Cell, Vol. 119, 457–468, November 12, 2004, Copyright ©2004 by Cell Press

TROSPA, an *Ixodes scapularis* Receptor for *Borrelia burgdorferi*

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Summary

The Lyme disease agent *Borrelia burgdorferi* naturally persists in a cycle that primarily involves ticks and mammals. We have now identified a tick receptor (TROSPA) that is required for spirochetal colonization of *Ixodes scapularis*. *B. burgdorferi* outer surface protein A, which is abundantly expressed on spirochetes within the arthropod and essential for pathogen adherence to the vector, specifically bound to TROSPA. *TROSPA* mRNA levels in ticks increased following spirochete infestation and decreased in response to engorgement, events that are temporally linked to *B. burgdorferi* entry into and egress from the vector. The blockade of TROSPA by TROSPA antisera or by the repression of *TROSPA* expression via RNA interference reduced *B. burgdorferi* adherence to the *I. scapularis* gut in vivo, thereby preventing efficient colonization of the vector and subsequently reducing pathogen transmission to the mammalian host. Identification of an *I. scapularis* receptor for *B. burgdorferi* is the first step toward elucidating arthropod ligands that are required for survival of spirochetes in nature.

Introduction

In the United States, the life cycle of *Borrelia burgdorferi*, the Lyme disease agent, primarily involves a tick, *Ixodes scapularis*, and *Peromyscus leucopus,* the white-footed mouse (Nadelman and Wormser, 1998; Steere, 2001). *B. burgdorferi* enters the *I. scapularis* gut when larvae feed on infected mice and are then transtadially maintained through the molts. During tick engorgement, the spirochetes within the gut replicate, migrate through the vector, and are transmitted to the murine dermis, where *B. burgdorferi* multiply locally before disseminating to diverse tissues (Malawista, 2000; Nadelman and Wormser, 1998; Steere, 2001; Steere et al., 2004).

B. burgdorferi adapt to the transition between the arthropod vector and mammalian host in part by preferential gene expression. For instance, during early mammalian infection, spirochetes express decorin and fibronectin binding proteins that facilitate attachment to host tissues (Guo et al., 1998; Probert and Johnson, 1998). In contrast, the gene for outer surface protein (Osp) A is preferentially expressed by *B. burgdorferi* within the *I. scapularis* gut (de Silva et al., 1996) and downregulated during transmission to the vertebrate host (de Silva et al., 1996; Ohnishi et al., 2001). The temporal pattern of *ospA* gene expression within ticks suggests that OspA plays a role in spirochete persistence in the vector.

Recent advances in spirochete genetics have also advanced our knowledge of vector-pathogen interactions during the colonization and transmission of microbes through arthropods. As an example, *B. burgdorferi* OspC is induced during tick feeding, and *Borrelia* mutants with deletions in *ospC* displayed defects in salivary gland invasion as well as in the establishment of infection in mammals (Grimm et al., 2004; Pal et al., 2004). In addition, OspA has been shown to be essential for *B. burgdorferi* to persist within the tick (Yang et al., 2004). The targeted ablation of OspA in an infectious isolate of *B. burgdorferi* did not interfere with the spirochete's ability to infect and cause disease in mice, but the OspA-deficient spirochetes failed to colonize and survive within *I. scapularis*—and, in particular, these *B. burgdorferi* could not bind to the tick gut (Yang et al., 2004).

The vector ligands that participate in *B. burgdorferi*-tick interactions have not yet been identified. Since the tick gut is the principal site where *B. burgdorferi* persist in the vector, and the spirochetes almost exclusively reside within this tissue during the long intervals between the tick blood meals (de Silva and Fikrig, 1995), it is likely that *B. burgdorferi* has evolved mechanisms to specifically interact with arthropod receptors within the gut. We have now characterized an *I. scapularis* protein that binds to OspA and determined its importance in spirochete-vector interactions.

Results

Cloning of the OspA Receptor within *I. scapularis*

Since OspA binds to a protein receptor within the *I. scapularis* gut (Pal et al., 2000), an *I. scapularis* cDNA library was constructed in a yeast-*Escherichia coli* shuttle vector and screened using a LexA-OspA fusion pro-

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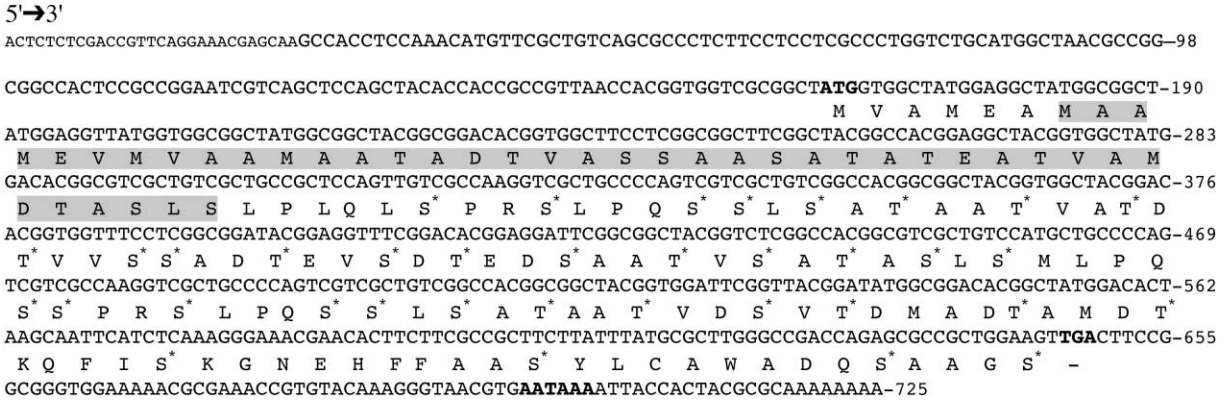

Figure 1. Cloning of *TROSPA*

Nucleotide and predicted amino acid sequences of *TROSPA* (accession number AY189148). The sequences were obtained from OspA interactive yeast two-hybrid overlapping clones except for the first 29 nucleotides (indicated in small letters), which were derived from clones obtained from 5′ RACE and cDNA library screening. The in-frame putative TROSPA translational start codon, stop codon, and polyadenylation signal are indicated in bold. A putative transmembrane domain and several O-glycosylation sites are indicated by shading and asterisks, respectively.

tein as bait. We isolated three OspA interactive clones, which represented overlapping sequences of one common tick cDNA, designated as the gene encoding the tick receptor for OspA (*TROSPA*). The observed protein-protein interaction was specific, as TROSPA did not interact with control antigens such as LexA alone (without any fusion partner) or a LexA-Lamin fusion protein in this yeast two-hybrid assay. The GC-rich *TROSPA* sequence obtained from the two-hybrid screen contained an open reading frame (ORF) of 617 nucleotides with a 3′-untranslated region that included a polyadenylation signal sequence. To define the full-length *TROSPA* mRNA, we performed 5′ RACE (rapid amplification of cDNA ends) and direct screening of two different *I. scapularis* cDNA libraries with a *TROSPA* probe (see Experimental Procedures). Sequencing of isolated cDNA clones from the 5′ RACE or library screening yielded an additional 29 upstream nucleotides beyond the 5′ terminus of the cDNA identified from the two-hybrid screen (Figure 1). To rule out the possibility that the 5′ RACE or cDNA library screening could not obtain additional *TROSPA* sequence due to the secondary structure of the highly GC-rich *TROSPA* mRNA, an *I. scapularis* genomic DNA library was constructed and probed with *TROSPA* cDNA. One phage clone contained a 20 Kb genomic DNA insert harboring the entire *TROSPA* gene. Comparison of the genomic and cDNA sequences identified the presence of three exons and two introns within the *TROSPA* gene (accession number AY640046). The genomic sequence had typical promoter elements such as a TATA box (gTATAaaa) and transcription factor recognition sequences either upstream of the *TROSPA* transcription initiation site at the 5′ end of exon 1 or within intron 1 (data not shown).

Nucleotide sequence analysis revealed that the full-length *TROSPA* cDNA contained a KOZAK (Kozak, 1987) compatible ATG codon at position +164 at the 5′ end, which encoded a TROSPA protein with a predicted molecular mass of 16.1 kDa. *TROSPA* nucleotide and protein sequences displayed no significant homologies to genes in several databases (BLAST search, GenBank and EMBL nonredundant databases, October 2004). The deduced TROSPA amino acid sequence had several randomized repeats of up to 17 residues with an unusually high number of potential posttranslational modification signals, including O-glycosylation sites such as GalNAc motifs (n = 38, threshold > 0.5), O-(β)-GlcNAc motifs (n = 27, threshold > 0.5), and phosphorylation sites (n = 21, threshold > 0.5) with a putative transmembrane helix between residues 6 and 40 (TMpred Prediction) (Figure 1).

