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Expression of *Anaplasma marginale* Ankyrin Repeat-Containing Proteins during Infection of the Mammalian Host and Tick Vector▽†

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Transmission of tick-borne pathogens requires transition between distinct host environments with infection and replication in host-specific cell types. *Anaplasma marginale* illustrates this transition: in the mammalian host, the bacterium infects and replicates in mature (nonnucleated) erythrocytes, while in the tick vector, replication occurs in nucleated epithelial cells. We hypothesized that proteins containing ankyrin motifs would be expressed by *A. marginale* only in tick cells and would traffic to the infected host cell nucleus. *A. marginale* encodes three proteins containing ankyrin motifs, an AnkA orthologue (the AM705 protein), AnkB (the AM926 protein), and AnkC (the AM638 protein). All three *A. marginale* Anks were confirmed to be expressed during intracellular infection: AnkA is expressed at significantly higher levels in erythrocytes, AnkB is expressed equally by both infected erythrocytes and tick cells, and AnkC is expressed exclusively in tick cells. There was no evidence of any of the Ank proteins trafficking to the nucleus. Thus, the hypothesis that ankyrin-containing motifs were predictive of cell type expression and nuclear localization was rejected. In contrast, AnkA orthologues in the closely related *A. phagocytophilum* and *Ehrlichia chaffeensis* have been shown to localize to the host cell nucleus. This difference, together with the lack of a nuclear localization signal in any of the AnkA orthologues, suggests that trafficking may be mediated by a separate transporter rather than by endogenous signals. Selection for divergence in Ank function among *Anaplasma* and *Ehrlichia* spp. is supported by both locus and allelic analyses of genes encoding orthologous proteins and their ankyrin motif compositions.

Tick-borne pathogens in the genera *Anaplasma* and *Ehrlichia* must invade and replicate in two very distinct environments, hematopoietic cells within a mammalian host and both midgut and salivary gland cells within the arthropod vector. We, and others, have hypothesized that this transition between hosts requires expression of unique proteomes (11, 19, 23, 27). This is supported by proteomic approaches, unbiased as to location or function, which identified both marked upregulation and unique expression of bacterial proteins in the tick vector relative to the mammalian host (23, 27). In our recent study using *Anaplasma marginale*, all 15 proteins shown to be upregulated in tick cells had been originally annotated as hypothetical proteins, consistent with the significant percentage of proteins of unknown function in the genera *Anaplasma* and *Ehrlichia* (1–3, 5, 8, 13, 17, 23). A second approach to discovery of proteins upregulated or uniquely expressed in the tick vector is predictive, based on specific differences between the host environments and cell types. For *A. marginale*, a striking difference is that between the infection of nonnucleated cells in the mammalian host and the infection of nucleated cells in the tick vector. Unlike most other members of the genera *Anaplasma* and *Ehrlichia*, which infect nucleated hematopoietic cells, *A. marginale* invades and replicates in mature erythrocytes in the mammalian host (4). Upon acquisition by a feeding tick, *A. marginale* invades and replicates in, sequentially, midgut and salivary gland epithelial cells (9, 28, 29), a progression common among the tick-borne *Anaplasma* and *Ehrlichia* spp. Consequently, we proposed that while bacterial proteins that localize to the host cell nucleus during intracellular infection would be expressed in both the mammalian and tick cell environments for most bacteria in these two genera, *A. marginale* would express these proteins only in the tick vector.

Two orthologous ankyrin repeat-containing proteins have been shown to traffic to the host cell nucleus during infection: they are *E. chaffeensis* p200 and *A. phagocytophilum* AnkA. *E. chaffeensis* p200 localizes to the nucleus and binds Alu-Sx DNA motifs (32). *A. phagocytophilum* AnkA similarly localizes to the host cell nucleus, binds chromatin-regulatory regions, and downregulates cytochrome B-245 (*CYBB*) and other host defense genes (10, 12, 22). The AnkA-mediated downregulation of host defense genes is consistent with *A. phagocytophilum* survival not only in mammalian neutrophils but also in the phagocytic midgut epithelial cells of ticks. In contrast, an *A. marginale* AnkA orthologue would be expected to be dispensable for survival and replication in the mature erythrocytes of the mammalian host and thus specifically expressed in the tick vector.

In the present study, we tested whether the *A. marginale* AnkA orthologue is uniquely expressed or significantly upregu-

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lated in the cells of the tick vector and determined if AnkA localized to the nucleus of tick cells. In addition, we screened the *A. marginale* genome for additional ankyrin repeat-containing proteins as candidates for host cell nuclear localization and global regulators and tested whether these localized to the nucleus and were specifically expressed in the tick vector. We present the results of these studies and discuss the findings in the context of the pathogen-host vector interaction.

MATERIALS AND METHODS

Identification and conservation of *A. marginale* genes encoding ankyrin repeat motifs. We identified *A. marginale* ankyrin repeat motif-encoding genes by using two approaches. First, the NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/cdd) was searched for ankyrin repeat domains in all sequenced *A. marginale* strains, and second, the genomic architecture analysis of SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1) was used to search for ankyrin repeat domains in all sequenced *Anaplasma* and *Ehrlichia* species (26). Ankyrin domain-containing genes were then BLAST searched against genome sequences to determine whether a homologue was present in each of the strains used in the study. The strains examined included *A. marginale* St. Maries (CP000030) and Florida (CP001079), *A. marginale* subsp. *centrale* (CP001759), *A. phagocytophilum* HZ (CP000235), *E. ruminantium* Gardel (CR925677), Welgevonden (CR767821), *E. chaffeensis* Arkansas (CP000236), and *E. canis* Jake (CP000107).

