MAJOR ARTICLE

Fibronectin Facilitates the Invasion of *Orientia tsutsugamushi* into Host Cells through Interaction with a 56-kDa Type-Specific Antigen

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Background. *Orientia tsutsugamushi,* the causative agent of scrub typhus, is an obligate intracellular bacterium. The pathogen's mechanism of cellular invasion is poorly characterized.

Methods. Through ligand immunoblots, glutathione S-transferase (GST) pull-down assays, and in vitro inhibition assays of intracellular invasion, a bacterial ligand was identified and was shown to interact with fibronectin (Fn) to enhance the intracellular invasion of *O. tsutsugamushi*.

Results. *O. tsutsugamushi* can bind to immobilized Fn in vitro, and exogenous Fn stimulates bacterial invasion of mammalian host cells. Bacterial invasion in the presence of Fn was abrogated by the addition of Arg–Gly–Asp peptides or by an anti–$\alpha5\beta1$ integrin antibody. Through a ligand immunoblot and GST pull-down assay, a 56-kDa type-specific antigen (TSA56) was identified as the bacterial ligand responsible for the interaction with Fn. Antigenic domain III and the adjacent C-terminal region (aa 243–349) of TSA56 interacted with Fn. Furthermore, we found that the enhanced invasion of the pathogen was abrogated by the addition of purified recombinant peptides derived from TSA56.

Conclusion. Fn facilitates the invasion of *O. tsutsugamushi* through its interaction with TSA56.

Orientia tsutsugamushi, an obligate intracellular bacterium, is the causative agent of scrub typhus [1], a disease characterized by fever, rash, eschar, pneumonitis, meningitis, and disseminated intravascular coagulation [2]. It can lead to severe multiorgan failure if left untreated, with mortality rates between 1% and 40%, depending on the area and the strain of *O. tsutsugamushi* encountered [2]. Scrub typhus is confined geographically; it extends from far eastern Russia and northern Japan in the north, to northern Australia in the south, and to Pakistan and Afghanistan in the west [1]. An estimated 1 billion people in this area are at risk for scrub typhus, and an estimated 1 million new cases arise annually [3]. Scrub typhus can be effectively treated with antibiotics, such as doxycycline and chloramphenicol. However, reinfection and relapses occur frequently because of the wide variety of antigenically distinct serotypes [4]. In addition, decreased efficacy of antibiotic treatments has been recently reported in several cases [5, 6]. In spite of the increasing numbers of patients and recurrent outbreaks of scrub typhus in areas of endemicity [6–8], a vaccine of satisfactory effectiveness has not yet been developed.

Bacterial infection of host cells is mediated primarily by the interactions between bacterial surface components, complementary host receptors, and extracellular matrix molecules. Fibronectin (Fn) is considered the most important extracellular matrix protein involved in the adherence and entry of bacteria into host cells [9–11]. Fn is an ~250-kDa glycoprotein with 2 homologous subunits joined near the C-terminal ends by 2 disulfide bonds [12]. The Fn monomer contains multiple functional domains, including 2 heparin-binding regions (designated Hep-1 and Hep-2), a gelatin-binding domain, and a central cell-binding domain that contains the Arg–Gly–Asp (RGD) motif required for the interaction with integrins on the cell surface [13]. Numerous bacterial pathogens can bind to Fn, including *Escherichia coli* [14], *Mycobacterium avium* [15], *Neisseria gonorrhoeae* [10], *Staphylococcus aureus* [16], *Streptococ-*

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cus pyogenes [11], *Campylobacter jejuni* [17], *Bartonella henselae* [18], and *Treponema* species [19, 20]. Bacterial adhesion to Fn is mediated by a ligand-receptor interaction, and different binding sites on Fn may be used by different bacterial species [16, 19, 21].

As an obligate intracellular bacterium, *O. tsutsugamushi* must be internalized into host cells through high-affinity binding to host cell receptors. Currently, the mechanism of cellular invasion of *O. tsutsugamushi* is poorly characterized [22–24]. In the present study, we determined that *O. tsutsugamushi* can bind to Fn and that this interaction facilitates bacterial invasion. We also identified and characterized a 56-kDa type-specific antigen (TSA56) on the surface of *O. tsutsugamushi* that mediates its specific interaction with Fn.

METHODS

Cell culture. L929 cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco BRL) that was supplemented with 10% fetal bovine serum (Gibco BRL), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies).

