The *Rickettsia conorii* Autotransporter Protein Sca1 Promotes Adherence to Nonphagocytic Mammalian Cells

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The pathogenesis of spotted fever group (SFG) *Rickettsia* species, including *R. conorii* and *R. rickettsii*, is acutely dependent on adherence to and invasion of host cells, including cells of the mammalian endothelial system. Bioinformatic analyses of several rickettsia genomes revealed the presence of a cohort of genes designated *sea* genes that are predicted to encode proteins with homology to autotransporter proteins of Gram-negative bacteria. Previous work demonstrated that three members of this family, rOmpA (Sca0), Sca2, and rOmpB (Sca5) are involved in the interaction with mammalian cells; however, very little was known about the function of other conserved rickettsial Sca proteins. Here we demonstrate that *sca1*, a gene present in nearly all SFG rickettsia genomes, is actively transcribed and expressed in *R. conorii* cells. Alignment of Sca1 sequences from geographically diverse SFG *Rickettsia* species showed that there are high degrees of sequence identity and conservation of these sequences, suggesting that Sca1 may have a conserved function. Using a heterologous expression system, we demonstrated that production of *R. conorii* Sca1 in the *Escherichia coli* outer membrane is sufficient to mediate attachment to but not invasion of a panel of cultured mammalian epithelial and endothelial cells. Furthermore, preincubation of a recombinant Sca1 peptide with host cells blocked *R. conorii* cell association. Together, these results demonstrate that attachment to mammalian cells can be uncoupled from the entry process and that Sca1 is involved in the adherence of *R. conorii* to host cells.

Spotted fever group (SFG) *Rickettsia* species are Gram-negative obligate intracellular bacteria that are the etiologic agents of many severe emerging infectious diseases that occur throughout the world, including Rocky Mountain spotted fever (RMSF) and Mediterranean spotted fever (MSF), which are caused by *R. rickettsii* and *R. conorii*, respectively. These bacteria are transmitted to human hosts through the salivary gland contents of infected ticks and occasionally lice or mites. Expansion of the bacterial population and horizontal cell-to-cell transmission results in localized dermal and epidermal necrosis and the characteristic eschar or tache noir (58). Once established in the host, SFG *Rickettsia* infects primarily the endothelial lining of the vasculature (36, 46, 55). Damage to this tissue and infiltration of perivascular mononuclear cells often cause fluid leakage and the diagnostic macropapular dermal rash (6, 10, 24). While these unusual symptoms are good predictors for appropriate diagnosis and treatment, they are often accompanied by nondozen mal symptoms and flu-like symptoms, and often do not occur at all (6). Even in areas of the United States where awareness of RMSF is high, approximately 60% to 75% of patients receive an alternate diagnosis during their first visit for medical care (26, 39). Misdiagnosis of SFG *Rickettsia* infection is associated severe manifestations, including acute renal failure, pulmonary edema, interstitial pneumonia, neurological manifestations, and other multiorgan manifestations (6, 24).

The mortality rates for untreated Mediterranean and Rocky Mountain spotted fevers are estimated to be as high as 20%, but appropriate treatment drastically decreases the risk (9, 11, 23, 35). The severity of these diseases and the potential for aerosol transmission have led to classification of *Rickettsia* species as category B and C priority pathogens by the U.S. Centers for Disease Control and Prevention (CDC) (40).

Rickettsiae are strict intracellular parasites that require host cells to replicate. In order to survive, the bacteria must invade and reside exclusively in mammalian or arthropod host cells (60). Intracellular bacteria escape from vacuoles (44, 56, 65) and move intra- and intercellularly by means of actin-based motility (20, 21, 25, 50), which leads to infection of neighboring cells and possibly to release into the vasculature. SFG rickettsiae must, therefore, perform a series of regimented pathogenic steps in widely diverse environments in order to survive and thrive in their hosts.

A critical initial step in SFG rickettsiae pathogenesis is bacterial recognition of and attachment to target cells. In *vivo*, SFG rickettsiae are first exposed to and primarily infect the host endothelium, but they are known to enter a wide spectrum of normally nonphagocytic cells in *vitro* (7, 42, 46, 47, 53, 61, 63, 64, 66, 67). This entry can be divided into two distinct events, adherence and invasion. *Rickettsia* can adhere to all types of cells that have been tested, including endothelial cell ghosts that lack any form of chemical membrane gradients (66). This process does not absolutely require energy as adherence still occurs if either the bacteria or the host cells are killed. How-
ever, adherence is at least partially temperature dependent and appears to require receptors present in cholesterol-enriched membrane segments (29, 34, 41, 57, 61).