The searches identified the same three *A. marginale* genes, each containing multiple ankyrin domain repeats: AM705 with 10 ankyrin repeat domains, AM926 with 2 ankyrin repeats, and AM638 with 9 ankyrin repeats. The conservation of these genes and the encoded proteins among *A. marginale* strains was determined by alignment using ClustalW (AlignX; Invitrogen) for the completely sequenced *A. marginale* St. Maries and Florida (1, 3) and for *A. marginale* subsp. *centrale* (13). The Mississippi, Puerto Rico, and Virginia strains of *A. marginale* had been pyrosequenced previously (3). The Puerto Rico AnkA was in the pyrosequenced data (GenBank accession NZ_ABOQ01000029; locus tag AmarPR_010100002855). AnkA was missing in the pyrosequence data for the Mississippi and Virginia strains, and AnkB and AnkC were missing for all three strains; these genes were specifically amplified, amplicons cloned into pCR4-TOPO vector (Invitrogen) and sequenced using the M13 forward primer and the BigDye version 3.1 cycle sequencing kit from Applied Biosystems with an ABI 3130XL Genetic Analyzer. AM705 and AM638 were amplified, cloned, and sequenced in 10 and 25 overlapping segments, respectively. To control for errors in amplification and sequencing, the St. Maries strain was handled identically and the sequences for each gene compared to the reference complete genome sequence (CP000030), which was based on a bacterial artificial chromosome (BAC) approach (1).

Quantitative expression levels of ankyrin repeat-containing proteins in infected mammalian and tick cells. The expression of the three *A. marginale* ankyrin repeat-bearing proteins in the different host environments was determined using quantitative Western blots (23). Briefly, a large region of each open reading frame was expressed as a recombinant protein and the purified protein used to immunize mice to generate specific monoclonal antibodies for quantitative detection of each protein in infected cells. Predicted B-cell epitope-bearing regions of each gene were identified (http://tools.immuneepitope.org) and the encoding open reading frames amplified using the following primer sets: for AM705 (2,100-bp fragment), 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGATGACGATACACCATTG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTACCAGCCTCTGGACAGGTA-3′; for AM926 (750-bp fragment), 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGACTTTCTTTTGGGAGAAGTAA-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTACCCCTCTTGTTCTTCTTC-3′; and for AM638 (1,650-bp fragment), 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCCTCACACTGCGACATA-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTATTCGCGTGCAGCGTCTTC-3′. The PCR cycling conditions were 10 cycles of melting at 94°C for 30 s and annealing at 35°C (AM705), 41°C (AM926), or 39°C (AM638) for 30 s with extension at 72°C for 2 min (AM705 and AM638) or 1 min (AM926), followed by 25 cycles of melting at 94°C for 30 s and annealing at 70°C for 30 s and extension at 72°C for 2 min (AM705 and AM638) or 1 min (AM926). Cloning and expression utilized the Gateway expression system (Invitrogen), and the recombinant His-tagged fusion proteins were affinity purified as previously described (23).

Monoclonal antibodies were generated by immunizing mice with recombinant proteins followed by hybridoma fusion and limiting dilution cloning as previously described (23). Briefly, 50 μg of each recombinant protein emulsified in Titermax Gold adjuvant (CytRx) was used to immunize mice subcutaneously. Three days prior to hybridoma fusion, mice were boosted intravenously with 50 μg of protein without adjuvant. Hybridoma supernatants were screened by Western blotting for reactivity by using the St. Maries strain of *A. marginale* isolated from infected ISE6 cells or infected erythrocytes. Quantitative Western blotting was performed by first normalizing *A. marginale* organisms isolated from infected ISE6 cells and infected erythrocytes by using two independent methods. First, the number of bacteria was quantified by real-time PCR based on the single-copy *msp5* gene, as described previously (9), and lysates from equal numbers (10^7) of bacteria from each host cell were loaded. Second, the Western blots were probed with monoclonal antibody ANAF16C1, reactive with Msp5, as an internal standard (30). Msp5 is constitutively expressed at a high level in both infected mammalian erythrocytes and infected ISE6 cells (23). Uninfected ISE6 cells and uninfected erythrocytes were used as negative antigen controls. The proteins were resolved by electrophoresis using 4 to 20% precast polyacrylamide gels (Bio-Rad). After transferring the proteins to a nitrocellulose membrane, we probed for the expression of AM705, AM926, and AM638 proteins with monoclonal antibodies 149/312, 148/42.17, and 150/103, respectively, and antibody binding was detected by using the Western Star chemiluminescence system (Applied Biosystems). An isotype-matched monoclonal antibody TRYP1E1 (reactive with a *Trypanosoma brucei* protein) was used as a negative antibody control.

***In situ* expression of ankyrin repeat-containing proteins in *Dermacentor andersoni*.** Ticks infected with the St. Maries strain were used to detect *in situ* expression of all three ankyrin repeat-containing proteins by immunohistochemistry. Briefly, a calf that was Msp5 seronegative by competitive enzyme-linked immunosorbent assay (C-ELISA) was inoculated intravenously with the St. Maries strain (7, 14). Male *D. andersoni* ticks were acquisition fed on the calf for 7 days during acute infection (bacteremia of $\geq 10^8$ *A. marginale* organisms per ml) (9). The ticks were removed and incubated at 26°C and 96% relative humidity for an additional 7 days to allow complete digestion of the blood meal and replication in the midgut epithelium, followed by transmission feeding on a second seronegative calf for 7 days. Upon removal, the ticks were immediately fixed in 10% formaldehyde and later embedded in paraffin. Immunohistochemistry was performed as previously described (28) on serial 4-μm deparaffinized sections of the ticks by using 15 μg of each monoclonal antibody/ml. Uninfected ticks treated identically were used as negative antigen controls. An isotype-matched monoclonal antibody, TRYP1E1 (reactive with a *T. brucei* protein), was used as a negative antibody control. Binding was detected with horseradish peroxidase-labeled anti-mouse antibody (Dako), and Mayer's hematoxylin was used as a counterstain.