Localization of TROSPA in the Tick Gut

Since the initial *TROSPA* cDNA sequence encoded a functional OspA binding protein, this cDNA was expressed in *E. coli* (Figure 2A, lane 1) and used as an antigen to develop TROSPA antisera (Figure 2A, lane 2) for localization and functional studies. In Western blots, the TROSPA antisera reacted with a tick gut protein that migrated with an approximate molecular mass of 55 kDa, both in one- and two-dimensional gels (Figure 2B, lane 1 and 2C, left panel). TROSPA appeared to be gut specific, as *I. scapularis* salivary gland lysates or hemolymph proteins did not significantly react with TROSPA antisera (data not shown). Since TROSPA antisera raised against the recombinant peptide recognized a 55 kDa gut protein, we next examined whether the 55 kDa protein was native TROSPA and if the protein was post-translationally modified. Nymphal gut proteins were separated on a two-dimensional gel, and the TROSPA antisera-reactive 55 kDa tick protein was isolated and digested in-gel with trypsin, followed by liquid chromatography-mass spectrophotometry/mass spectrophotometry (LC-MS/MS) analysis for identification of the charged TROSPA peptides (see Experimental Procedures). As summarized in Supplemental Table S1 (at http://www.cell.com/cgi/content/full/119/4/457/DC1/), we consistently obtained a mass match for the triply charged TROSPA peptide (938.77) on the TROSPA antisera-reactive 55 kDa gut protein, strongly suggesting the presence of native TROSPA.

Because of the higher molecular mass of native TROSPA when compared to the recombinant peptide sequence, we next determined whether TROSPA was a glycoprotein. Treatment of gut proteins with a variety of

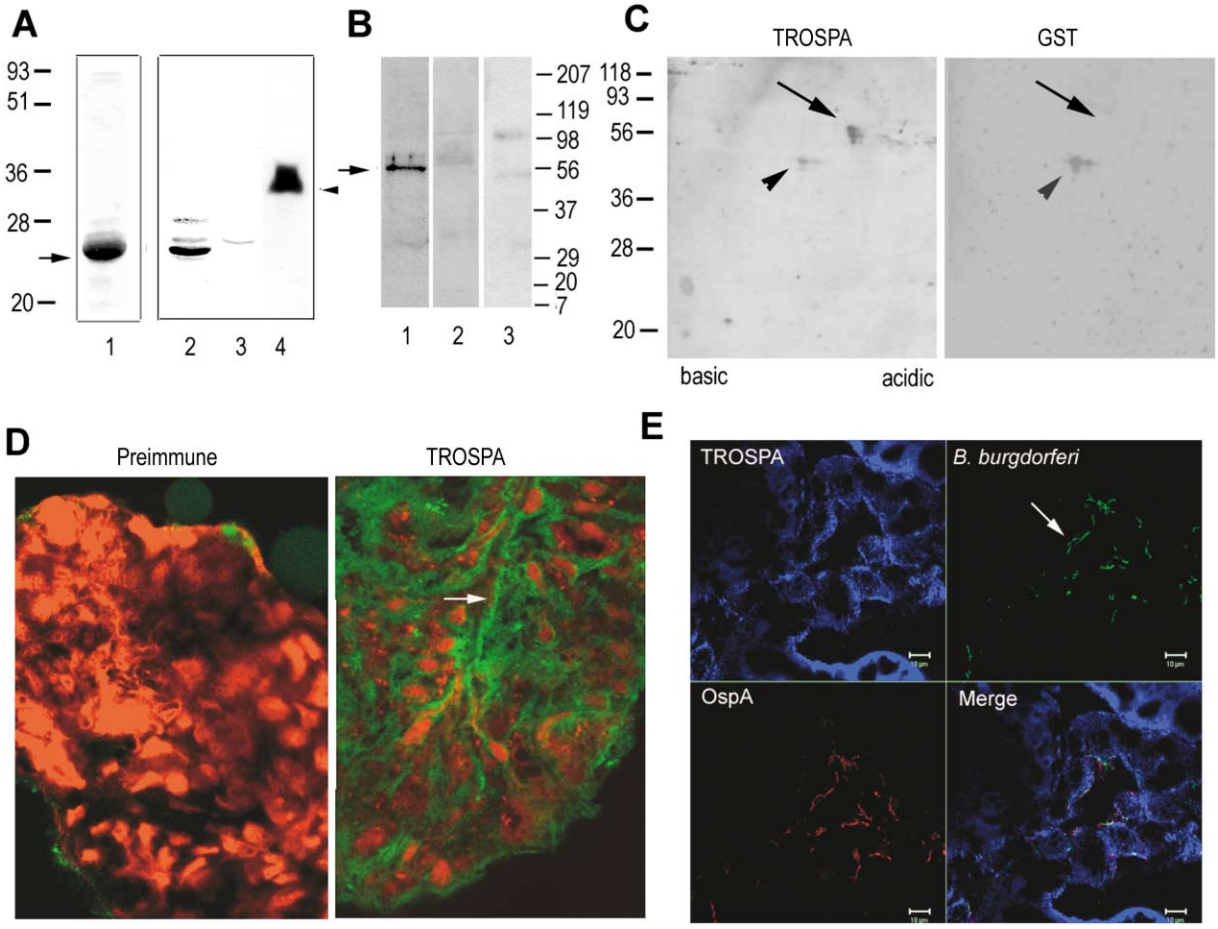

Figure 2. Localization of TROSPA in the Tick Gut

(A) Production of recombinant TROSPA and recognition by TROSPA antisera. Expression of an OspA interactive yeast two-hybrid clone (spanned from nucleotide +49 to +724 of *TROSPA* cDNA) in *Escherichia coli* and purification of TROSPA after cleavage of the GST fusion partner (Coomassie blue staining, lane 1, arrow). Western blotting of TROSPA, as shown in lane 1, by a TROSPA antisera (lane 2) or control GST antisera (lane 3) is shown. Glycosylated TROSPA (arrowhead) produced in *Drosophila* S2 cell detected with anti-V5 antibody (lane 4). Glycosylated TROSPA (as shown in lane 4) was also strongly recognized by TROSPA antisera (data not shown).

(B) TROSPA antisera recognized a specific tick gut protein in the Western blot. Proteins in TGE were resolved into a 12% SDS-PAGE gel, transferred onto nitrocellulose, and blotted with 1:1000 dilution of TROSPA antisera (lane 1) or controls: GST antisera (lane 2) and preimmune sera (lane 3). The arrow indicates a tick protein of approximate molecular mass of 55 kDa that strongly reacted with TROSPA antisera. Molecular mass markers (kDa) are indicated on the right.

(C) Recognition of the specific native 55 kDa tick gut protein (as shown in [B]) by TROSPA antisera in a two-dimensional gel (arrow, left panel). The blot was reprobed with GST antisera, which do not recognize native TROSPA (arrow, right panel). Arrowhead indicates tick proteins recognized by TROSPA as well as GST antisera. Molecular mass markers (kDa) are indicated on the left.

(D) Confocal immunofluorescence localization of native TROSPA in the *I. scapularis* gut. Binding of preimmune or TROSPA antisera was detected using a FITC-labeled rabbit IgG; tick nuclei were stained with propidium iodide (red). TROSPA antisera reactive fluorescence (green) was most prevalent in the tissue areas surrounding nuclei and on luminal surfaces (arrow). Images were examined using a Zeiss LSM 510 confocal 63× objective lens and presented as a single image for clarity.

(E) Colocalization of *B. burgdorferi* and TROSPA within the gut of an unfed nymphal *I. scapularis*. Spirochetes, OspA, and TROSPA were detected using FITC-labeled anti-*B. burgdorferi* goat IgG, Alexa 568-labeled anti-mouse IgG, and Alexa 647-labeled anti-rabbit IgG, respectively. Images were examined using the confocal 40× objective lens. Note attached spirochetes (green) producing OspA (red) within tick gut cells expressing TROSPA (blue).

glycosidases reduced the level of TROSPA antisera-reactive 55 kDa gut protein when compared to untreated controls. However, the antisera failed to recognize any protein of smaller molecular mass, suggesting that deglycosylated TROSPA is unstable in ticks (data not shown). When we expressed recombinant TROSPA in a *Drosophila* cell line, we found that TROSPA was post-translationally modified in arthropods (Figure 2A, lane 4). Unlike recombinant TROSPA produced in *E. coli*, *Drosophila*-expressed TROSPA selectively reacted with a glycoprotein detection kit based on Periodic Acid-Schiff method, thereby demonstrating that TROSPA undergoes glycosylation in arthropods (see Supplemental Figure S1A on the *Cell* web site). Confocal immunofluorescence studies showed TROSPA labeling on the surface of *Drosophila* cells, indicating that the protein was predominantly associated with the plasma membrane of transfected cells (Supplemental Figure S1B).