In contrast to adherence, rickettsial invasion is an active process that has been defined as “induced phagocytosis.” Several studies of rickettsia invasion have shown that this process is morphologically and mechanically related to a “zipper-like” invasion strategy, whereby localized receptor-ligand interactions induce cytoskeletal arrangements around the bacterium (20, 21, 25, 32, 49). More recent detailed analyses of interactions induce cytoskeletal arrangements around the bacterium (20, 21, 25, 32, 49). More recent detailed analyses of internalization mechanisms revealed that R. conorii invasion is dependent on the actin nucleating protein complex, Arp2/3, as well as many host signaling events, including those mediated by c-Cbl, clathrin, caveolin 2, Cdc-42, phosphoinositide 3-kinase, well as many host signaling events, including those mediated by c-Cbl, clathrin, caveolin 2, Cdc-42, phosphoinositide 3-kinase, Src, and other kinases (5, 20, 25, 32). Since adherence and invasion are absolutely vital for survival of SFG Rickettsia and since these pathogenic processes are not sheltered from intraacellular protection, they provide pronounced targets for therapeutic interposition.

Bioinformatic analysis of SFG Rickettsia has identified a family of predicted outer surface proteins designated Sca (surface cell antigen) proteins (2). These proteins, including Sca1, belong to a family of Gram-negative proteins called autotransporters, many of which are virulence factors (27, 28). Members of this protein family have modular structures, including an N-terminal signal sequence, a central passenger peptide, and a C-terminal “translocation module” (β-peptide) (28). Following translation, the peptide is initially secreted across the inner membrane using information in the N-terminal signal sequence. The C-terminal peptide then inserts into the outer membrane to form a β-barrel-rich transmembrane pore through which the passenger peptide passes, which exposes the passenger peptide to the extracellular environment (28). Four predicted rickettsial autotransporter proteins, Sca0 (rOmpA), Sca1, Sca2, and Sca5 (rOmpB), are conserved across the spotted fever group (>95% identity) genome segments (29 to 327) [rGST-Sca1(29-327)] under control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. pGEX-HIS is a derivative of pGEX-2TKP that encodes an N-terminal GST tag, a C-terminal four-glycine linker, and a six-histidine tag flanking the pGEX-HIS expression cassette.

Amino acid alignment. Amino acid sequences of R. conorii Malish7 (gene identification number, gi:15891942), R. rickettsii Sheila Smith (gi:15782888), R. japonica YM (gi:51557577), R. africae ES-5 (gi:16747112), and R. australis Phillips (gi:51557593) were aligned using Clustal W/ Mac Vector 9.5.2 (Mac Vector, Cary, NC) with an open gap penalty of 10.0 and an extend gap penalty of 0.2. The degrees of identity and similarity of Sca1 sequences were determined using the calculated alignment. Therefore, the numbers in amino acid designations below do not refer to any single protein but refer to the total alignment.

Gene manipulations. Insertion of the R. conorii sca1 open reading frame into pET22b was performed by directional restriction endonuclease-mediated insertion. Briefly, sca1 was PCR amplified from R. conorii Malish7 genomic DNA using primers sca1-F (5′-ACCATTGGAATGTTAAACAGAAACA) and sca1-R (5′-CTTAAAGGTAACTACACCACCAACCACACTAA) and TA cloned into pcR2.1 (Invitrogen, Carlsbad, CA) to produce pSca1-100. The sca1 insertion was removed by digestion with NotI and BamHI (New England Biolabs, Ipswich, MA) and ligated into similarly digested pET22b with quick ligation (New England Biolabs) to make pSca1-200. Positive clones were screened by PCR, and the entire sca1 insertion was sequenced to verify accurate insertion in frame with 5′ peB signal and 3′ His sequences. Construction of pYCO (pET22b:ompB) is described elsewhere (5). pYCT contains ompB bp 105 to 4002 (amino acids 35 to 1334) corresponding to the rOmpB “passenger domain.” The gene fragment was PCR amplified from chromosomal DNA using the primers described previously for pYCI1 (5) and was directionally cloned into pET-22b using NotI and XhoI sites.

pGST-Sca1(29-327) was constructed by restriction enzyme-mediated insertion into pGEX-2TKP (acquired from T. Kouzarides). The sca1 insertion fragment was amplified from R. conorii Malish7 genomic DNA using primers GST-F (5′-GGATCACGACATCTTTTGGG) and Sca1-R (5′-CTCAAGCTGCAGCTGACCTC) and ligated into pCR1. The fragment was ligated with the BamHI and XhoI restriction enzymes and ligated into pGEX-2TKP to create a peptide encoding recombinant Shine-Dalgarno 5′-translational (GST)-Sca1(amino acids 29 to 327) [rGST-Sca1(29-327)] under control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter.