Bacterial inoculum. *O. tsutsugamushi* was purified using a modification of a Percoll gradient purification method [25]. The *O. tsutsugamushi* strain Boryong was infected and propagated in L929 cells. After 3–4 days of infection, infectivity was determined by an indirect immunofluorescence technique (see below) [26]. When an infection rate of >90% was achieved, cells were harvested by centrifugation at 6000 *g* for 20 min. The pellet was resuspended with 6.5 mL of Tris-sucrose (TS) buffer (33 mmol/L Tris-Cl [pH 7.4] and 0.25 mol/L sucrose). Resuspended cells were homogenized with a Polytron homogenizer (Wheaton) for 100 strokes and centrifuged at 200 *g* for 5 min. The supernatant was mixed with 40% Percoll medium (Pharmacia) in TS buffer and centrifuged at 25,000 *g* for 60 min. The bacterial band was collected and centrifuged at 77,000 *g* for 30 min. *O. tsutsugamushi* were collected and washed 3 times with TS buffer. The *O. tsutsugamushi* pellet was resuspended in DMEM and stored in liquid nitrogen until use. The infectivity titer of the inoculum was determined as described elsewhere [26], with modification, and infected-cell-counting units (icu) were calculated as follows: (total number of cells used for infection) $\times$ (percentage of infected cells) $\times$ (dilution rate of the *O. tsutsugamushi* suspension)/100 [26]. For all of the infection assays, 2.5×10^6 icu of *O. tsutsugamushi* were used to infect L929 cells cultured in 24-well plates.

Binding of* O. tsutsugamushi *to immobilized Fn. Glass coverslips (12 mm in diameter) in 24-well tissue culture plates were coated with 100 μg/mL human plasma Fn, vitronectin (Vn; Calbiochem), or bovine serum albumin (BSA; Sigma) overnight at 4°C. After being washed 3 times with PBS, unbound sites were blocked with 5% BSA (wt/vol) in PBS, and 200-μL aliquots of *O. tsutsugamushi* (at 2.5×10^6 icu) were added to the coverslips and incubated at 37°C for 1 h. To remove unbound bacteria, the coverslips were washed extensively 3 times with PBS. Bound bacteria were visualized by an indirect immunofluorescence assay, and the bacteria in 3 randomly chosen fields were counted. To further confirm the binding of *O. tsutsugamushi* to immobilized Fn, we performed dot blot analysis on nitrocellulose membranes. The membrane was coated with 50 μL of increasing concentrations of Fn (2–300 μg/mL) or with BSA (50 μg/mL) using the Minifold 1 dot blot system (Schleicher & Schuell) and subsequently blocked with 5% skim milk. The coated membrane was then incubated with 5 mL (2.5×10^6 icu/mL) of the bacterial solution for 1 h at 37°C. After being washed 3 times with a Tris-buffered solution (Tris-buffered saline [TBS], 25 mmol/L Tris–HCl, 3 mmol/L KCl, and 140 mmol/L NaCl [pH 7.4]) containing 0.05% Tween 20, the membrane was incubated with an anti-TSA56 monoclonal antibody (clone KI37) [27] and subsequently with an alkaline phosphatase–conjugated goat anti–mouse IgG secondary antibody (Sigma). Fn-bound *O. tsutsugamushi* were detected using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche) as the chromogenic substrate.

Indirect immunofluorescence. Immunofluorescence techniques were used to visualize *O. tsutsugamushi* [26]. Infected monolayers of L929 cells on 24-well tissue culture plates were collected by trypsin treatment 1 h after infection. Uninfected or surface-bound bacteria were removed by washing 3 times with PBS. The infected cells were then fixed in cold absolute methanol for 15 min at 4°C and stained as described elsewhere [26]. Infected *O. tsutsugamushi* were incubated with an anti-TSA56 monoclonal antibody followed by fluorescein isothiocyanate–conjugated rabbit anti–mouse IgG antibody (Sigma). Cells were examined with an Olympus FV1000 laser confocal microscope (Olympus). Images of cellular sections were produced every 200 nm, and all images were analyzed and processed using Olympus Fluoview software (Olympus). The number of internalized bacteria was counted in 100 cells selected randomly and expressed as the average ($\pm$SD) number of intracellular bacteria per cell for 3 separate experiments.

Expression and purification of recombinant proteins. Recombinant TSA56 and its truncated forms were fused with glutathione S-transferase (GST) and purified. The signal peptide (residues 1–19) and the putative transmembrane region (residues 430–532), which has low immunogenicity [28], were excluded from the protein constructs. Six truncated forms of TSA56 were generated on the basis of the antigenic domains (ADs) revealed by previous serologic analysis and conformational prediction [28, 29]. GST-TSA56_F1$^{19\text{-}114}$, GST-TSA56_F2$^{142\text{-}203}$, and GST-TSA56_F3$^{243\text{-}429}$ included AD I, AD II, and AD III, respectively, as shown in figure 4*A*. GST-TSA56_F3.1$^{243\text{-}349}$ and GST-TSA56_F3.2$^{330\text{-}429}$ were constructed to further refine the Fn-binding domain. GST-TSA54_F4$^{19\text{-}429}$ included all 3 of the ADs. Each construct was amplified by polymerase chain reaction (PCR) with the primers listed in table 1

Table 1. Primers used in this study.