Subcellular localization of ankyrin repeat-containing proteins. Confluent ISE6 cells were inoculated with the St. Maries strain and monitored by microscopic examination of cytospin preparations (stained with Giemsa stain) until 80 to 90% of cells were infected. The cells were fixed with 10% formaldehyde and incubated overnight at room temperature. After centrifugation at 5,000 × *g* for 2 min, half the supernatant was removed and the cells were resuspended in an equal volume of 0.2% agarose. The cells were then paraffin embedded and serial 4-μm sections processed as described for immunohistochemical staining with the following modifications. After antigen retrieval, the sections were blocked by applying four drops of Image-IT FX signal enhancer (Invitrogen) and incubated for 30 min at room temperature in a humid environment. Sections were individually incubated with 100 μl of each monoclonal antibody for 30 min. Following rinsing, the sections were incubated with 100 μl Alexa Fluor 488 goat anti-mouse antibody (5 μg/ml) for 30 min. After an additional rinse, coverslips were mounted using DAPI (4′,6-diamidino-2-phenylindole) slow-fade mounting medium (Invitrogen). Uninfected ISE6 cells were treated identically and used as negative antigen controls. Monoclonal antibody ANAR49, reactive with *A. marginale* major surface protein 2 (Msp2) (6), was used as a control for identification of a nonnuclear translocated protein. Monoclonal antibody TRYP1E1 was used as a negative antibody control. For epifluorescence microscopy, slides were viewed and photographed by using an Axio Imager.M1 microscope (Carl Zeiss) equipped with an X-Cite 120 Fl illuminating system (EXFO Photonic Solutions). Digital images were captured using an AxioCam MRm digital camera connected to a desktop computer running AxioVision (version 4.8.1.0). Images were processed using the ImageJ-based open source processing package Fiji (version 1.6.0_16). Rotations were generated for three-dimensional projections of z-stacks as follows: image stacks were obtained using optimal *z*-axis spacing (250 nm) for a Plan-Apochromat 63×/1.4 oil M27 objective (Carl Zeiss Imaging, Inc.) and exposure times set to maximize fluorescence intensity histograms without pixel saturation. Original z-stack image files were imported for processing into