Immunolocalization studies using confocal microscopy confirmed that TROSPA antisera reacted strongly

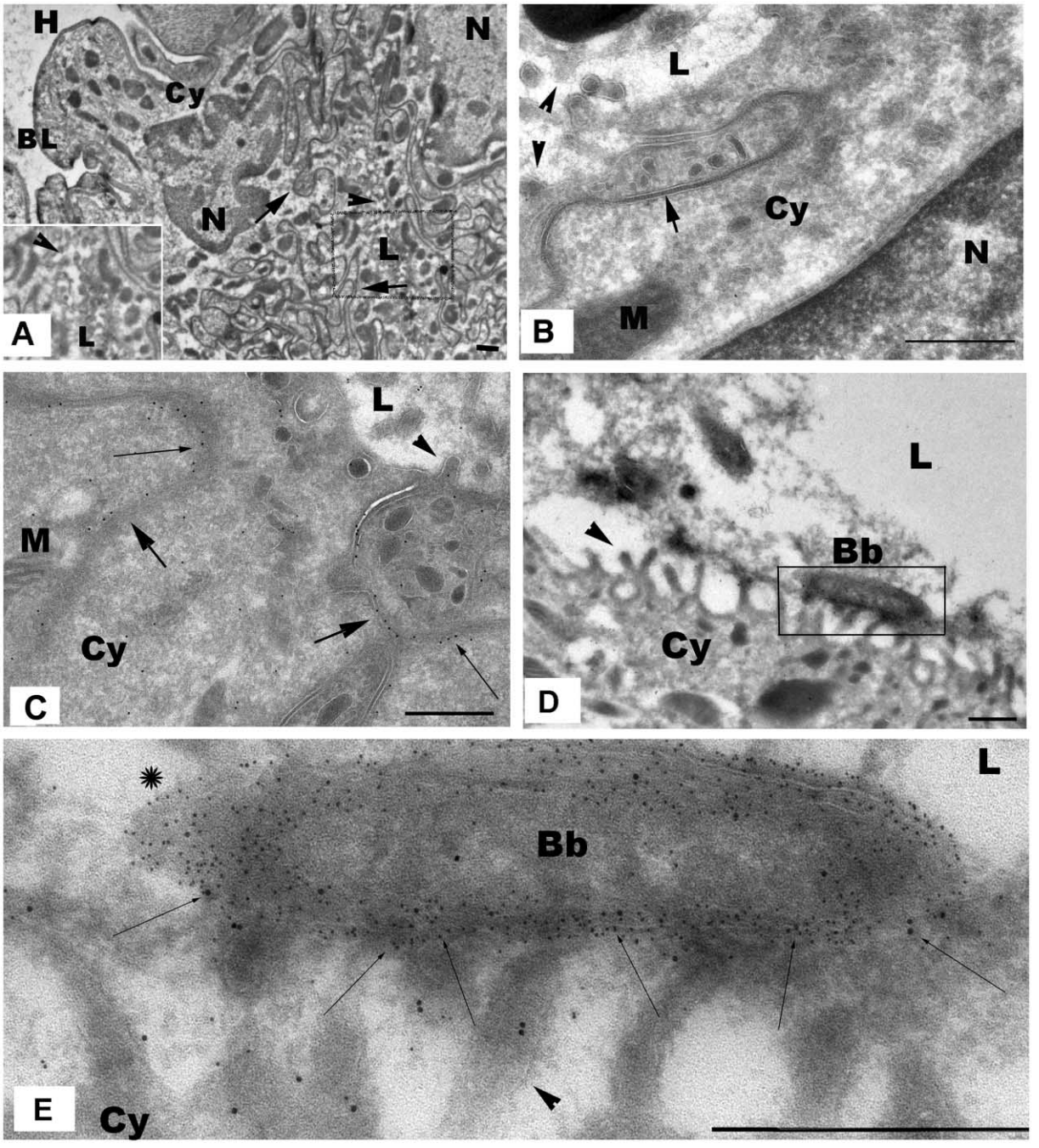

Figure 3. Immunogold Localization of Native TROSPA and *B. burgdorferi* within *I. scapularis* Gut

Bb, *Borrelia burgdorferi*; BL, basal lamina; Cy, cytoplasm; H, hemocoel side; L, lumen; M, mitochondria; N, nucleus. Short arrows point to convoluted epithelial plasma membranes of adjacent cells forming tight junctions. Luminal microvilli are indicated by arrowheads. Scale bar, 500 nm.

(A) A low-power electron micrograph of the morphology of the unfed *I. scapularis* nymphal gut. Note high convolution of plasma membranes toward a narrow lumen. Inset shows 2-fold magnification of the luminal area indicated by a box.

(B) A section of gut stained with preimmune sera. Note the absence of gold particles, indicating that preimmune sera does not recognize an antigen in the *I. scapularis* gut.

(C) Localization of gold particles indicating existence of native TROSPA (thin arrows) along the junction of plasma membranes (short arrow) of epithelial cells. Some gold particles were also detected within the cytoplasm and luminal microvilli.

(D) A low-power electron micrograph showing attached *B. burgdorferi* to the gut microvilli in a partially fed (48 hr) *I. scapularis* nymphal gut.

(E) Colocalization of *B. burgdorferi* and TROSPA within tick gut. Magnified view of the attachment site of *B. burgdorferi* (area indicated by box in [D]) to the microvilli of tick gut epithelial cells expressing TROSPA. Spirochetes were labeled with antisera raised against whole *B. burgdorferi* N40 (small, 5 nm gold particles, asterisk). TROSPA was detected using a rabbit TROSPA antisera (larger, 10 nm gold particles, thin arrows).

with the gut tissue of *I. scapularis* nymphs—TROSPA was predominantly localized in the intercellular spaces and luminal surface of the gut (Figure 2D). We further studied the distribution of TROSPA and *B. burgdorferi* within the infected tick gut. *B. burgdorferi* produce OspA in the vector, and immunofluorescence studies using TROSPA and OspA antibodies demonstrated colocalization of *B. burgdorferi* and TROSPA within the tick gut (Figure 2E). We next used immunoelectron microscopy to examine the precise subcellular localization of TROSPA within the *I. scapularis* gut. The gut is mostly comprised of one to three tiers of epithelial cells resting on a thick basal lamina and the epithelial membrane toward the apical surface project short microvilli interdigitating in a narrow lumen (Figure 3A). The adjacent cells are separated by a highly folded plasma membrane forming "tight junctions" (short arrows, Figure 3A–3C), which maintain the integrity of the gut epithelial barrier between cells (Sauer and Hair, 1986; Sonenshine, 1993). In the *I. scapularis* gut, we detected heavy labeling of TROSPA along such tight junctions (579 $\pm$ 110 gold particles/μm^2) (Figure 3C). Organelle structures resembling Golgi bodies or endoplasmic reticulum were not obvious within the cells; however, we detected a low level of TROSPA labeling within the cytoplasm (36 $\pm$ 13 gold particles/μm^2) or within microvilli (115 $\pm$ 48 gold particles/μm^2) (Figure 3C). Negligible labeling ($<$1 gold particles/μm^2) was noted in cytoplasmic organelles such as the mitochondria, lysosomes, and residual bodies or within the nucleus. As expected, preimmune rabbit sera (control) did not show significant reactivity to tick gut epithelial cells ($<$1 gold particles/μm^2) (Figure 3B). Colocalization of *B. burgdorferi* and TROSPA using immunoelectron microscopy indicated specific TROSPA labeling within the tick gut where spirochetes are closely associated with the luminal microvilli (Figures 3D and 3E). Some TROSPA labeling was also detected on the surface of the bacterium, suggesting the presence of the soluble form of TROSPA in ticks (Figure 3E).

OspA Specifically Binds to Recombinant TROSPA

We next assessed whether TROSPA binds to OspA by performing a series of in vitro binding assays. The first set of assays evaluated the interaction between TROSPA and recombinant OspA (Figures 4A–4C). Labeled OspA specifically bound to both recombinant TROSPA as well as native protein(s) in nymphal *I. scapularis* gut extracts (Figure 4A). Binding kinetics of a fixed concentration of OspA with increasing amounts of TROSPA demonstrated that the OspA-TROSPA binding was saturable (Supplemental Figure S2). An independent, surface plasmon resonance (SPR)-based assay further demonstrated high affinity of OspA-TROSPA interaction with a calculated K_d of 42.6 $\pm$ 16.3 nM (Supplemental Figure S3). OspA did not bind to control proteins including maltose binding protein, bovine serum albumin, or other recombinant *I. scapularis* proteins such as Salp16 or Salp15, which are expressed selectively in the salivary glands (Das et al., 2001); or Salp25D, which is expressed in both the salivary glands (Das et al., 2001) and gut (our unpublished data) (Figure 4A). Recombinant *I. scapularis* proteins used in the assay covered a wide range of charges and molecular masses such as Salp16 (pI 4.7/16 kDa), Salp25D (pI 5.7/25 kDa), and Salp15 (pI 9.7, 14.7 kDa) (Das et al., 2001). *B. burgdorferi* OspB is 53% identical and 63% similar to OspA and also expressed within ticks; however, FITC-labeled OspB did not bind to TROSPA (data not shown). Competition assays further demonstrated the specificity of the OspA-TROSPA interaction. Unlabeled OspA significantly inhibited the binding of labeled OspA to TROSPA in a concentration-dependent fashion ($p < 0.01$, Figure 4B). Finally, either pretreatment of immobilized tick gut extracts prepared from nymphal *I. scapularis* with antisera to TROSPA or preincubation of labeled OspA with recombinant TROSPA both significantly ($p < 0.01$) inhibited binding of labeled OspA to nymphal tick gut lysates (Figure 4C).