TSA56 product, primer	Primer sequence (5'→3')
TSA56_F1	
TSA56_F1 forward	GCCGAATTCGCGATAGAATTGGAGGATGAAGTAGG
TSA56_F1 reverse	GCCCTCGAGTTAACTCATAGTAGGCTGAGGTGGTG
TSA56_F2	
TSA56_F2 forward	GCCGAATTCCCTATAAGTATAGCTGATCGTGAC
TSA56_F2 reverse	GCCCTCGAGTTACAAACTCCTCCATTGCTCATGG
TSA56_F3	
TSA56_F3 forward	GCCGAATTCGTAGTTGGTCTTGCTGCATTATCAAA
TSA56_F3 reverse	GCCCTCGAGTTAACTCAGATCAAACTCTGTTTCTT
TSA56_F3.1	
TSA56_F3.1 reverse	GCCCTCGAGTTAGTTGCTGCCCTTGCCCCTGCTG
TSA56_F3.2	
TSA56_F3.2 forward	GCCGAATTCATGATACACTTGAATTTTGTCATG
TSA56_F4	
TSA56_F4 forward	GCCGAATTCGCGATAGAATTGGAGGATGAAGTAGG
TSA56_F4 reverse	GCCCTCGAGTTAACTCAGATCAAACTCTGTTTCTT

NOTE. TSA56, 56-kDa type-specific antigen.

and genomic DNA from *O. tsutsugamushi*. The resulting PCR products were cloned into pGEX4T-1 (Amersham Pharmacia) at the *Eco*RI and *Xho*I sites. All constructs were sequenced in accordance with standard methods. Expression and purification of GST or its fusion proteins were performed according to the manufacturer's instructions (Amersham Pharmacia).

Invasion-inhibition assay. For the inhibition assays, the RGD peptide (0.5 mmol/L; Sigma) or the anti–$\alpha 5\beta 1$ integrin

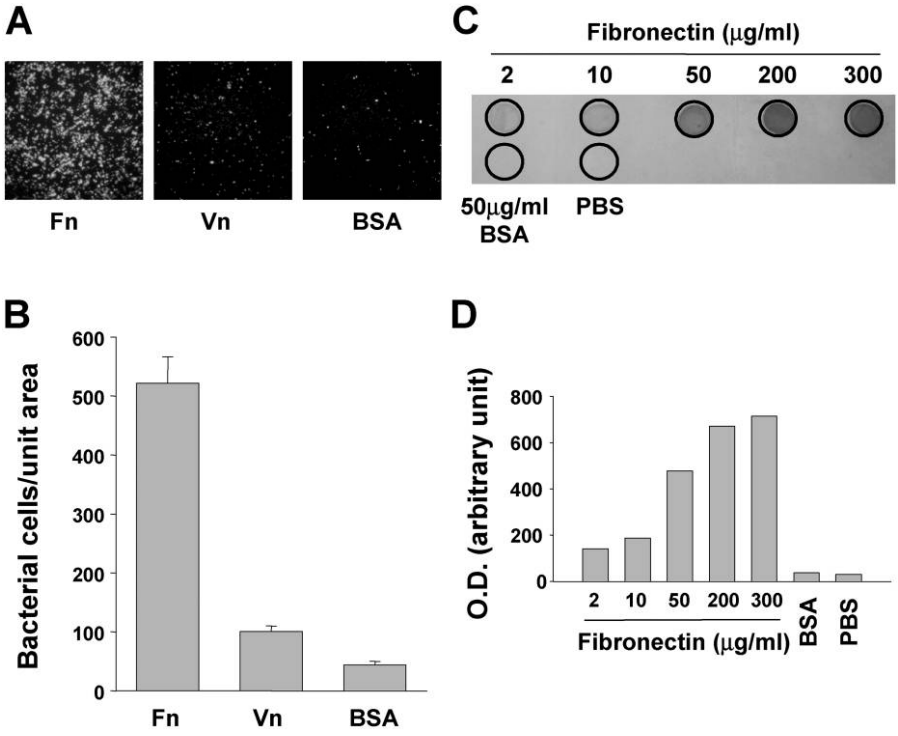

Figure 1. Binding of *Orientia tsutsugamushi* to immobilized fibronectin (Fn). *A,* Representative microscopic fields showing the binding of purified *O. tsutsugamushi* to Fn-, vitronectin (Vn)–, or bovine serum antigen (BSA)–coated glass slides. Bacteria were visualized by immunofluorescence, as described in Methods. *B,* The average (±SD) no. of bacteria in randomly selected microscopic fields, counted in 3 separate experiments. *C,* Binding of *O. tsutsugamushi* to polyvinylidene fluoride membranes coated with increasing concentrations of Fn. The bound bacteria were detected by an immunoblot assay. *D,* Optical densities of each spot, shown in arbitrary units.