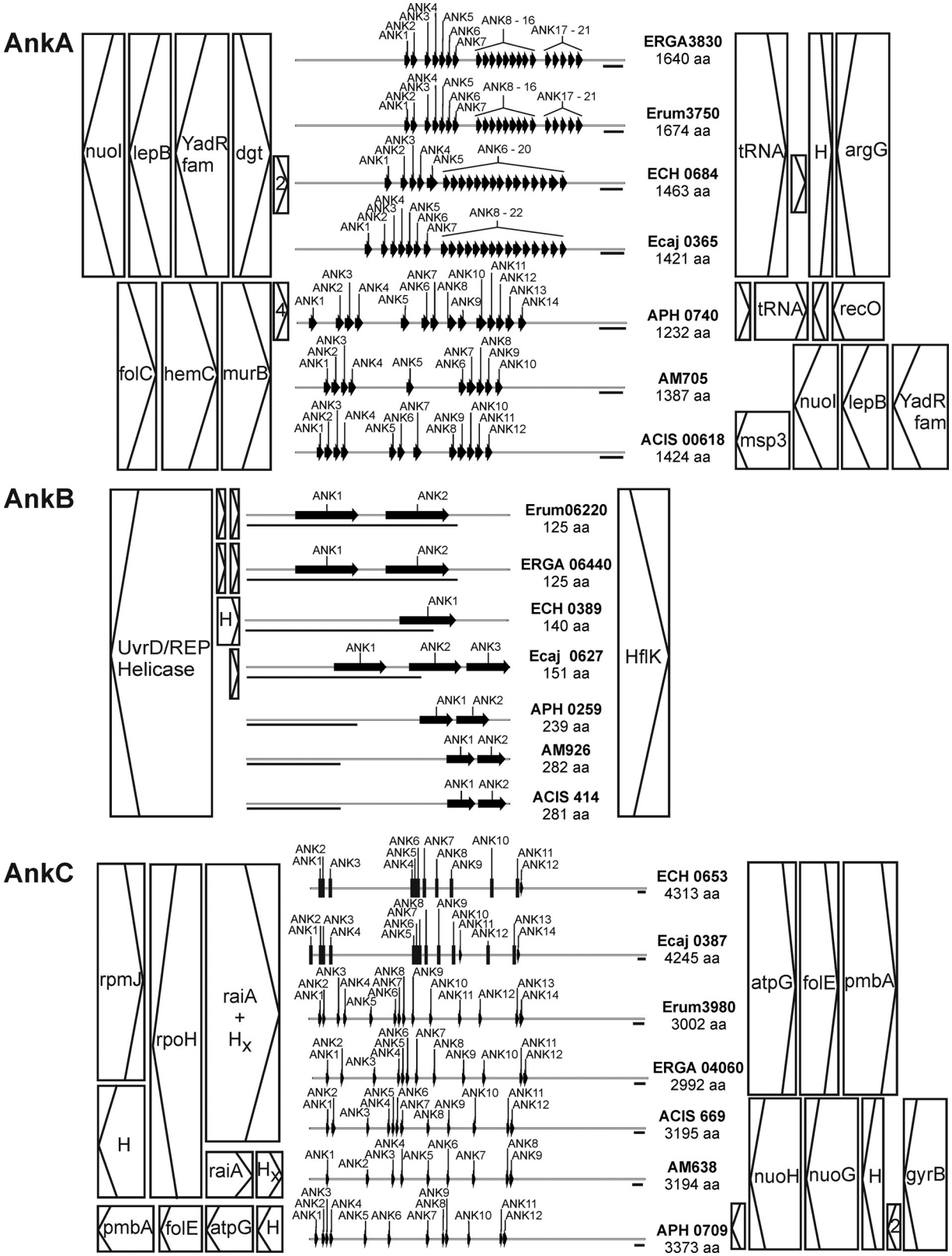

FIG. 1. Locus synteny and ankyrin motif content of Ank orthologues broadly conserved in the genera *Anaplasma* and *Ehrlichia*. The genomic contexts of genes encoding the three orthologous Ank proteins in the Gardel strain of *Ehrlichia ruminantium* (ERGA), the Welgevonden strain of *E. ruminantium* (Erum), the Arkansas strain of *E. chaffeensis* (ECH), the Jake strain of *E. canis* (Ecaj), the HZ strain of *Anaplasma phagocytophilum* (APH), the St. Maries strain of *A. marginale* (AM), and the Israel vaccine strain of *A. marginale* subsp. *centrale* (ACIS) are indicated by the flanking boxes. Boxes contain an arrowhead indicating the directionality of the gene, a gene name or type (e.g., tRNA), or an H for a hypothetical coding sequence; boxes without lettering represent small (<500-bp) open reading frames without annotation. The size of the Ank protein is indicated below the gene identifier (e.g., AM705, 1,387 amino acids [aa] in length), and the numbers and positions of the ankyrin motifs are indicated by dark arrows. Black bars below each Ank orthologue represent 100 amino acids in length; numbers within the boxes (e.g., 2 and 4) indicate multiple small orfs. H_x refers to the identical hypothetical coding sequence in more than one site.

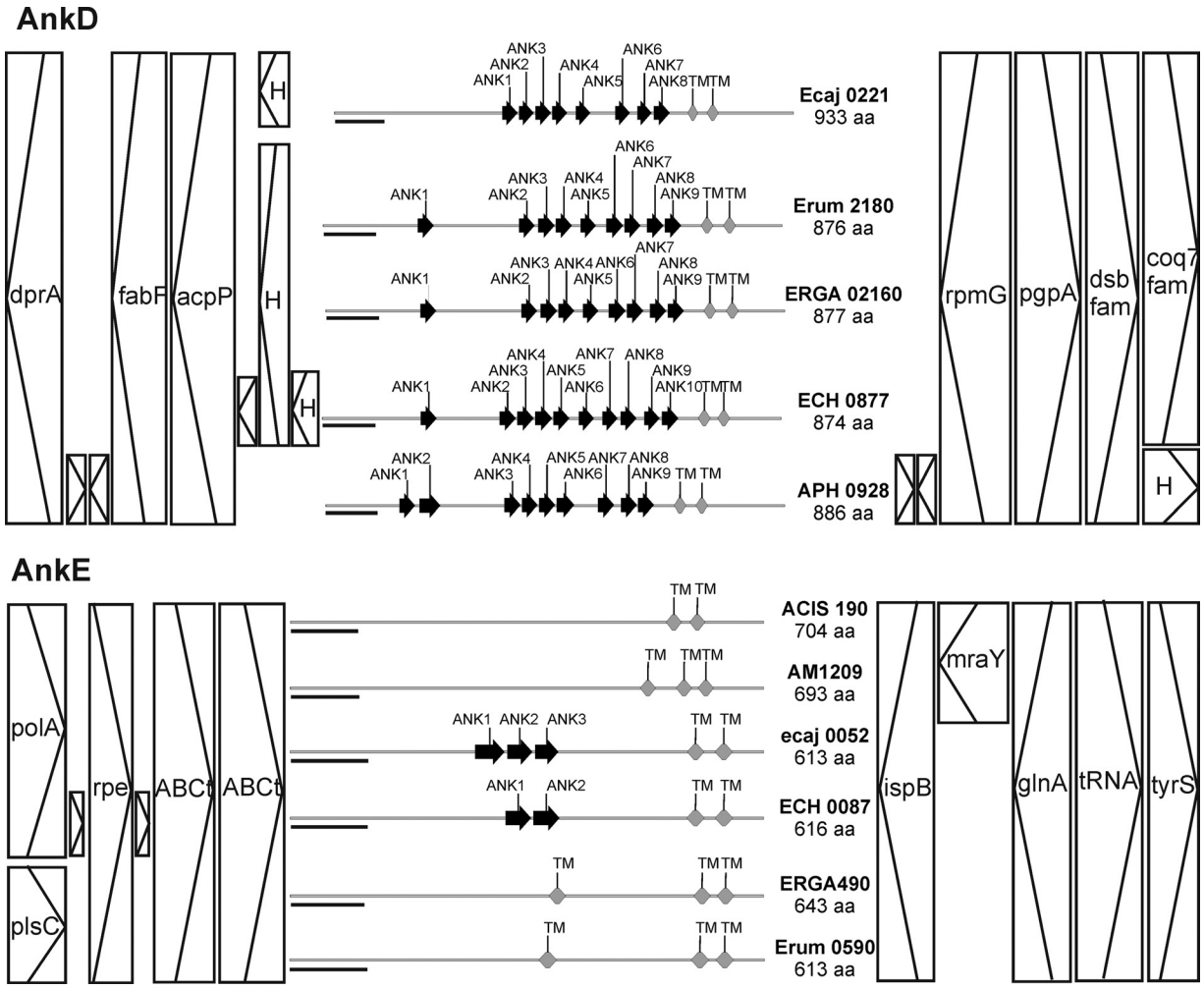

FIG. 2. Locus synteny and ankyrin motif content of Ank orthologues variably conserved in the genera *Anaplasma* and *Ehrlichia*. The genomic contexts of genes encoding AnkD and AnkE are indicated by the flanking boxes; there is no AnkD orthologue in *A. marginale* sensu lato and no AnkE orthologue in *A. phagocytophilum*. The examined strains and the representation of the flanking genes, ankyrin motifs, and size markers are the same as in Fig. 1. Light gray diamonds marked with "TM" indicate predicted transmembrane domains.

the ImageJ-based open source processing package Fiji (64 bit, version 1.45). First, each fluorescence channel was mildly deblurred (fast Fourier transformation autopreconditioning, theoretical point spread function, and 5 iterations; "Parallel Iterative Deconvolution 3D" plug-in, version 1.11, and "Diffraction PSF 3D" plug-in, version 2). Deblurred image stacks were merged and pseudocolored (with magenta being Alexa Fluor 488 and cyan being DAPI), and a 360-degree *y* axis rotation with interpolation was created for each 3-dimensional projection ("3D Project" stack function) and then exported in avi format with jpeg compression.