In the second series of assays, we tested the binding of soluble TROSPA to immobilized spirochetes. *B. burgdorferi* lysates (Figure 4D) or intact unfixed *B. burgdorferi* (Figures 4E and 4F) were immobilized on microtiter wells or glass slides, respectively, and incubated with TROSPA. Binding of TROSPA to *B. burgdorferi* was detected using antisera against TROSPA. TROSPA specifically bound to OspA-producing *B. burgdorferi* lysates (Figure 4D) or to intact spirochetes (Figure 4E). Markedly less binding of TROSPA to the OspA-deficient *B. burgdorferi* was noted when compared to OspA-expressing *B. burgdorferi* (Figures 4D and 4F). Additionally, an OspA antibody also inhibited the binding of TROSPA to wild-type *B. burgdorferi* (Figure 4D). *B. burgdorferi* FlaB antibody (Pal et al., 2000) did not significantly inhibit the binding of TROSPA to wild-type *B. burgdorferi* (data not shown). Taken together, these experiments demonstrate the specificity of the TROSPA-OspA interaction.

Expression of *TROSPA* within Ticks

We then determined whether *TROSPA* was regulated during tick development, feeding, or *B. burgdorferi* infestation. To measure the levels of *TROSPA* mRNA during tick development, quantitative RT-PCR was performed using β-*actin* as an internal control (Figure 5A). *TROSPA* was highly expressed in immature stages of ticks such as larva (7.5 $\pm$ 0.2 *fg*/μg total RNA) and nymphs (3.0 $\pm$ 0.5 *fg*/μg total RNA). *TROSPA* expression was detectable in adults (0.21 $\pm$ 0.03 *fg*/μg total RNA); however, the level was significantly lower than that in larva or nymphs ($p < 0.01$) (Figure 5A).

To correlate *TROSPA* expression in ticks to *B. burgdorferi* binding within the tick gut, we next studied whether a lower level of TROSPA within adult ticks also resulted in their diminished capacity to bind OspA or the OspA-producing intact bacteria. We used equal amounts of prepared gut lysates from nymphal and adult ticks to assess the binding of OspA or *B. burgdorferi*. The decreased level of *TROSPA* mRNA in adults (Figure 5A) correlated with diminished binding of OspA or OspA-producing intact *B. burgdorferi* to the gut lysates from adult ticks in comparison to nymphs ($p < 0.01$, Figure 5B).

Before migrating to mammals, *B. burgdorferi* spend long intervals in the gut of unfed *I. scapularis* nymphs. Therefore, we tested the possibility that *B. burgdorferi* influences *TROSPA* expression, as it could be advantageous for the spirochete to induce the tick to produce abundant TROSPA. We performed quantitative RT-PCR

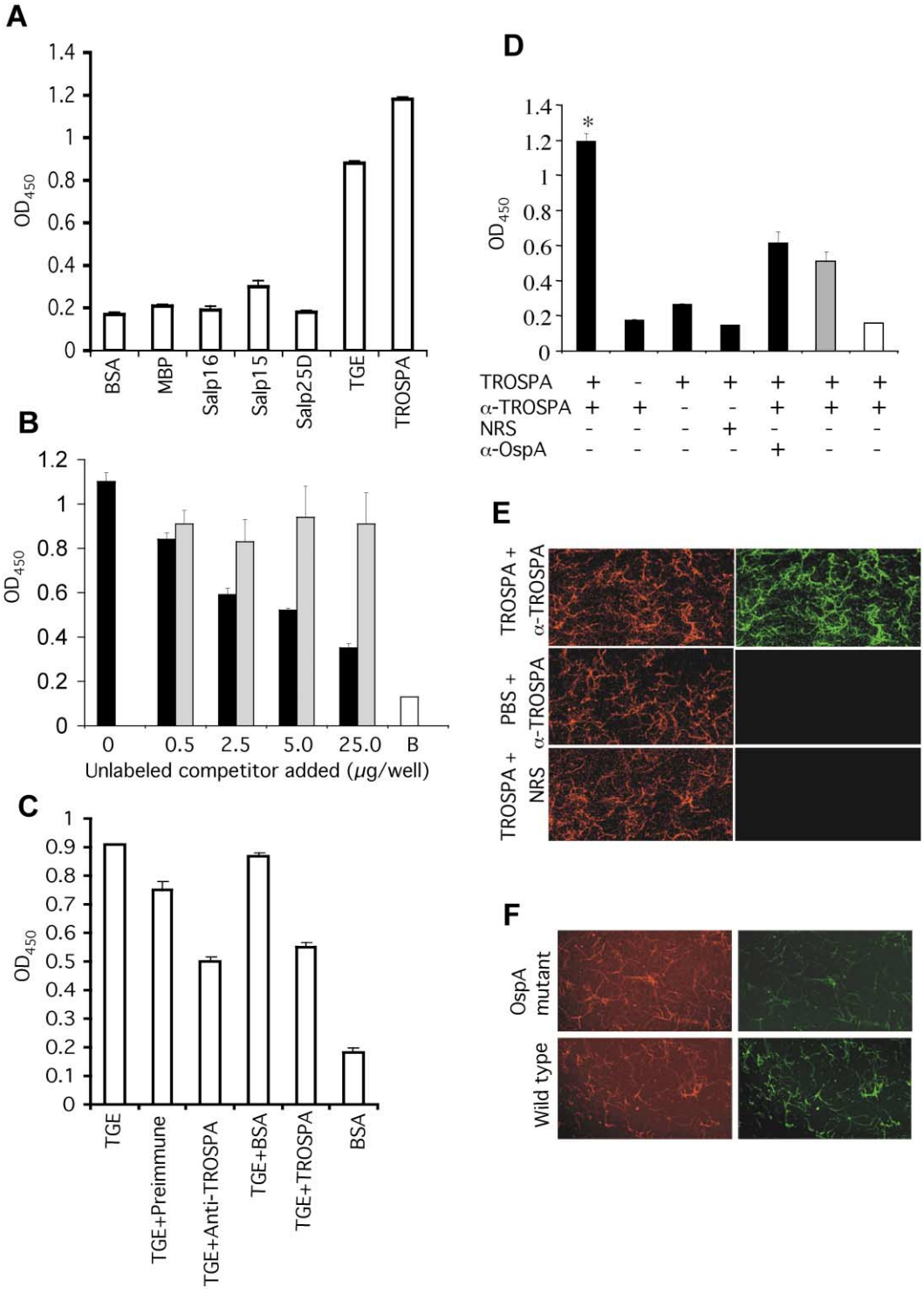

Figure 4. Interaction between OspA and TROSPA In Vitro

Bars represent mean ± SE from three experiments. (A) OspA directly binds to TROSPA. Bovine serum albumin (BSA); recombinant maltose binding protein (MBP); recombinant tick proteins Salp16, Salp15 and Salp25D; tick gut extracts (TGE); or recombinant TROSPA were immobilized onto microtiter wells (5 μg/ml), probed with FITC-labeled OspA, and recognized by HRP-labeled anti-FITC IgG. Difference between TROSPA or TGE with controls was significant, $p < 0.01$. (B) Inhibition of the OspA-TROSPA interaction using increasing amounts of unlabeled OspA as competitor. Recombinant TROSPA were immobilized onto microtiter wells (150 ng/well) and probed with FITC-labeled OspA (1 μg/well) in the absence (bar marked 0) or presence of increasing amounts (0.5–25 μg/well) of unlabeled OspA (black bars) or a control *B. burgdorferi* outer surface protein, BmpA (pI 5.2/36.9 kDa) (gray bars). Unlabeled OspA significantly inhibited the binding of labeled OspA to TROSPA at all concentrations used ($p < 0.01$ or lower) in comparison to BmpA, which was unable to inhibit the binding. A similar result was also obtained when BSA was used as a control competitor instead of BmpA (data not shown). Background binding of FITC-labeled OspA to PBS-coated

on equal amounts of cDNA prepared from *B. burgdorferi*-infested or naive *I. scapularis* nymphs. We detected a significant increase in *TROSPA* transcripts in infected compared to uninfected ticks ($p < 0.03$, Figure 5C). The increase in *TROSPA* mRNA was attributed to the presence of viable spirochetes as confirmed by the detection of *B. burgdorferi ospA* and *flaB* mRNAs by RT-PCR. Gut epithelial cells of infected or naive ticks were similar in number and structure (data not shown), suggesting that the observed difference in *TROSPA* transcripts was due to the presence of the spirochetes.

We next studied expression of *TROSPA* during tick feeding using cDNA prepared from unfed or fully engorged nymphs. We detected a significant decrease ($p < 0.01$) in the amount of *TROSPA* mRNA in nymphal ticks after feeding (Figure 5C). Since we found a downregulation of *TROSPA* during tick feeding, as opposed to a *TROSPA* upregulation in unfed ticks by spirochetes (Figure 5C), we assessed whether *TROSPA* mRNA levels were affected in ticks feeding on spirochete-infected or naive mice. *TROSPA* mRNA levels in these fed nymphs showed no significant differences (data not shown), demonstrating that downregulation of *TROSPA* during feeding is not influenced by spirochete infection.