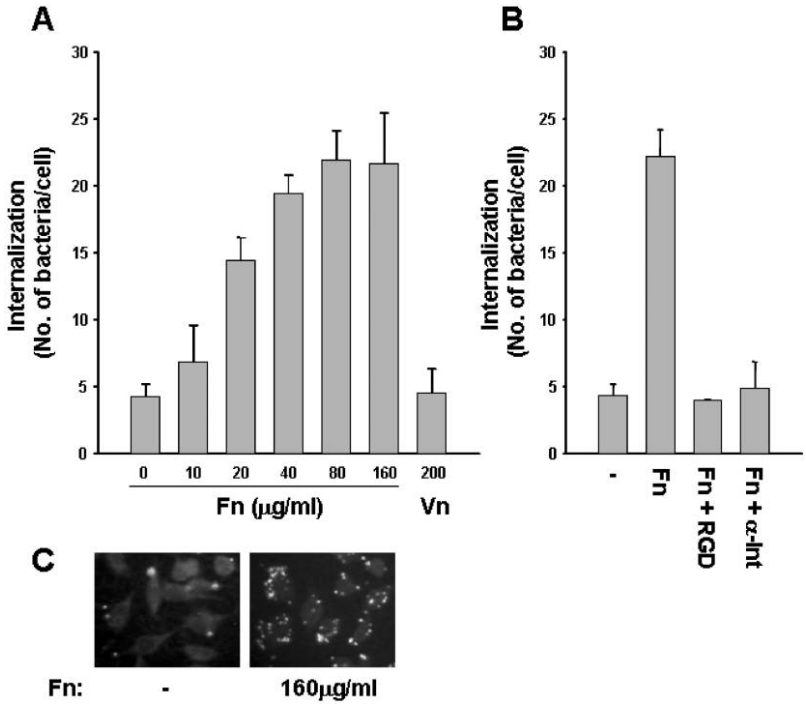

Figure 2. *A,* Enhanced invasion of *Orientia tsutsugamushi* into L929 cells resulting from the addition of exogenous fibronectin (Fn). Internalized bacteria were counted in 3 separate experiments and are expressed as the average (±SD) no. of bacteria per host cell. Vitronectin (Vn; 200 μg/mL) was used as a control. *B,* Significant reduction in the enhanced internalization of *O. tsutsugamushi* in the presence of Fn (80 μg/mL) resulting from the addition of the Arg–Gly–Asp (RGD) peptide or the anti–α5β1 integrin (α-Int) antibody to the incubation medium. *C,* Enhanced infection of *O. tsutsugamushi* in the presence of Fn as visualized by confocal microcopy, done as described in Methods.

monoclonal antibody (100 μg/mL; MAB2514; Chemicon) was added to L929 cell cultures for 15 min before infection with *O. tsutsugamushi*. To test the inhibitory effect of recombinant TSA56 proteins on the invasion of *O. tsutsugamushi*, Fn (80 μg/mL) was preincubated for 1 h at 4°C with various amounts of each GST-TSA56 fusion protein or BSA, as indicated below. The preincubated Fn solutions were then incubated with *O. tsutsugamushi* inoculum and used for infection.

Binding of Fn to* O. tsutsugamushi *extracts and TSA56. Bacterial extracts prepared by sonication on ice were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% skim milk in TBS containing 0.05% Tween 20 and incubated with a solution of 100 μg/mL human Fn for 18 h at 4°C with gentle agitation. Unbound Fn was removed by washing 3 times with TBS containing Tween 20. Fn bound to the immobilized bacterial extract was detected using a mouse anti–N-terminal Fn antibody (Chemicon) and a peroxidase-conjugated anti-mouse IgG secondary antibody (Sigma) with enhanced chemiluminescence (Pierce). To verify the interaction between Fn and TSA56, 5 μg of GST-TSA56 or GST protein alone was separated by SDS-PAGE and transferred onto a PVDF membrane. A ligand immunoblot assay was then performed, using a solution of 100 μg/mL Fn. For GST pull-down assays, 10 μg of purified GST or GST-TSA56 fusion proteins was bound to glutathione-Sepharose beads and incubated with 100 μg/mL human Fn in PBS for 1 h at 4°C with gentle agitation. Any complexes of the fusion proteins and Fn were collected by centrifugation at 500 *g* for 3 min, and then the unbound Fn was removed by extensive washing 5 times with PBS containing 0.05% Tween 20. Bound Fn was detected by SDS-PAGE and immunoblot assays using the mouse anti–N-terminal Fn antibody.