Nucleotide sequence accession numbers. The sequenced gene products (with GenBank numbers) identified in this study were as follows: *A. marginale* strain Mississippi AnkA (JF712893), AnkB (JF712895), and AnkC (JF712898), Virginia strain AnkA-1 (JF712891), AnkA-2 (JF712892), AnkB (JF712894), and AnkC (JF712897), and Puerto Rico strain AnkB (JF712896) and AnkC (JF712899).

RESULTS

Identification and conservation of *A. marginale* genes encoding ankyrin repeat motifs. A genome-wide screen of the annotated St. Maries genome identified three genes encoding proteins with the ankyrin repeat motif. AM705 encodes an 146-kDa protein containing 10 ankyrin repeats that is orthologous to both *A. phagocytophilum* AnkA (e value 10^{-55}) and *E. chaffeensis* p200 (e value 10^{-17}). In addition, there are AnkA orthologues in *A. marginale* subsp. *centrale*, *E. canis*, *E. chaffeensis*, and *E. ruminantium*; the locus positions are syntenic at the 5′ ends among the *Anaplasma* spp. but divergent between *A. marginale* sensu lato and *A. phagocytophilum* at the 3′ ends (Fig. 1). There is 5′ and 3′ synteny among *Ehrlichia* spp. but no clear synteny between the *Anaplasma* and *Ehrlichia* genera. AM926 encodes a 31-kDa protein with two ankyrin repeats; the carboxy-terminal positions of the motifs are conserved among *A. marginale*, *A. marginale* subsp. *centrale*, and *A. phagocytophilum*. There are orthologues in each of the examined *Ehrlichia* spp., and the locus structure is widely conserved at both the 5′ and 3′ ends among all *Anaplasma* and *Ehrlichia* spp. (Fig. 1). AM638 encodes a 348-kDa protein with 9 ankyrin repeats, with orthologues in each of the other examined species. Interestingly, while there is locus synteny at the 3′ end between *A. marginale* sensu lato and *A. phagocytophilum* and independently among the *Ehrlichia* spp., the 5′ synteny is shared only among *A. marginale* sensu lato and the *Ehrlichia*

TABLE 1. Conservation (percent amino acid identity) of ankyrin motif-containing proteins among *Anaplasma* and *Ehrlichia* spp.

Protein	% amino acid identity[a]									
	A. marginale (sensu stricto)					*A. marginale* subsp. *centrale*	*A. phagocytophilum*	*E. chaffeensis*	*E. canis*	*E. ruminantium*
	St. Maries	Florida	Mississippi	Puerto Rico	Virginia					
AnkA (AM705)	100	95	94	94	94	49	19	8	9	10
AnkB (AM926)	100	99	99	100	99	94	34	39	35	40
AnkC (AM638)	100	98	98	98	98	63	26	16	15	19
AnkD (APH928)	NP	NP	NP	NP	NP	NP	100	19	19	20
AnkE (AM1209)	100	100	100	100	100	69	NP	12	12	10

[a] The St. Maries strain of *A. marginale* (GenBank CP000030) was used as the reference for AnkA, AnkB, AnkC, and AnkE. The HZ strain of *A. phagocytophilum* (GenBank CP000235) was used as the reference for AnkD. NP, not present.

spp., with *A. phagocytophilum* representing the outlier (Fig. 1). The identification of these three ankyrin-containing proteins is consistent with the prior bioinformatic analysis of Rikihisa and Lin (24); these proteins are here designated *A. marginale* AnkA (AM705), AnkB (AM926), and AnkC (AM638). There are two additional proteins containing ankyrin motifs in *Ehrlichia* spp. which lack orthologues in one or more *Anaplasma* spp. or, if orthologues are present, they do not have ankyrin motifs identified by the currently available algorithms. AnkD is present in all examined *Ehrlichia* spp. and in *A. phagocytophilum* with conservation of locus position (Fig. 2) but is absent in both *A. marginale* sensu stricto and *A. marginale* subsp. *centrale*. In contrast, AnkE has orthologues and locus synteny in all examined species except *A. phagocytophilum* but has ankyrin motifs only in *E. canis* and *E. chaffeensis* (Fig. 2).

Conservation of AnkA, AnkB, and AnkC among *A. marginale* strains. AnkB and AnkC are highly conserved in their amino acid sequences, including the ankyrin repeat domains, among *A. marginale* sensu stricto strains; the AnkA ankyrin repeats are also conserved among strains; however, there is greater sequence divergence among sensu stricto strains than for either AnkB or AnkC (Table 1). In contrast, only AnkB is highly conserved between sensu stricto stains and *A. marginale* subsp. *centrale* (Table 1).

Quantitative expression levels of ankyrin repeat-containing proteins in infected mammalian and tick cells. Using monoclonal antibodies specific to each ankyrin-repeat containing protein, we probed lysates of 10^7 *A. marginale* isolated from either infected ISE6 cells or infected erythrocytes for expression. All three proteins, AnkA, AnkB, and AnkC, were expressed in bacteria isolated from at least one of the host cell types (Fig. 3). The level of AnkA in bacteria from infected erythrocytes was higher than the level in bacteria from infected ISE6 cells; densitometric analysis of three independent replicates revealed statistically significantly higher levels (106 ± 0.7 versus 93.7 ± 1.2 [means ± standard deviations in relative density units]; $P = 0.0001$ [unpaired Student's t test]). In contrast, AnkC was expressed only in bacteria isolated from ISE6 cells, with no detectable AnkC in 10^7 bacteria from infected erythrocytes (Fig. 3). AnkB was expressed in bacteria isolated from both cell types, with no significant difference in levels based on densitometric analysis of three independent replicates (114 ± 3.9 versus 117.3 ± 1.3; $P = 0.2$). *A. marginale* Msp5, constitutively expressed in both host cell types (23), was used as an internal standard for equal numbers of loaded bacteria (Fig. 3), and there was no significant difference in the Msp5 levels as measured by densitometry.

