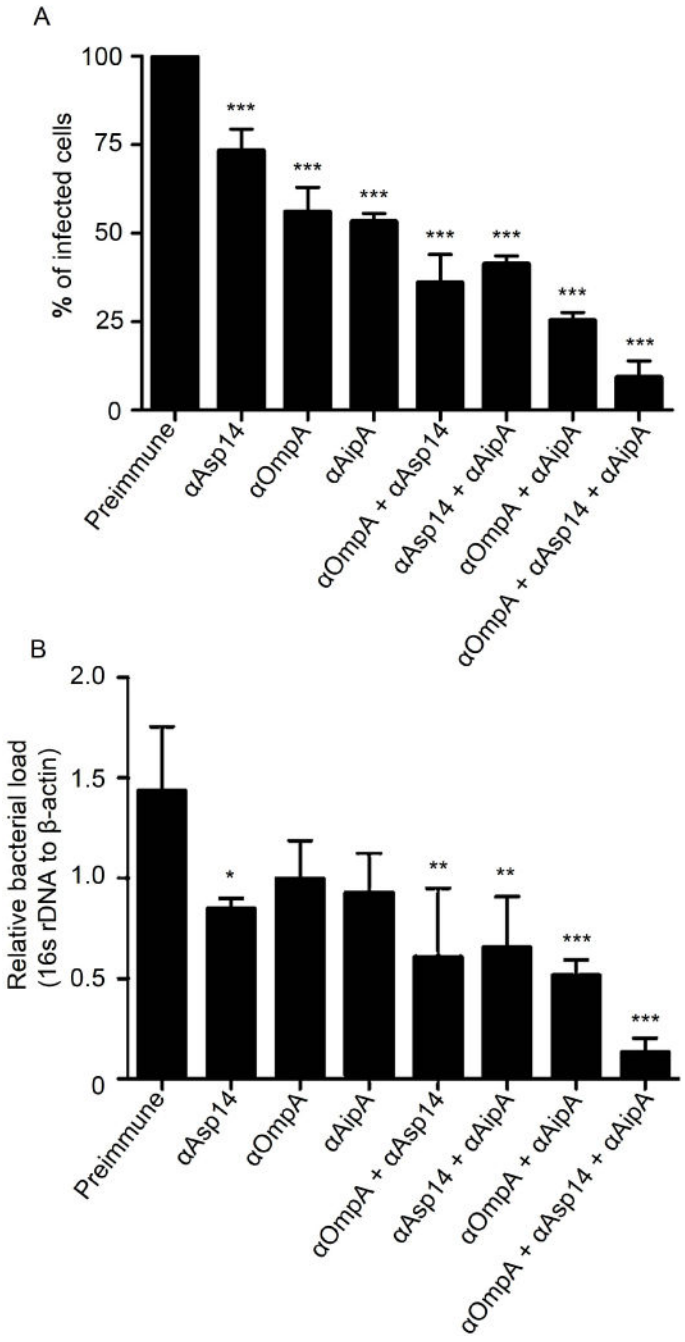

Figure 6. A combination of antisera targeting AipA, OmpA, and Asp14 blocks *A. phagocytophilum* infection of host cells

DC bacteria were incubated with preimmune serum or antiserum targeting $AipA_{1–87}$, OmpA, and/or Asp14 and then incubated with HL-60 cells. (A) The cells were fixed and screened by confocal microscopy to assess the percentage of infected cells. Results shown are relative to host cells that had been treated with preimmune serum and are the means ± SD for three experiments. (B) DNA isolated from the cells was subjected to quantitative PCR analyses. Relative DNA loads of *A. phagocytophilum* 16s rRNA gene were normalized to DNA loads of the human β-actin gene. Results shown are the means ± SD of triplicate

samples and are representative of three independent experiments with similar results. Statistically significant (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$) values relative to the bacterial load of host cells that had been incubated with preimmune antisera are presented.