RESULTS

Binding of* O. tsutsugamushi *to Fn. We examined the ability of *O. tsutsugamushi* to adhere to Fn immobilized onto a solid surface. In contrast to the few bacterial cells that bound to the BSA- or Vn-coated coverslips, a significantly ($P < .005$) greater number of bacteria were retained on coverslips coated with Fn (figure 1*A*). The number of adherent bacteria was quantified in randomly selected microscopic fields, and we found that *O. tsutsugamushi* bound to Fn at a rate ~5-fold that of binding to Vn or BSA (figure 1*B*). Purified *O. tsutsugamushi* were then incubated with a nitrocellulose membrane coated with increasing concentrations of Fn (2–300 μg/mL) or with BSA (50 μg/mL) and detected using anti-TSA56 antibody. The optical density of Fn-bound *O. tsutsugamushi* increased in an Fn dose–dependent manner (figure 1*C*). In addition, the optical density of bacteria

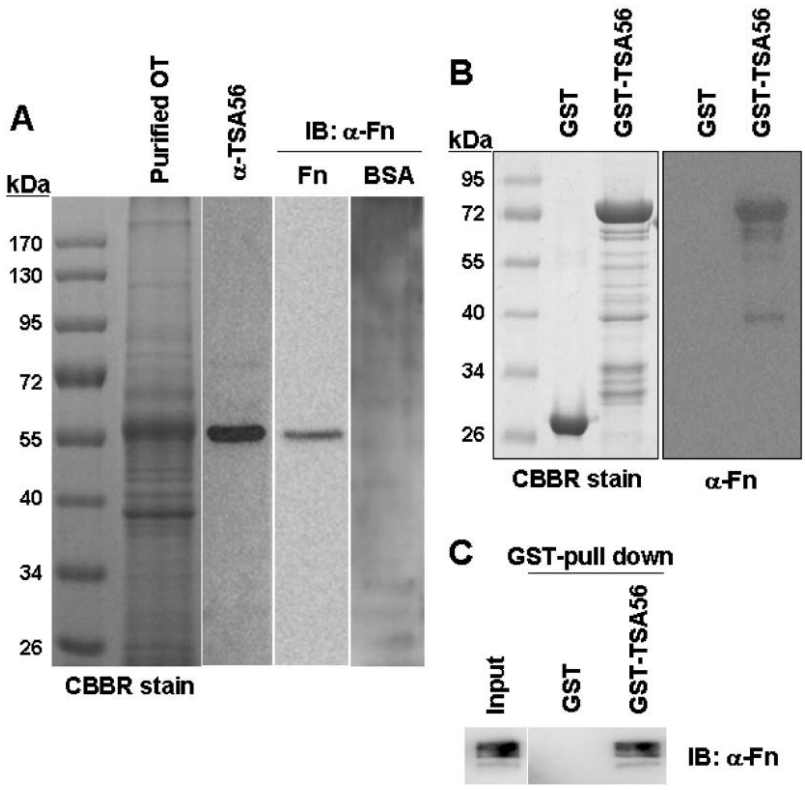

Figure 3. Binding of fibronectin (Fn) to a 56-kDa type-specific antigen (TSA56) from *Orientia tsutsugamushi*. *A,* Binding of Fn to a 56-kDa protein. The second lane from the left shows whole bacterial lysates (purified *O. tsutsugamushi* [OT]) separated by SDS-PAGE and stained with Coomasie brilliant blue R-250 (CBBR). Proteins from these gels were transferred onto polyvinylidene fluoride membranes and probed with either an anti-TSA56 antibody *(center lane)* or an anti-Fn antibody after incubation with Fn or bovine serum antigen (BSA) *(right 2 lanes). B,* Interaction of Fn with TSA56, as shown by a ligand-binding blot using a purified glutathione S-transferase (GST)–TSA56 fusion protein. Fn interacted with TSA56 but not with the GST control. *C,* Interaction of Fn with TSA56, as confirmed by GST pull-down assays. IB, immunoblotting.

incubated with Fn was 10-fold higher than that of an area coated with an equal concentration of BSA (figure 1*D*).

Enhanced invasion of* O. tsutsugamushi *by exogenous Fn. Other studies have shown that the presence of Fn increases the invasiveness of several pathogenic bacteria into host cells [10, 11, 30]. We therefore investigated whether exogenous Fn could enhance the infectivity of *O. tsutsugamushi*. Purified bacteria were incubated with increasing concentrations of Fn or Vn and then added to L929 cultures in the absence of serum. The incubation of *O. tsutsugamushi* with Vn (≤200 μg/mL) did not significantly change the infection rate of L929 cells, but incubation with Fn increased the number of infected cells dramatically (figure 2*A* and 2*C*). The infection rate was dependent on the concentration of Fn, with the maximum bacterial infection observed at a concentration of ~80 μg/mL. Half-maximal infection was achieved at ~15 μg/mL Fn.