***In situ* expression of ankyrin repeat-containing proteins in *Dermacentor andersoni*.** The quantitative Western blot analysis shown in Fig. 3 confirmed expression of AnkA and AnkB in *A. marginale*-infected erythrocytes obtained from *in vivo* infection of a natural mammalian host. To confirm expression in the natural tick vector, sections of transmission-fed adult male *D. andersoni* ticks were probed using immunohistochemistry. AnkA, AnkB, and AnkC were detected in the salivary gland acinar cells (Fig. 4). Uninfected ticks, handled identically but fed on an uninfected calf, were negative when probed with monoclonal antibodies specific to each protein, as were in-

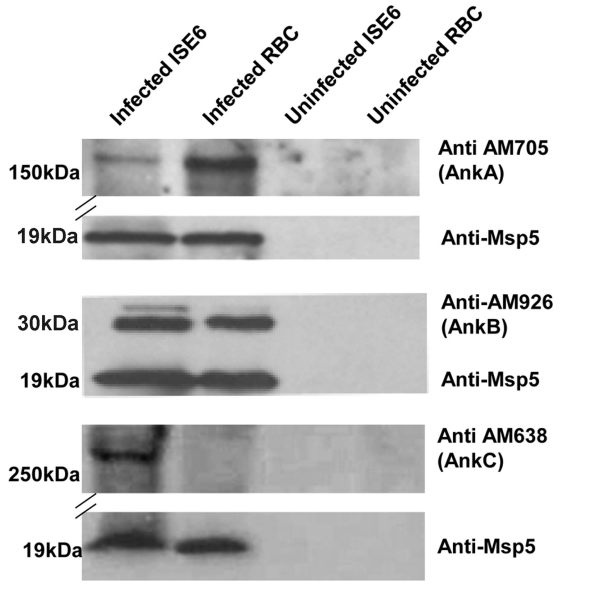

FIG. 3. Expression of *Anaplasma marginale* ankyrin repeat-containing proteins in infected mammalian and tick cells. *A. marginale* ($10^{[7 \pm 0.05]}$ organisms) isolated from infected ISE6 tick cells, *A. marginale* ($10^{[7 \pm 0.05]}$ organisms) isolated from infected erythrocytes (red blood cells [RBC]), uninfected ISE6 cells, and uninfected erythrocytes were probed with antibodies specific for the AM705 (AnkA), AM926 (AnkB), or AM638 (AnkC) protein and, in the same blot, with monoclonal antibody ANAF16C1, specific for the constitutively expressed Msp5.

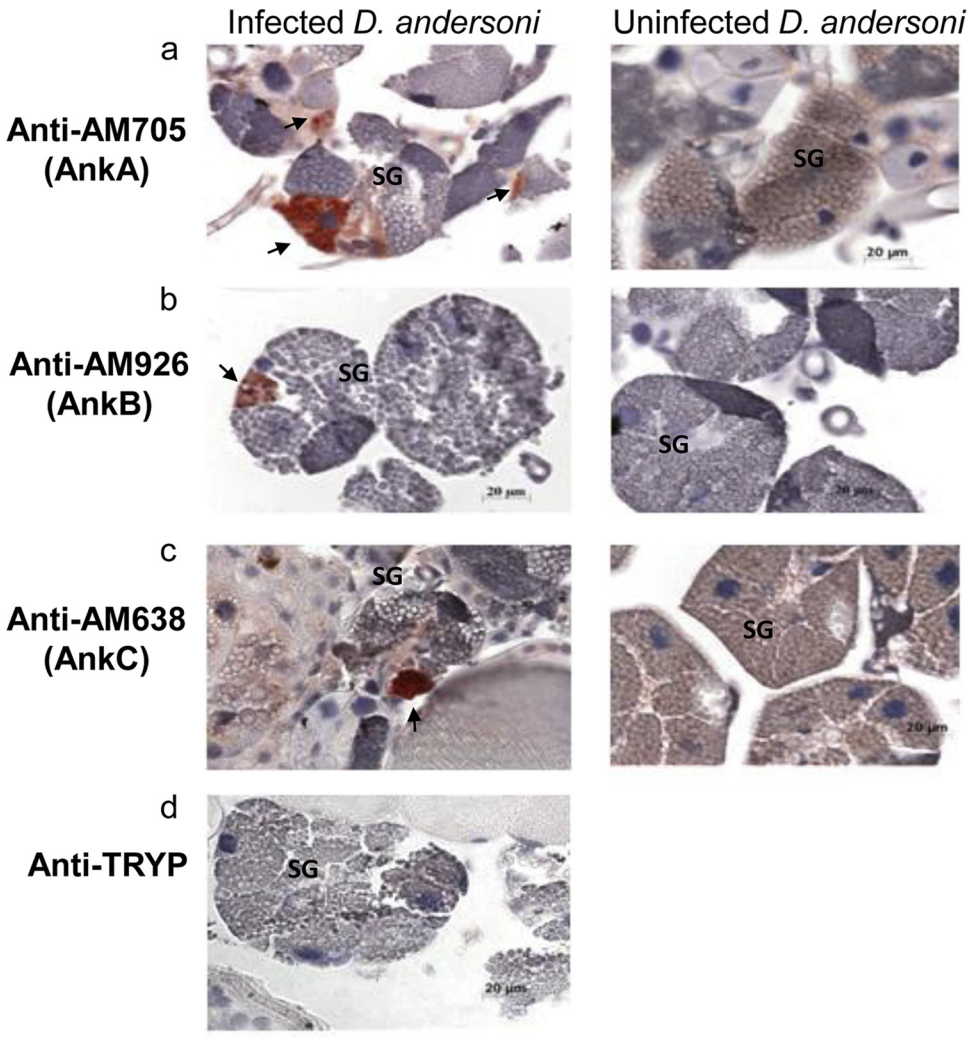

FIG. 4. Expression of *Anaplasma marginale* ankyrin repeat-containing proteins in infected *Dermacentor andersoni*. Serial sections of salivary glands (SG) of infected or uninfected ticks were probed with monoclonal antibody 149/312, 148/42.17, or 150/103, specific to the AM705 (AnkA), AM926 (AnkB), or AM638 (AnkC) protein, respectively. Monoclonal antibody TRYP1E1 (TRYP) was used to probe salivary glands from infected ticks as a negative antibody control.

fected ticks probed with the negative-control antibody, TRYP1E1 (Fig. 4).