In this study, we examined sca1 transcription and demonstrated that Sca1 is present on the surface of R. conorii isolated from infected mammalian cells. Using a heterologous system to express Sca1 at the outer membrane of Escherichia coli, we determined that Sca1 expression is sufficient to mediate adherence to nonphagocytic mammalian cells. Likewise, a soluble Sca1 peptide is able to inhibit association of virulent R. conorii bacteria with their cognate host cells. We predict that the Sca1 function is likely conserved among diverse SFG Rickettsia species due to the high degrees of identity and similarity of Sca1 sequences. Interestingly, Sca1 expression on the surface of E. coli is not sufficient to mediate invasion of mammalian cells, suggesting that while Sca1 may play a critical role in the association of SFG rickettsiae with target cells, other interactions are required to induce bacterial internalization.
were separated by agarose gel electrophoresis in the presence of ethidium bromide, and the accuracy of amplification was verified by DNA sequencing.

**Protein preparation, sequencing, Western blotting, and immunofluorescence.** Isolated *R. conorii* Malish7 preparations were boiled in SDS-PAGE buffer, electrophoresed in 4 to 20% gradient acrylamide gels (Invitrogen), and stained with Coomassie brilliant blue. A portion of a separated gel corresponding to proteins with molecular masses of approximately 120 to 250 kDa was extracted and subjected to microcappillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) peptide sequencing (Taplin Biological Mass Spectrometry Facility, Harvard Medical School, Boston, MA).

Affinity-purified rabbit anti-Sca1 antibody was produced using the *R. conorii* Sca1 peptide sequence 14TEQSQNTYTPESTEC157 (Gen Script, Piscataway, NJ). This antibody was used to assess the presence of Sca1 in *E. coli* BL21(DE3)[pSca1-200]. Outer membrane proteins of *E. coli* were prepared as described previously (51). Briefly, 10 ml of induced *E. coli* BL21(DE3) with corresponding plasmids was centrifuged and resuspended in 1 ml phosphate-buffered saline (PBS) with 1× Complete protease inhibitor cocktail (Roche, Basel, Switzerland). The cells were lysed by sonication, and the debris was pelleted by centrifugation at 1,000 × g to remove unbroken cells. The supernatant was treated with 0.5% Sarkosyl to solubilize the inner membrane and was centrifuged again at high speed to precipitate the outer membrane. The outer membrane preparation was subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with anti-Sca1 or anti-rOmpB, followed by goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (Sigma, St. Louis, MO). Immunoblots were developed using chemiluminescence and exposure to film.

Recombinant GST-Sca1 containing amino acids 29 to 327 [rGST-Sca1 (29-327)] or GST-His (pGEX-His) protein was produced in *E. coli* BL21-CodonPlus (DE3) [pGEX-6P1] or [pGEX-5X-2]. Following induction at 30°C with 0.5 mM IPTG, bacteria were harvested by centrifugation and lysed by sonication in PBS. Lysates were cleared by centrifugation and loaded onto 5-ml TRAP FF column (GE Healthcare, Piscataway, NJ) columns using an ATKA fast protein liquid chromatography (FPLC) with a 1-ml UV absorbance monitor and a Frac900 fraction collector (GE Healthcare). The columns were washed and subjected to increasing concentrations of PBS, 30 mM glutathione elution buffer, and protein purity was assessed by SDS-PAGE and mass brilliant blue staining. Protein concentrations were determined by calculating the mean observed results of Bradford and bicinchoninic acid (BCA) protein assays (Pierce, Rockford, IL). Fractions containing purified protein were dialyzed against PBS, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol (pH 7.5). Aliquots were snap-frozen with liquid nitrogen prior to cryogenic storage at −80°C and were thawed on ice immediately prior to use in cell-based assays. Sca1(29-327) antisera was produced from rGST-Sca1(29-327) after removal of the GST tag. Briefly, recombinant protein preparations dialyzed against PBS, 10% glycerol (pH 7.5) were incubated overnight at room temperature with 10 μg/ml thrombin (GE Healthcare) to liberate the GST peptide. Each resulting solution was passed repeatedly over GST-TRAP FF and HiTrap benzamidine FF columns (GE Healthcare) to remove the GST peptide and thrombin, respectively. The remaining GST-free Sca1(29-327) protein with Freund’s adjuvant was utilized to immunize a New Zealand White rabbit. Any contaminating anti-GST antibodies were removed from the Sca1(29-327) antiserum by repeated passage over an rGST peptide-loaded GST-TRAP FF column. The *E. coli* cell surface reactivity of the antibody was eliminated by incubation with fixed *E. coli* BL21(DE3)[pET22b], followed by centrifugation to precipitate any antibodies bound to the *E. coli*.

rOmpB(35-1335) was produced in *E. coli* BL21(DE3) harboring pYC7 and was purified under denaturing conditions. Briefly, following induction at 30°C with 0.5 mM IPTG, bacteria were harvested by centrifugation, resuspended in PBS, and disrupted with a French press. Insoluble components were isolated by ultracentrifugation and solubilized in 8 M urea-PBS (pH 8.0). rOmpB(35-1335) was purified from the urea-soluble material by Ni-nitrilotriacetic acid (NTA) affinity chromatography.