It has been well documented that RGD, the core amino acid motif in Fn, binds to integrin receptors, including α5β1 integrin, in the plasma membrane of mammalian cells [10, 12]. We next tested the possible involvement of the Fn RGD motif and integrin interactions in Fn-enhanced *O. tsutsugamushi* infection by blocking cell adhesion to Fn with an Arg–Gly–Asp-Ser (RGDS) peptide or an antibody against α5β1 integrin. As shown in figure 2*B,* inclusion of the RGDS peptide (0.5 mmol/L) or an anti–α5β1 integrin antibody (100 μg/mL) into the infection assay strongly inhibited the internalization of Fn-treated *O. tsutsugamushi*. The RGDS peptide and anti–α5β1 integrin antibody treatments reduced the number of infective bacteria to Fn-untreated control levels. Taken together, these data suggest that Fn facilitates the infection of *O. tsutsugamushi* through its interaction with the α5β1 integrin receptor.

Identification of the bacterial ligand required for Fn interaction. To identify potential Fn-binding proteins on the surface of *O. tsutsugamushi,* we performed ligand immunoblot assays. Protein extracts from *O. tsutsugamushi* were separated by SDS-PAGE and transferred onto PVDF membranes. Fn or BSA was incubated with the membrane and detected using an anti-Fn antibody. In contrast to BSA, which did not show any signal, Fn bound a specific protein with a molecular mass of 56 kDa, as shown in figure 3*A*. This protein is the same size as the major outer membrane protein of *O. tsutsugamushi,* TSA56 [28, 31]. To investigate further whether TSA56 interacts with Fn, we made TSA56-GST fusion constructs without the signal peptide and putative transmembrane domains (GST-TSA56_F4) (figure

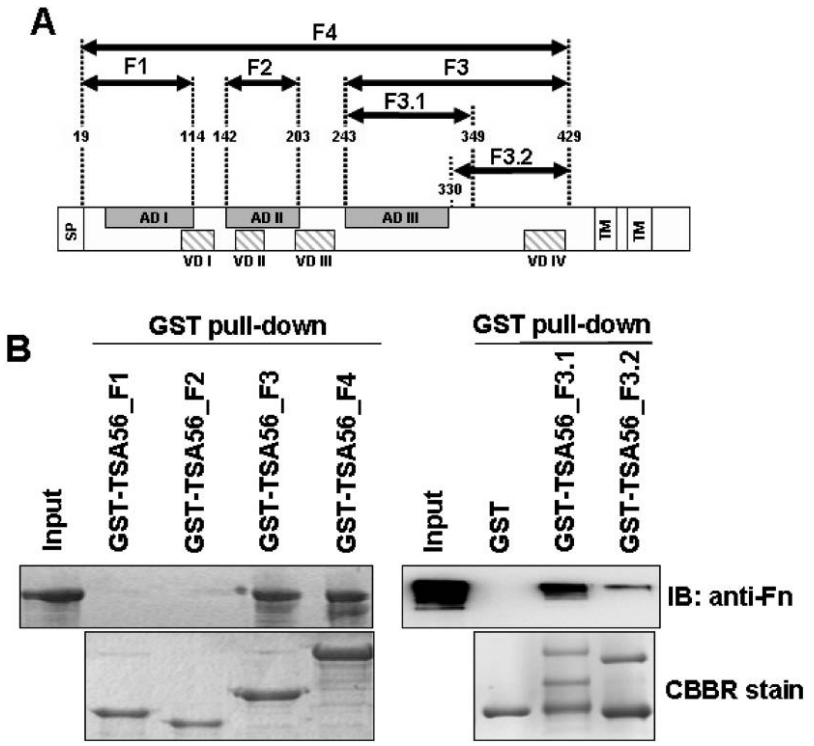

Figure 4. Mapping of the fibronectin (Fn)–binding domain in a 56-kDa type-specific antigen (TSA56). *A,* Schematic representation of the TSA56 sequence in which the positions of the signal sequence (SP), antigenic domains (ADs), variable domains (VDs), and transmembrane domains (TMs) are indicated. Each TSA56 fragment used for the glutathione S-transferase (GST) pull-down assays is marked. *B,* Interaction of Fn with TSA56 fragments. GST fusion proteins of TSA56 fragments were expressed, purified *(bottom panels),* and used for GST pull-downs followed by immunoblotting (IB) with an anti-Fn antibody *(top panels).* CBBR, Coomasie brilliant blue R-250.