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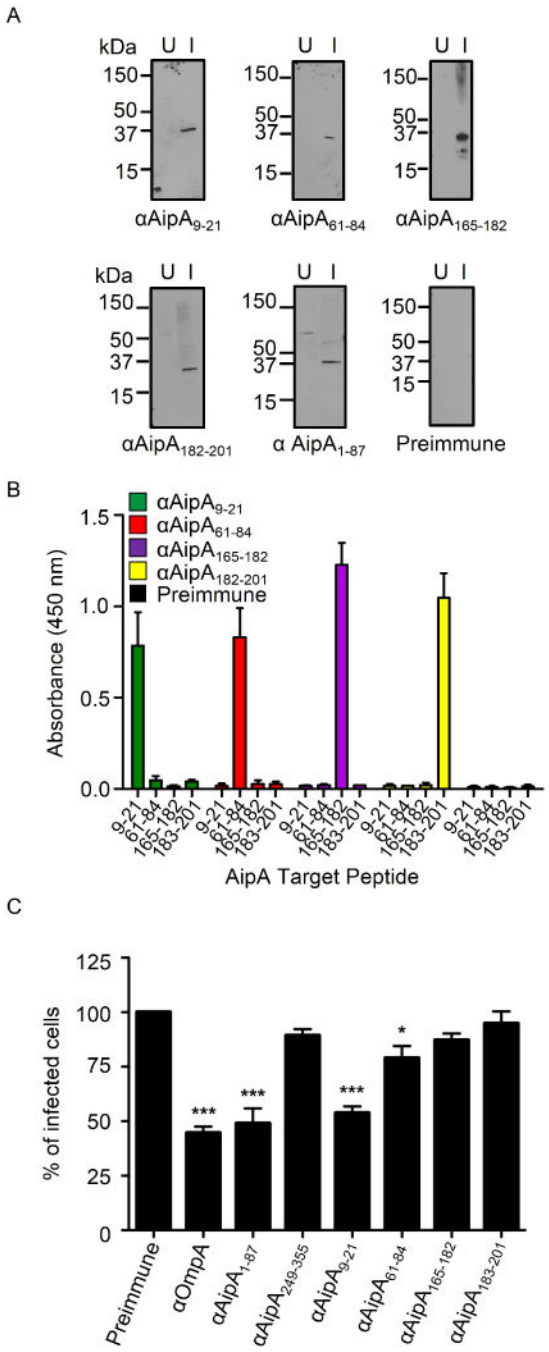

Figure 7. AipA residues 9–21 are critical for establishing infection in host cells

(A) Western blot analyses in which rabbit antiserum targeting $AipA_{9–21}$, $AipA_{61–84}$, $AipA_{165–182}$, $AipA_{183–201}$, $AipA_{1–87}$, or preimmune rabbit serum was used to screen whole cell lysates of uninfected (U) and *A. phagocytophilum* infected HL-60 cells (I). Data are representative of two experiments with similar results. (B) ELISA in which $AipA_{9–21}$, $AipA_{61–84}$, $AipA_{165–182}$, and $AipA_{183–201}$ antibodies were used to screen wells coated with peptides corresponding to AipA residues 9–21, 61–84, 165–182 and 183–201. Each antiserum only recognized the peptide against which it had been raised. Results shown are

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the mean (± SD) of triplicate samples. Data are representative of three experiments with similar results. (C) Pretreatment of *A. phagocytophilum* with $AipA_{9–21}$ antiserum inhibits infection of HL-60 cells. DC bacteria were pretreated with antiserum specific for $AipA_{9–21}$, $AipA_{61–84}$, $AipA_{165–182}$, $AipA_{183–201}$, $AipA_{1–87}$, $AipA_{249–355}$, OmpA, or preimmune serum for 30 min. Next, the treated bacteria were incubated with HL-60 cells for 60 min. After removal of unbound bacteria, host cells were incubated for 24 h and subsequently examined using Msp2 (P44) antibody and confocal microscopy to assess the percentage of infected cells. Results shown are relative to preimmune serum-treated host cells and are the means ± SD for six experiments. Statistically significant (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$) values are indicated.