TROSPA Antibodies Interfere with Tick-*B. burgdorferi* Interactions

We then examined the biological significance of the OspA-TROSPA interaction using a murine model of Lyme borreliosis to determine whether TROSPA antisera could influence the colonization of the *I. scapularis* gut in vivo by *B. burgdorferi*. Groups of five C3H mice were intradermally challenged with *B. burgdorferi* N40 (10^5 spirochetes/mouse). At 3 weeks, all the mice had swelling of the tibiotarsal joints, and spirochete infection was confirmed by culture. TROSPA antisera or preimmune rabbit sera (control) was administered to different groups of animals. Twenty-four hours later, ten uninfected *I. scapularis* nymphs or 20 larvae were allowed to feed to repletion on each mouse. The engorged ticks were dissected, and the luminal contents were examined for *B. burgdorferi*. Equal numbers of viable spirochetes were detected in preimmune or TROSPA antisera-treated groups, suggesting that TROSPA antisera treatment did not interfere with the migration of *B. burgdorferi* into the tick gut (data not shown). While TROSPA antisera reacted with the luminal surface of the fed gut, no significant alterations in gut morphology were observed by confocal microscopy of nymphs treated with preimmune or TROSPA antisera.

While the number of spirochetes, which arrived in the tick gut, was not affected by the presence of TROSPA antisera, we did detect a significant difference in *B. burgdorferi* numbers that were tightly associated with the gut tissue. The role of TROSPA antisera in *B. burgdorferi* adherence to the tick gut was examined at 24, 48, 72, and 96 hr after feeding in engorged ticks that had fed on mice treated with control or TROSPA antisera. Gut tissue was washed free of luminal contents or unbound spirochetes and assessed by immunofluorescence confocal microscopy. A large number of *B. burgdorferi* in the control groups (preimmune serum) was found in close proximity to the gut luminal tissue (Figure 6A, left panel). In contrast, TROSPA antisera-treated samples had very few spirochetes associated with the gut at 48 hr (Figure 6A, right panel, and Figure 6B, $p < 0.001$). This reduction in spirochete numbers in TROSPA antisera-treated samples was noted at all time points analyzed (data not shown). This observed reduction in *B. burgdorferi* number in antisera-treated ticks was further evaluated by RT-PCR analyses of *ospA* and *flaB* transcripts for detection of viable spirochetes, either from fed larval (data not shown) or nymphal ticks (Figure 6C), after seven days of engorgement. Significantly lower numbers of viable spirochetes were evident in the TROSPA antisera-treated group. Results similar to those shown in Figures 6A and 6B were obtained when some of the larvae and nymphs from the preimmune or TROSPA antisera-treated group were allowed to molt into nymphs or adults respectively and then analyzed by confocal microscopy and RT-PCR.

We next assessed whether diminished spirochete colonization of the tick gut in the TROSPA antisera-treated group (Figures 6A and 6B) also resulted in a decreased capacity to transmit infection to naive mice. TROSPA antisera or preimmune rabbit sera (control) were passively transferred into groups of five C3H mice that had been infected with *B. burgdorferi* N40 (10^5 spirochetes/mouse) for 3 weeks. Larval ticks (50 ticks/group) were allowed to feed to repletion on these mice and kept in the laboratory to molt into nymphs. Newly molted nymphs (from the TROSPA antisera- or control sera-treated, three ticks/group) were then allowed to feed to repletion on groups of naive C3H mice. Murine skin samples were collected 3 days following tick feeding and assessed

wells (white bar marked B) is indicated. (C) TROSPA antisera or recombinant TROSPA protein interferes with OspA binding to native protein(s) in TGE. Microtiter wells were coated with TGE and probed with FITC-labeled OspA in the absence (bar marked TGE) or presence of competitors including preimmune sera (TGE + Preimmune), TROSPA antisera (TGE + Anti-TROSPA), BSA (TGE + BSA), recombinant TROSPA (TGE + TROSPA). Background binding of FITC-labeled OspA to BSA-coated wells (bar marked BSA) is also shown. $p < 0.001$ between TGE and (TGE + Anti-TROSPA), $p < 0.007$ between TGE and (TGE + TROSPA), whereas difference between TGE and controls (TGE + Preimmune or TGE + BSA) is insignificant, $p > 0.05$. (D) TROSPA binding is specific to *B. burgdorferi*. Soluble proteins from wild-type *B. burgdorferi* (black bar), an OspA-deficient *B. burgdorferi* (gray bar), or BSA (white bar) were coated onto microtiter wells. Bound TROSPA was detected using TROSPA antisera (α-TROSPA) or normal rabbit sera (NRS) followed by HRP-conjugated secondary antibody. Specificity of binding was tested by addition (indicated by +) or absence (indicated by −) of TROSPA protein, OspA antibody (α-OspA), or TROSPA antiserum. *, difference (first bar) with controls (bars 2–7) is significant, $p < 0.002$. (E) TROSPA directly binds to the surface of intact *B. burgdorferi*. Unfixed *B. burgdorferi* were immobilized onto glass slides and incubated in the presence or absence of recombinant TROSPA. Binding was detected using TROSPA antisera or normal rabbit sera (NRS) followed by incubation with anti-rabbit IgG-FITC. Samples were counterstained with propidium iodide (left panels) to visualize spirochetes. Images were collected using a 40× objective lens on the confocal microscope. (F) TROSPA binds poorly to the surface of intact OspA-deficient *B. burgdorferi*. Unfixed *B. burgdorferi* were immobilized onto glass slides, incubated with TROSPA, and processed similarly as described in (E).

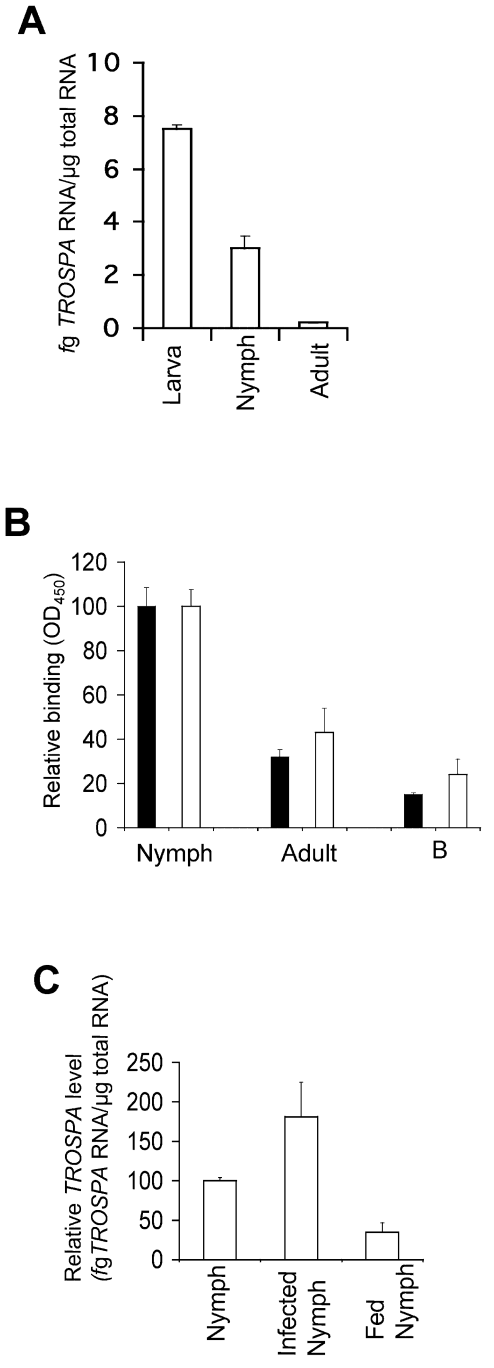

Figure 5. Expression of *TROSPA* within *I. scapularis*

Bars represent mean ± SE from three experiments. (A) *TROSPA* is downregulated during the development of *I. scapularis*. Total RNA was prepared from larva (n = 100), nymph (n = 25), or adults (n = 10), and amounts of *TROSPA* were measured by quantitative PCR. Differences between *TROSPA* amounts in larva or nymph with adult is significant, $p < 0.01$. (B) Expression of *TROSPA* correlated with *B. burgdorferi* OspA or intact *B. burgdorferi* binding to the tick gut. Lysates from adult (n = 10) or nymphal guts (n = 50) were prepared, and equal amount of protein was used to assess binding of FITC-labeled OspA (black bars) or intact spirochetes (white bars). Bound OspA was measured using a HRP-labeled anti-FITC IgG, whereas bound *B. burgdorferi* were detected using a rabbit antiserum raised against *B. burgdorferi* and HRP-labeled anti-rabbit IgG. Low *TROSPA* expression in adult ticks (A) is reflected in low OspA and *B. burgdorferi* binding capacity of adult guts. Background binding of OspA or *B. burgdorferi* to FCS (control) is also shown (B). Binding of nymphs to OspA or *B. burgdorferi* was considered as a 100% value. Differences of OspA or *B. burgdorferi* binding between nymphs and adults are significant, $p < 0.01$. (C) *TROSPA* expression is altered in different physiological states of nymphal *I. scapularis*. The presence of *B. burgdorferi* within unfed *I. scapularis* enhanced the amount of *TROSPA* transcripts ($p < 0.03$), whereas the amount of *TROSPA* RNA decreased during tick feeding ($p < 0.01$). Total RNA was prepared from naive (n = 100), *B. burgdorferi*-infested (n = 100), or fed nymphs (n = 25) and assessed by quantitative RT-PCR. Amount of *TROSPA* transcripts within unfed naive nymphs was considered as the 100% value.

for *B. burgdorferi* transmission by quantitative PCR. TROSPA antisera significantly reduced (approximately 75%) the migration of spirochete into mice, indicating that diminished colonization of the tick gut reduced the number of spirochetes that were transmitted to the mammalian host (Figure 6D, $p < 0.001$). *B. burgdorferi* begin to multiply once they reach murine skin (Malawista, 2000; Nadelman and Wormser, 1998; Steere et al., 2004), and viable spirochetes were recovered from murine skin samples at 2 weeks following tick feeding.