4*A*) [28]. We purified the fusion protein from *E. coli* and used it in a ligand immunoblot assay (figure 3*B*). We found that Fn bound specifically to GST-TSA56_F4 and not to the GST control. We also performed GST pull-down assays using the GST-TSA56_F4 fusion protein to confirm the specific interaction between TSA56 and Fn. The GST control did not interact with Fn (figure 3*C*).

Identification of the Fn-binding domain in TSA56. To determine the TSA56 domain required for Fn interaction, we constructed GST-truncated fusion proteins that contained at least 1 of the 3 ADs of TSA56 (figure 4*A*) [28]. After GST pull-down (figure 4*B*), we found that Fn bound to GST-TSA56_F3, which contained AD III, and to GST-TSA56_F4. However, GST-TSA56_F1 and GST-TSA56_F2, which contained AD I and AD II, respectively, did not interact with Fn. These data indicate that a 187-aa C-terminal region of TSA56 interacts with Fn. To refine the binding domain of TSA56 further, the F3 region was divided into 2 fragments (GST-TSA56_F3.1 and GST-TSA56_F3.2). F3.1 contained AD III, and F3.2 consisted of the C-terminal portion of F3 (figure 4*A*). As shown in figure 4*B (right panels),* GST-TSA56_F3.1 strongly interacted with Fn, in contrast to the GST control, which failed to interact. GST-TSA56_F3.2 also had a modest interaction with Fn but was weaker than GST-TSA56_F3.1. The amino acid (residues 330–349) overlap of the 2 constructs may be involved in Fn interaction in addition to AD III. These results indicate that AD III and 19 adjacent C-terminal residues of TSA56 are sufficient for Fn binding.

Inhibition of intracellular invasion of* O. tsutsugamushi *by preincubation with TSA56 fragments. Because TSA56 binds to Fn, we investigated whether recombinant TSA56 would inhibit Fn-enhanced invasion of *O. tsutsugamushi*. Fn was preincubated with increasing concentrations of recombinant TSA56 or BSA for 1 h and then mixed with *O. tsutsugamushi* before being added to L929 cells (figure 5*A*). A dose-dependent reduction in infectivity was observed when Fn was preincubated with increasing concentrations of TSA56. The infectivity rate in the presence of 100 μg/mL TSA56 was 25% of the control (without preincubation with TSA56). However, preincubation of Fn with $\leqslant$200 μg/mL BSA did not affect the rate of internalization. To define further the domains of TSA56 responsible for this inhibition, these infection assays were repeated with the truncated TSA56 fusion proteins (figure 5*B*). The purified fusion proteins (GST-TSA56_F4 and GST-TSA56_F3) that interacted with Fn also reduced the infectivity of *O. tsutsugamushi* in a dose-dependent manner, whereas GST-TSA56_F1 and GST-TSA56_F2 had no effect. Collectively, the AD III and adjacent C-terminal region of TSA56 was sufficient for Fn interaction and inhibition of Fn-enhanced invasion of bacteria.

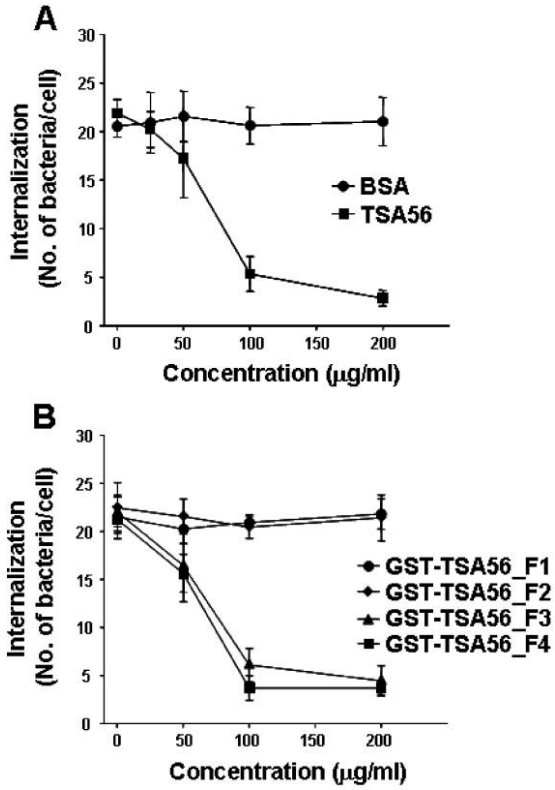

Figure 5. Effect of a 56-kDa type-specific antigen (TSA56) on internalization of *Orientia tsutsugamushi* into L929 cells. *A,* Infection assays using preincubated fibronectin (Fn). Fn was preincubated with increasing concentrations of purified TSA56 or bovine serum antigen (BSA). In each assay, pretreated Fn and *O. tsutsugamushi* were incubated with L929 cells for 1 h. Internalized bacteria were counted in 3 separate experiments and are represented as the average (±SD) no. of bacteria per host cell. *B,* Infection assays performed in the presence of glutathione S-transferase (GST) fusion proteins containing TSA56 fragments and analyzed as in panel A.