Subcellular localization of ankyrin repeat-containing proteins. To test whether any of the three proteins was translocated to the host cell nucleus, as has been shown for both *A. phagocytophilum* AnkA and *E. chaffeensis* p200 (22, 32), infected ISE6 cells were probed with each monoclonal antibody and stained with DAPI for nuclear identification and examined by fluorescence microscopy. Monoclonal antibody ANAR49 was used to detect Msp2, an integral outer membrane protein, as a marker for a nontranslocated protein (Fig. 5). AnkA, AnkB, and AnkC all localized to the bacteria within the cytoplasmic vacuole, similar to Msp2, with no evidence of either nuclear translocation or translocation outside the vacuole (Fig. 5). This observation was confirmed by visualization in three dimensions in which rotation permitted confirmation that vacuolar and nuclear localizations were distinct for all three Anks (for AnkA, AnkB, and AnkC, see Fig. S1, S2, and S3 in the supplemental material, respectively). There was no reactivity of any of the anti-*A. marginale* antibodies with uninfected ISE6 cells and no reactivity of the negative-control monoclonal antibody TRYP1E1 with infected ISE6 cells (Fig. 5).

DISCUSSION

Ankyrin repeats are common in eukaryotic cells (18) and, although initially thought to be relatively uncommon in prokaryotes, have been reported with increasing frequency in a diverse set of alpha-, beta-, and gammaproteobacteria (24). Although the ankyrin domain has most commonly been linked to protein-protein interactions in the host cell cytosol, seminal studies with both *A. phagocytophilum* AnkA and *E. chaffeensis* p200 identified host cell nuclear localization with chromatin and DNA binding (10, 12, 22, 32). In a directed search for *A. marginale* proteins uniquely expressed or specifically upregulated in the tick vector, we hypothesized that an *A. marginale* AnkA orthologue would be expressed only in the tick vector, where the host cells are nucleated, and not in mature nonnu-

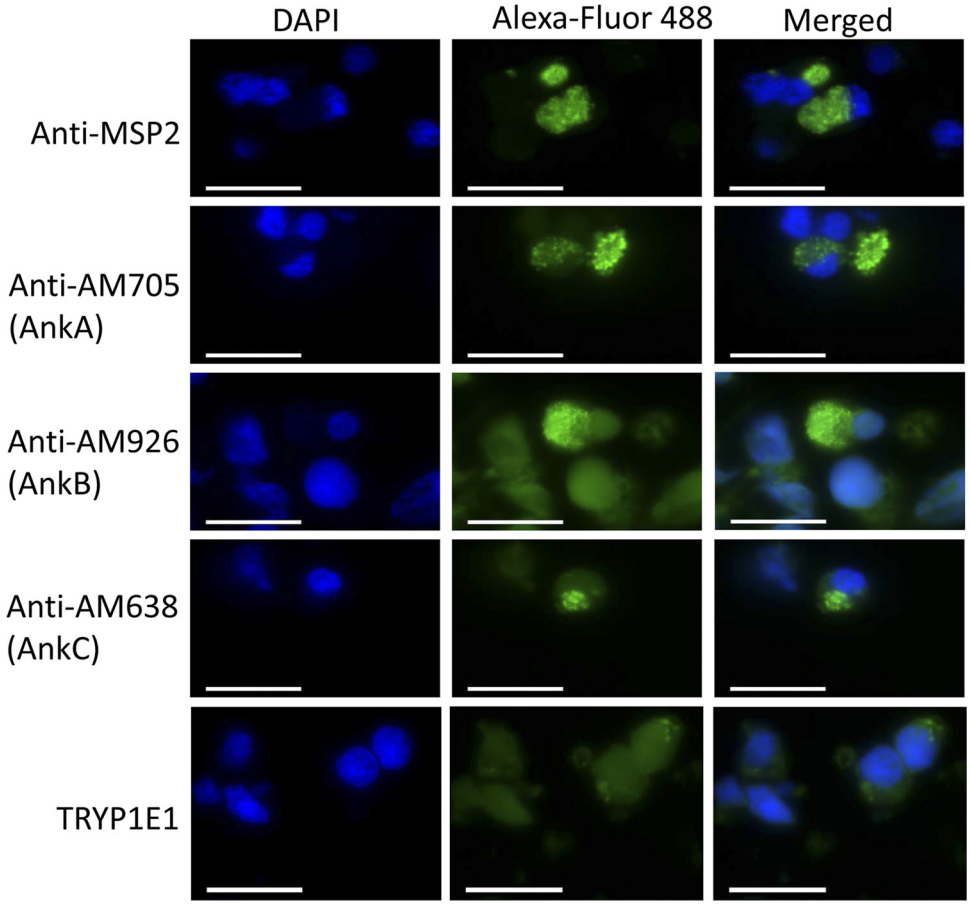

FIG. 5. Subcellular localization of *Anaplasma marginale* ankyrin repeat-containing proteins. *A. marginale*-infected ISE6 tick cells were probed with monoclonal antibodies specific for the AM705 (AnkA), AM926 (AnkB), or Am638 (AnkC) protein and identified using Alexa Fluor 488-conjugated goat anti-mouse antibody (green) and epifluorescence microscopy. Host cell nuclei were stained with DAPI (blue). Monoclonal antibody ANAR49 was used to label Msp2, an integral *A. marginale* outer membrane protein, as a positive control for intravacuolar bacteria. Monoclonal antibody TRYP1E1, reactive with *Trypanosoma brucei*, was used as a negative antibody control. The white bars represent 20 μm.