TROSPA-Deficient Ticks Have Reduced *B. burgdorferi* Colonization

To confirm the role of TROSPA in mediating *B. burgdorferi* colonization within the tick gut, we generated TROSPA-deficient nymphal ticks using RNA interference (RNAi) (see Experimental Procedures). *TROSPA* dsRNA and *salp15* (Das et al., 2001) dsRNA were synthesized in vitro. *salp15* was chosen as a control for the TROSPA RNAi studies because it is a well-characterized *I. scapularis* gene (Das et al., 2001). Injection of *salp15* dsRNA results in downregulation of *salp15*, which, however, does not interfere with the tick feeding (data not shown). Equal volumes of the respective dsRNA or buffer were delivered directly into the gut of naive *Ixodes* nymphs (50 ticks/group) via microinjection (Pal et al., 2004). Ticks were allowed to rest for 3 hr and placed on C3H mice (three mice/group) that were infected with *B. burgdorferi* N40 (10^5 spirochetes/mouse) for 3 weeks. At 48 hr following the onset of feeding, RT-PCR or Western blot assessment indicated a dramatic reduction in the levels of *TROSPA* mRNA and protein (Figures 6E and 6F) within *TROSPA* dsRNA-treated tick guts, compared to *salp15* dsRNA-treated or buffer-treated groups. In contrast, both the mRNA and protein levels of two control genes—*actin* (Figures 6E and 6F) and *salp25D* (data not shown)—remained unaltered in both dsRNA- and buffer-treated groups.

We then assessed whether colonization of spirochetes was affected by RNAi-mediated TROSPA deficiency. Guts isolated from *TROSPA* dsRNA, *salp15* dsRNA, or buffer-treated ticks were assessed for spirochete-tick gut associations as described above (Figure 6A). A significant reduction in the number of *B. burgdorferi* closely associated with the gut was noted in *TROSPA* dsRNA-treated ticks (Figures 6G and 6H, $p < 0.001$), as compared to *salp15* dsRNA or buffer-treated ticks confirming a definitive role for TROSPA in facilitating colonization of the tick gut by spirochetes.

Discussion

Tissue adherence capabilities of pathogenic bacteria are essential for their survival and persistence within host tissues (Finlay and Cossart, 1997). *B. burgdorferi* prominently produce OspA within the vector (de Silva et al., 1996; Schwan and Piesman, 2000), OspA mediates spirochete-*I. scapularis* adherence (Pal et al., 2000), and the targeted ablation of OspA prevents *B. burgdorferi* colonization of ticks in vivo, highlighting the importance of OspA-based binding of the spirochetes to the *I. scapularis* gut (Pal et al., 2001; Yang et al., 2004). It is likely that this event involves both vector and pathogen gene products (Munderloh and Kurtti, 1995). In the present study, we have identified an arthropod receptor, TROSPA, that is required for the successful colonization of ticks by spirochetes.

Several lines of evidence demonstrate that *I. scapularis* TROSPA is a specific ligand for *B. burgdorferi* OspA. The OspA-TROSPA interaction suggested by the yeast two-hybrid screen was directly confirmed in a series of in vitro assays where OspA selectively bound TROSPA but not to other tick proteins. TROSPA antisera interfered with OspA binding to native tick gut proteins; however, partial inhibition of this attachment indicated that other pathways might also exist (Figure 4B). Recombinant TROSPA also directly adhered to the *B. burgdorferi* surface or whole-cell lysates, and an OspA antibody inhibited this interaction. Finally, compared to OspA-producing wild-type spirochetes, TROSPA bound poorly to OspA-deficient *B. burgdorferi*.

The natural cycle of the Lyme disease spirochete reflects concomitant temporal expression of *ospA* and *TROSPA*. Inside ticks, *B. burgdorferi* copiously express *ospA* (de Silva et al., 1996; Schwan and Piesman, 2000), and, at the same time, *I. scapularis* express abundant *TROSPA*. Following tick engorgement, *TROSPA* expression decreases, paralleling the downregulation of *ospA* by *B. burgdorferi*. Both these changes may help spirochete detachment from the gut for transmission to a new host (de Silva et al., 1996; Schwan and Piesman, 2000). In nature, spirochete colonization of immature stages of *I. scapularis* is essential for the continuity of the *B. burgdorferi* life cycle in mice and ticks. In contrast, although adult ticks are colonized by spirochetes, they do not feed upon mice or participate in the perpetuation of the *B. burgdorferi*-mouse life cycle (Nadelman and Wormser, 1998; Steere, 2001). Adult *I. scapularis* expressed significantly less *TROSPA* than larvae or nymphs and bound less *B. burgdorferi* in vitro than nymphs, suggesting that either a lower level of TROSPA is sufficient for adults to maintain *B. burgdorferi* or low TROSPA levels in adults could be compensated by other binding pathways.

Although *B. burgdorferi* spends a significant amount of time inside *I. scapularis*, the spirochetes have not previously been shown to influence the vector. We therefore determined whether *B. burgdorferi* altered *I. scapularis* gene expression. Most interestingly, we observed a significant increase in the expression of *TROSPA* when *I. scapularis* were infested with spirochetes compared to ticks that did not harbor *B. burgdorferi*. *TROSPA* upregulation could potentially increase the OspA-TROSPA-mediated colonization of ticks by *B. burgdorferi*.

We finally evaluated whether blocking of TROSPA within *I. scapularis* interferes with the life cycle of *B. burgdorferi*. In vivo studies using a murine model of Lyme borreliosis show that TROSPA antisera significantly reduced the ability of spirochetes to colonize both larval and nymphal *I. scapularis*. Fewer *B. burgdorferi* persisted throughout the postfeeding phases in antisera-treated groups, and these spirochetes had a diminished capability to be transmitted to a new host. These findings demonstrate that TROSPA-based tissue colonization is an essential prerequisite for *B. burgdorferi* to persistently infect ticks and for their subsequent migration to new host. A similar result was obtained in RNAi studies where we have shown that selective knock down of TROSPA directly interferes with colonization of spirochetes within the tick gut. We noted no significant changes in gut morphology, feeding behavior, or molting of ticks treated either with TROSPA antisera or *TROSPA* dsRNA, suggesting that the effects of the dsRNA or antisera on colonization were not due to indirect effects on tick biology but directly through TROSPA-OspA interaction. A small proportion of spirochetes, however, were still able to colonize, persist, and then be transmitted to a naive host, indicating that TROSPA may not be the only ligand utilized by spirochete in ticks.

In summary, we present here direct evidence for the existence of a vector molecule, which is actively involved in spirochete colonization and persistence within ticks. Many arthropod-borne pathogens follow a complex enzootic life cycle involving a wide variety of hosts, which is mediated, at least partly, by expression of a distinct set of receptors in different tissue environments. Identification of TROSPA as an important ligand at the *B. burgdorferi*-tick gut interface establishes a paradigm for exploring how these interactions support long-term persistence of pathogens within the vector. In nature, large numbers of human pathogens persist within selective arthropod vectors. Understanding the fine molecular details of vector-pathogen interactions will lead to the development of novel therapeutic or vaccine strategies that can be rationally designed to target the pathogen life cycle within the arthropod host.

Experimental Procedures

Ticks, Bacteria, and Mice

The ticks used in this study originated from adult female *I. scapularis* ticks collected in the field. Development and rearing of various stages of ticks were performed in the laboratory as described (Pal et al., 2000). A low-passage (passage 3) virulent clonal isolate of *B. burgdorferi* N40 was used throughout (Pal et al., 2000); in vitro binding assays also employed an OspA-deficient *B. burgdorferi* (Yang et al., 2004). Six- to eight-week-old C3H/HeN mice were purchased from the National Institutes of Health.