DISCUSSION

Our knowledge of the interactions between bacterial pathogens and the extracellular matrix has significantly increased in recent years. In particular, there have been extensive advances in defining the molecular interactions of pathogens with Fn. Investigations into the binding of *S. aureus* and *Streptococcus* species to Fn have shed light on how these interactions affect host cell signaling and cellular physiology that result in bacterial uptake [9]. Furthermore, bacterial adhesins have become more attractive targets of therapeutics and vaccine development [32, 33]. In the present study, we have found that the scrub typhus pathogen, *O. tsutsugamushi,* can bind to Fn via a major antigenic membrane protein, TSA56. In addition, the interaction of TSA56 with Fn significantly enhances the intracellular invasion of *O. tsutsugamushi*. We further defined the domain of the TSA56 protein that is sufficient for Fn binding.

The intracellular invasion of host cells is critical for the subsequent growth and survival of *Rickettsia* species. However, the basic invasion mechanism of these obligate intracellular parasites has been poorly characterized. Recently, the first report of a receptor-ligand interaction required for *Rickettsia* internalization was published by Martinez et al. [34]. They found that *Rickettsia conorii* can interact with Ku70, a protein that shares homology with integrin A domains and binds to Fn [34]. In this case, the internalization process is dependent on the presence of cholesterol-enriched microdomains and ubiquitination of the cellular receptor. The authors also suggested that the binding of a specific *R. conorii* ligand, outer membrane protein B, to Ku70 may directly stimulate the internalization process or that *R. conorii* initially bind to Fn and utilize this interaction as a bridge to bind Ku70. Activation of Src family tyrosine kinases and focal adhesion kinases during *R. conorii* invasion into nonphagocytic host cells further suggested the potential association between integrin signaling and the *Rickettsia* invasion process [35].

In the present study, we found that the most abundant outer membrane protein of *O. tsutsugamushi,* TSA56, can directly bind to Fn and that this interaction enhanced the internalization process of the intracellular pathogen. In other studies, it has been reported that the bacterium induced phagocytosis in phagocytic and nonphagocytic host cells after attachment [36, 37]. The role played by Fn during this internalization process may be mediated through its interaction with integrins such as $\alpha5\beta1$, which may subsequently stimulate downstream signaling molecules and induce endocytosis in nonphagocytic host cells [9, 38]. We confirmed the significance of this interaction by using the RGD peptide and a blocking antibody against $\alpha5\beta1$ integrin. Inhibition of Fn-enhanced infection by the RGD peptide and the anti-integrin antibody strongly suggests that the interaction between the RGD motif in Fn and the $\beta1$ subunit of the integrin receptor may play a key role in stimulating the cellular uptake and invasion of bacteria [10, 38]. Integrin signaling during the invasion process of *O. tsutsugamushi* is currently being investigated.

Interestingly, antibodies against TSA56 have been reported to be sufficient to block the in vitro invasion of *O. tsutsugamushi* into L929 cells [39]. The surface-exposed ADs of TSA56 could be the main targets of those neutralizing antibodies [28, 29]. Because AD III is immunodominant in both mice and humans, this domain has been proposed as a potential target for peptide-based vaccine development [28]. On that basis of the structural model of TSA56 from in silico and serological analysis, we constructed several recombinant constructs of TSA56 to define the Fn-binding domain. Here, we have shown that AD III and the adjacent C-terminal region of TSA56 is the putative Fn-binding domain and may facilitate Fn-mediated infection with *O. tsutsugamushi*. It has been shown that the Fn-binding domains of several bacterial proteins are disordered in the absence of Fn and undergo a disordered-to-ordered transition on ligand binding [9]. We have found that several regions in TSA56 are predicted

to have disordered structures by web-based predictors (data not shown) [40], including sites within the Fn-binding region (aa 254–61 and 341–9). However, we failed to detect any conserved repeat motifs or sequences that have been reported for other Fn-binding domains [9, 17]. Additional studies to identify the minimum Fn-binding domain(s) within the Fn-binding region of TSA56 are currently under way. Taken together, our results suggest that the Fn-binding domain of TSA56 could be a potential target for therapeutic peptides or vaccine development by neutralizing the intracellular invasion of *O. tsutsugamushi*.

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