cleated erythrocytes of the mammalian host. Our search identified a clear AnkA/p200 orthologue in *A. marginale*, the AM705 protein, as well as two additional ankyrin domain-bearing proteins, AnkB (AM926) and AnkC (AM638). These identifications match those recently reported by Rikihisa and Lin in a bioinformatic search of sequenced genomes in the genera *Anaplasma* and *Ehrlichia* (24). AnkB (AM926) and AnkC (AM638) were originally annotated as hypothetical proteins (1); however, we have now shown that these and AnkA (AM705) are expressed as proteins during infection of the natural mammalian host, the natural tick vector, or both. Accordingly, we redesignated these proteins *A. marginale* AnkA (AM705), AnkB (AM926), and AnkC (AM638). All three have orthologues in the most closely related bacterial species in the genera *Anaplasma* and *Ehrlichia* with conservation of ankyrin motifs and partial to complete retention of locus synteny (Fig. 1). Based on the currently available genome sequences, the number of encoded ankyrin repeat-bearing proteins varies among the bacteria in the family *Anaplasmataceae*, from 3 in *A. marginale* to 60 in the wPip strain of *Wolbachia pipiens* (31).

The hypothesis that *A. marginale* AnkA is expressed only in the nucleated cells of the tick has been rejected: AnkA was expressed in mammalian erythrocytes and tick salivary gland acinar cells. The quantitative analysis using ISE6 cells indicated that AnkA is expressed in erythrocytes at levels higher than those in the tick cells. Whether this lower level in the ISE6 cells is reflective of levels in the actual tick tissues is unknown; however, our broader proteomic analysis has supported the predictive value of *A. marginale* expression in ISE6 cells for *D. andersoni* (23). In contrast, AnkC was expressed in ISE6 cells and *D. andersoni* salivary glands but not in the mammalian host. Collectively, with the unbiased proteomic analysis reported previously, 15 *A. marginale* proteins have been identified as uniquely expressed or significantly upregulated in tick cells, 9 proteins uniquely expressed or expressed at higher levels in the mammalian erythrocyte, and the majority (including AnkB, described in this study) expressed in both host cell environments (16, 20, 21, 23).

Unlike either *A. phagocytophilum* AnkA or *E. chaffeensis* p200, the *A. marginale* Ank proteins did not translocate to the nucleus (Fig. 5; see Fig. S1 to S3 in the supplemental material). None of the AnkA/p200 orthologues or the newly confirmed AnkB and AnkC proteins has a consensus nuclear localization signal. How *A. phagocytophilum* AnkA and *E. chaffeensis* p200

are translocated remains unknown; however, a requirement for an additional nuclear transporter would explain the difference between *A. marginale* AnkA and the orthologues in the other two species. *A. phagocytophilum* AnkA has also been shown to be translocated to the host cell cytosol in a type IV secretion system-dependent manner (15); in contrast, *A. marginale* AnkA, AnkB, and AnkC appear intimately associated with the bacterium itself with no evidence of secretion beyond the vacuole (Fig. 5; see Fig. S1 to S3 in the supplemental material). This suggests that while these proteins may be derived from a common ancestor, there has been divergence to effect different functions in the specific pathogen-host cell interactions. Survival of *A. phagocytophilum* and *E. chaffeensis* within professional phagocytic cells and within neutrophils and monocytes, respectively, likely requires a global downregulation of bactericidal mechanisms, while *A. marginale*, demonstrated to infect only mature erythrocytes *in vivo*, has no similar requirement. Although the retention of the ankyrin repeat domains provides a structural basis for protein-protein and protein-DNA interactions in *A. marginale*, trafficking of these proteins clearly differs from that of their orthologues. In addition, AnkD is lost from both *A. marginale* and *A. marginale* subsp. *centrale*, consistent with a role required in nucleated mammalian cells but not for infection of the nucleated cells of the tick vector. AnkE has even more restriction, with the presence and encoding of ankyrin motifs in only those ehrlichiae that infect mammalian monocytes.

Given the presumed function of Ank proteins in mediating intracellular events required for successful survival and replication and the conservation of orthologues across genera, the divergence among strains within a genus is unexpected. For sensu stricto *A. marginale*, this is most notable for AnkA, consistent with the observation of strain-specific polymorphism in *A. phagocytophilum* AnkA (24, 25). More remarkable are the differences in both AnkA and AnkC between sensu stricto *A. marginale* and *A. marginale* subsp. *centrale*. While the latter was originally isolated in South Africa and the examined sensu stricto strains were isolated in either North America or the Caribbean, geographic distance alone does not appear to explain the divergence as preliminary examination of AnkA, AnkB, and AnkC of an Australian strain indicates clustering with the North America/Caribbean strains. Whether this divergence in AnkA and AnkC underlies one or more of the phenotypic differences between *A. marginale* subsp. *centrale* and the sensu stricto strains (13), including virulence, is yet untested.

The sequence divergence in the ankyrin motif-containing proteins among *A. marginale* sensu lato strains and, more broadly, the variable retention of Ank orthologues among the tick-transmitted *Anaplasma* and *Ehrlichia* spp. are most consistent with specific selective pressures molding the genomes. Notably, these genes have been a site for genomic recombination reflected in the loss of synteny at one flanking edge of the gene. The retention or loss of locus synteny, orthologous genes, and encoded ankyrin motifs does not occur along taxonomic lines (which do accurately represent genetic relatedness for these genera [4]), suggesting that the Ank proteins do not represent temporal drift from a common ancestor over time but rather likely reflect specific adaptations to the host niches of both the mammalian and vertebrate hosts. This is supported by the recent work that correlated *A. phagocytophilum* AnkA genotypes with specific mammalian hosts (25). Understanding how these proteins function in both hosts represents a clear challenge, one that may provide insight into bacterial evolution as well as opportunities for improved pathogen control.

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