Construction of Tick cDNA and Genomic DNA Libraries

Tick RNA (400 μg) was isolated from 5000 unfed *I. scapularis* nymphs using an RNA isolation kit (Clontech) and used to construct an *I. scapularis* cDNA plasmid library into the EcoRI and NotI cloning site of the yeast-*E. coli* shuttle vector pYESTrp2 (prepared by Research Genetics-Invitrogen). The library had a titer of 1×10^6 cfu/100 ng, and over 90% of the clones contain inserts that range from 0.5 to 4 kb. Known tick genes such as *salp15, salp16*, *salp25D* (Das et al., 2001), and β-*actin* (Pal et al., 2004) were readily amplified from the library by PCR.

An *I. scapularis* genomic DNA library was prepared in a lambda

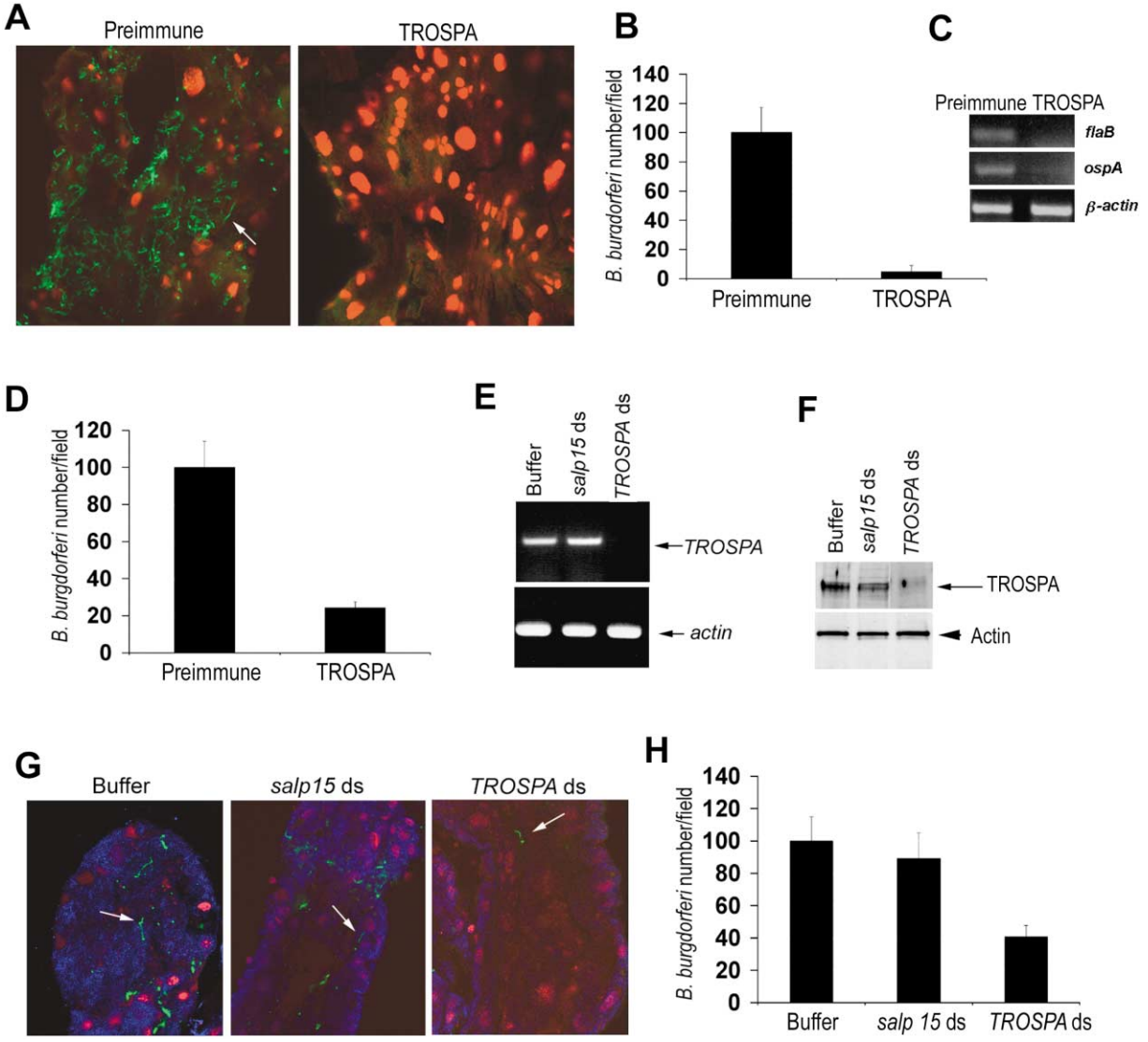

Figure 6. TROSPA Antisera and *TROSPA* dsRNA Interfered with the Colonization of *B. burgdorferi* within *I. scapularis* and Transmission to Murine Host

(A) TROSPA antisera interfere with the colonization of *B. burgdorferi* within the tick gut. Nymphal ticks were allowed to engorge on *B. burgdorferi*-infected mice that had been treated with TROSPA antisera or preimmune sera (control). Guts were analyzed by confocal microscopy at 48 hr post feeding. The spirochetes (arrow) were stained with a FITC-labeled goat anti-*B. burgdorferi* antibody (green), and the nuclei of the tick gut epithelial cells were stained with propidium iodide (red). Images were examined using the confocal 40× objective lens and presented as a single image for clarity. Data are the representation of three independent experiments.

(B) Quantitative assessment (relative number of *B. burgdorferi*) of the data presented in (A). Groups of five mice were infected with *B. burgdorferi* before administration of either preimmune or TROSPA antisera at 24 hr before tick placement (ten nymphs/mouse). Engorged ticks were examined at 48 hr after detachment using confocal microscopy to enumerate *B. burgdorferi* in direct contact with tick gut tissue. In each experiment, the entire gut diverticulum was examined, and counts were made from at least three fields from two separate gut diverticula. The average number of microscopic fields/diverticula was six; range, two to seven fields/diverticulum. Data are the number of spirochetes per field from three independent experiments (mean ± SE). Differences between preimmune- and TROSPA antisera-treated groups were highly significant ($p < 0.001$). Numbers of *B. burgdorferi* in preimmune sera-treated group (control) were considered as a 100% value.

(C) Detection of *B. burgdorferi* mRNA within engorged *I. scapularis*. TROSPA antisera or preimmune sera-treated nymphs (as described in [A]) were analyzed at 7 days post feeding by RT-PCR using *flaB* or *ospA* primers for the detection of viable *B. burgdorferi*. *I. scapularis* β-*actin* was used as a control to ensure the equal loading of RNA. An aliquot of prepared RNA from each group was subjected to RT-PCR in the absence of reverse transcriptase to ensure the absence of genomic DNA (data not shown).

(D) TROSPA antisera dramatically reduced the transmission of *B. burgdorferi* to mice. Larval ticks were allowed to feed to repletion on *B. burgdorferi*-infected groups of three mice (50 ticks/mouse) (similar to [A]) that had been treated with TROSPA antisera or preimmune sera (control) and allowed to molt into nymphs. Antisera-treated newly molted nymphs were allowed to engorge on groups of three naive mice (three ticks/mouse), and murine skin samples were assessed for spirochete transmission after 3 days of feeding. DNA was isolated from skin samples of different groups of mice, and *B. burgdorferi flaB* was measured using quantitative PCR. Data are the number of spirochetes per field from two independent experiments (mean ± SE). Difference in the spirochete transmission in TROSPA antisera-treated group with the control is highly significant ($p < 0.001$). *B. burgdorferi flaB* copy numbers in preimmune sera-treated group (control) was considered as a 100% value.

Fix II vector (Stratagene), which accepts DNA inserts ranging from 9 to 23 Kb. DNA (200 μg) isolated from nymphal ticks, partially digested with Sau3AI, filled on and cloned in the XhoI half site arms of the vector. The ligated DNA was packaged into phage as per the manufacturer's instructions and stored at −70°C. The primary library had 1.4×10^6 recombinant clones, and 97% of the phages contained an insert.

Yeast Two-Hybrid Screening

The *I. scapularis* cDNA library constructed in a yeast-*Escherichia coli* shuttle vector was screened using a LexA-OspA fusion protein as the bait as detailed in the Supplemental Experimental Procedures.

Sequence Analysis

The cDNA sequences were obtained by the dye terminator sequencing method performed at the HHMI Biopolymer and W.M. Keck Biotechnology Resource Laboratory at Yale University. Database searches were made using WWW BLAST server in NCBI and EMBL. Sequence analysis was performed using ExPASY proteomics tools (www.expasy.ch/tools) as well as Lasergene software (DNAstar).

Rapid Amplification of cDNA Ends

Amplification of 5′ upstream of yeast two-hybrid *TROSPA* cDNA was performed using either a SMART (*s*witching *m*echanisms at 5′ end of *R*NA *t*ranscript, Clontech) or RLM (*R*NA *l*igase-*m*ediated, Ambion) RACE procedures. RNA (10 μg of total) was isolated from *I. scapularis* nymphs and used in RACE reactions according to the manufacturer's instruction with the following *TROSPA* specific reverse primers: 5′-GACAACTGGAGCGGCAGCGACAGCGA-3′, 5′-TGTAGCTGGAGCTGACGATTC-3′, and 5′-TGGTGTAGCTGGAGCT-3′. Amplified RACE products were subcloned into pCR 2.1 vector (Invitrogen) and sequenced.

Hybridization Screening of *I. scapularis* cDNA and Genomic DNA Libraries

We screened the tick genomic DNA library, the pYESTrp2 cDNA library, and an additional 48 hr fed nymphal *I. scapularis* cDNA library made in the phagemid vector pBK-CMV (Stratagene) constructed in the laboratory of Aravinda de Silva. The libraries were screened with a 689 bp *TROSPA* cDNA probe using Gene Image and CDP-Star kit as described in the manufacturer's protocol (Amersham-Pharmacia Biotech).

Protein Expression and Preparation of Polyclonal Antibody

Production of recombinant TROSPA in *E. coli* and *Drosophila* S2 cells and generation of polyclonal antibodies are detailed in the Supplemental Experimental Procedures.

Western Blotting

Western blotting was performed as described (Pal et al., 2001) using 1:500–5000 dilution of either of the following primary antibodies: rabbit GST antisera (Amersham-Pharmacia Biotech), murine or rabbit preimmune or TROSPA antisera, guinea pig anti-Salp25D (Das et al., 2001), or actin antisera (Sigma). Production of TROSPA in *Drosophila* S2 cell line was also detected using an HRP-conjugated anti-V5 antibody (Invitrogen). In some experiments, tick gut lysates were treated with a variety of glycosidases as detailed (Pal et al., 2000), and Western blotting was performed with TROSPA antisera as described above.

Two-Dimensional Gel Electrophoresis and Mass Spectrophotometry

The total gut proteins (100 μg) were resolved by two parallel two-dimensional SDS-PAGE gels (2D gel). The 2D gel was comprised of a nonlinear immobilized pH 3–10 gradient in the first dimension and 12% gel in the second dimension. Proteins from one gel were transferred electrophoretically onto polyvinylene difluoride membranes, which were probed with TROSPA antisera. The same blots were stripped and reprobed with GST antisera (control) to identify nonspecific hybridization. We contemplated that TROSPA antisera might contain a small amount of IgG directed toward GST, since trace amounts of GST could copurify with TROSPA used as antigen to raise antisera in rabbits. TROSPA immunoreactive proteins were detected by HRP-conjugated secondary antibody on an autoradiography film. The film was overlaid on the second 2D gel; the anti-TROSPA immunoreactive gel spot was isolated and characterized by tryptic peptide mass fingerprinting using LC-MS/MS analysis on a Waters Q-Tof API Mass Spectrometer at the HHMI Biopolymer and W.M. Keck Biotechnology Resource Laboratory at Yale.

Confocal and Immunoelectron Microscopy

Organs from nymphal ticks were prepared for confocal (Pal et al., 2001) and immunoelectron microscopy (Folsch et al., 2001) as detailed in the Supplemental Experimental Procedures.

In Vitro Binding Studies: Microtiter Assay, Immunoflorescence, and Surface Plasmon Resonance

In vitro binding of tick gut lysates or recombinant TROSPA with OspA as well as TROSPA binding to *B. burgdorferi* was performed as described (Pal et al., 2000) and further detailed in the Supplemental Experimental Procedures.

PCR

RT-PCR and quantitative PCR analysis of *I. scapularis TROSPA* or β-*actin*, *B. burgdorferi* flagellin (*flaB*) or *ospA*, and murine β-*actin* was performed as detailed in the Supplemental Experimental Procedures.

Passive Transfer of TROSPA Antisera and In Vivo Adherence Studies

Antisera transfer and in vivo adherence studies were performed as detailed before (Pal et al., 2001; Yang et al., 2004). Briefly, C3H mice

(E) Silencing of *TROSPA* transcripts induced by RNA interference. Data are the representation of three independent experiments. Nymphal ticks (50 ticks/group) were injected with *TROSPA* dsRNA (*TROSPA* ds), *salp15* dsRNA (*salp15* ds), or buffer alone (buffer) and fed onto *B. burgdorferi*-infected mice for 48 hr, and isolated guts were assessed by RT-PCR using primers to amplify a 515 bp *TROSPA* or a 400 bp β-*actin* fragment (arrow).

(F) Injection of *TROSPA* dsRNA selectively knocks down the native TROSPA protein. Guts from nymphal ticks, as described in (E), were probed with TROSPA or actin antisera in a Western blot. Position of 55 kDa native TROSPA denoted by arrow, whereas migration of 46 kDa actin is indicated by arrowhead.

(G) Detection of *B. burgdorferi*, which remained closely associated with gut tissue within *TROSPA* dsRNA, *salp15* dsRNA, or buffer-treated *I. scapularis*. Guts isolated from nymphal ticks, as described in (E), were processed for in vivo adherence studies using confocal immunofluorescence microscopy. The spirochetes (arrow) were stained with a FITC-labeled goat anti-*B. burgdorferi* antibody (green), TROSPA was labeled with a anti-rabbit IgG-Cy5 (blue), and the nuclei of the tick gut epithelial cells were stained with propidium iodide (red). Samples were examined using the confocal 40× objective lens, and one representative field from each RNAi group is presented as a single image for clarity.

(H) Quantitative assessment (relative number of *B. burgdorferi*) of the data presented in (G). *B. burgdorferi*, which remained in direct contact with tick gut tissue, were enumerated using confocal microscopy. In each experiment, counts were made from at least ten fields from five separate gut diverticulum. Data are the number of spirochetes per microscopic field from three independent experiments (mean ± SE). The average number of spirochetes/microscopic field in case of *TROSPA* dsRNA, *salp15* dsRNA, and buffer-treated ticks was 11 ± 2 (range, 1–37), 24 ± 4 (range, 7–68), and 27 ± 4 (range, 4–80), respectively. Differences of spirochete number between *TROSPA* dsRNA-treated group with either buffer-treated or *salp15* dsRNA treated groups were significant, $p < 0.001$. Numbers of *B. burgdorferi* in buffer treated group (control) were considered as 100% value.

were infected with *B. burgdorferi* N40 (10^5 spirochetes/mouse, five animals/group) for 3 weeks, and preimmune rabbit sera or TROSPA antisera were administered to groups of mice (250 μl intraperitoneally and 250 μl subcutaneously). Twenty-four hours later, 20 larva or ten *I. scapularis* nymphs were placed on each mouse. The animals were again treated with the respective sera on the next day. The ticks were allowed to feed to repletion and detach from the mice, which usually occurred between 72 and 96 hr. Guts from each group of nymphs were dissected under a microscope in PBS (20 μl/gut) after intervals of 24 hr until 96 hr following engorgement. Aliquots (5 μl) were examined for viable spirochetes under dark-field microscopy. A few larva or nymphs were subjected to RT-PCR 7 days following engorgement. Selected larva or nymphs from each group were also allowed to molt to nymphs or adults.

Organs from nymphal ticks were prepared for confocal microscopy as previously described by dissection of gut diverticula in PBS (Pal et al., 2001). Briefly, the lumen of each gut diverticulum was exposed and washed free of luminal contents or unbound spirochetes, placed on silylated glass slides, and fixed with acetone. The tissues were rinsed twice with PBS and incubated for 30 min in PBS-T with 5% normal goat serum (NGS) at room temperature. Organs were incubated with an affinity-purified FITC-labeled goat anti-*B. burgdorferi* antibody (KPL) at a dilution of 1:50 in PBS-T with 5% NGS at room temperature for 1 hr. The samples were counterstained with propidium iodide (50 μl of a 10 μg/ml solution), mounted in antifade reagent, and examined by scanning the entire organ in the confocal microscope.

RNA Interference

RNA interference techniques of nymphal *I. scapularis* were detailed in the Supplemental Experimental Procedures. Generation of dsRNA and microinjection into nymphal *I. scapularis* gut were performed as recently described (Narasimhan et al., 2004; Pal et al., 2004).

Statistical Analysis

Results are expressed as the mean ± standard error (SE). The significance of the difference between the mean values of the groups was evaluated by Student's t test or repeated-measure ANOVA with the Fisher's protected least significant difference test with Statview software (SAS Institute, Cary, NC). Binding analysis was performed using Origin software version 6.0 (OriginLab Corporation, MA).

Acknowledgments

We gratefully acknowledge the excellent efforts provided by Carolyn B. Marks for the immuno-EM study. We also sincerely thank Deborah Beck, Kathy DePonte, Kusumika Gharami, Kazi Mirajul Hoque, Nancy Marcantonio, Swapna Samanta, Michael Vasil, Terence Wu, and Kimberly Zichichi for their help. This work was supported by grants from the National Institutes of Health, Centers for Disease Control and Prevention, and American Heart Association. E.F. is the recipient of a Burroughs Wellcome Clinical Scientist Award in Translational Research.

Received: June 4, 2004
Revised: August 27, 2004
Accepted: October 27, 2004
Published: November 11, 2004

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Accession Numbers

The GenBank accession numbers for the *TROSPA* cDNA and genomic DNA sequences reported in this paper are AY189148 and AY640046, respectively.