Mouse anti-*R. conorii* hyperimmune serum was isolated from experimentally infected animals. Briefly, C3H/HeN mice were inoculated retroorbitally with 1.8 × 10⁹ PFU of *R. conorii* Malish7. These mice were monitored, and disease progression was scored. At day 11 postinfection, when the mice showed no further signs of illness, the mice were again infected retroorbitally with 7.9 × 10⁶ PFU of *R. conorii*. At day 21 postinoculation, all mice were sacrificed, and blood samples were obtained by cardiac puncture. Serum steel was obtained by centrifugal separation of blood components with Serum Gel S/1 tubes (Sarstedt, Nümbrecht, Germany) and filtering through 0.22-μm filters.

For Western immunoblot analysis, total *R. conorii* lysate or recombinant proteins [rGST-Sca1(29-327) and rOmpB(35-1335)-His] were separated by SDS-PAGE, transferred to nitrocellulose, probed with the appropriate antisera, and developed using chemiluminescence. Rabbit anti-Sca1(29-327) and normal rabbit sera were utilized at a dilution of 1:500.

For immunofluorescent or flow cytometric analysis of *R. conorii*, purified bacteria were air dried onto glass coverslips or left in solution before they were fixed with 4% paraformaldehyde. The bacteria were incubated with a 1:200 dilution of mouse anti-*R. conorii* hyperimmune serum as described above and a 1:200 dilution of anti-Sca1(29-327) or normal rabbit serum and then with a 1:1,000 dilution of secondary goat anti-mouse IgG-Alexa Fluor 546 antibody and a 1:1,000 dilution of secretory goat anti-ribbq IgG-Alexa Fluor 488 antibody. Cells were visualized with a Leica TCS SP2 AOBs laser scanning confocal microscope equipped with an acousto-optical beam splitter (AOBS) using a physical magnification of ×100 and an 80× digital zoom, which was controlled with LCS Leica confocal software. Bacteria were also analyzed with a BD LSR-II flow cytometer utilizing fluorescein isothiocyanate (FITC) and phycoerythrin (PE) parameters and FlowJo software. The analysis of Sca1 expression in relation to normal rabbit serum (FITC) was predicted on positive detection of mouse anti-*R. conorii* (PE) fluorescence.

**Cell association and invasion assays.** Cell association and invasion assays were performed as described previously (5, 33). Briefly, induced *E. coli* BL21(DE3) harboring pSca1-200, pYC7, or pET22b was added to a confluent monolayer of Vero, HeLa, or A499/926 cells in serum-free media. Portions of the bacterium-containing media were plated to determine the number of CFU that were added to each mammalian monolayer. Contact of the bacteria with each mammalian monolayer was initiated by centrifugation at 200 × g, and then the preparations were incubated at 37°C in the presence of 5% CO₂ for 20 and 60 min for the adherence and invasion assays, respectively. For invasion assays, infected cells were washed with PBS and then incubated for 2 h with complete medium supplemented with 100 μg/ml gentamicin to kill the extracellular bacteria. For all *E. coli* assays, infected cells were washed extensively in PBS, and bacteria were incubated with 0.1% Triton X-100 in sterile H₂O and then plated on LB agar to enumerate associated bacteria. The results were expressed as the percentage of the bacteria recovered based on the number of bacteria in the initial inoculum.

For *R. conorii* infection, confluent monolayers of Vero or A499/926 cells in 48-well tissue culture plates were pretreated with 800 μg/ml rGST-His or with rGST-Sca1(29-327) for 30 min prior to infection. *R. conorii* infectious particles were added to a monolayer of mammalian cells, and contact was induced by centrifugation at 500 × g. The cells were incubated for 20 min at 37°C in the presence of 5% CO₂ before extensive washing with PBS. The cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in H₂O and then plated on LB agar to enumerate associated bacteria. The results were expressed as the percentage of the bacteria recovered based on the number of bacteria in the initial inoculum.

For *R. conorii*, infected, confluent monolayers of Vero or A499/926 cells were washed with PBS, 1× Complete protease inhibitor cocktail (Roche, Basel, Switzerland), followed by a 1:500 dilution of goat anti-rabbit Alexa Fluor 488 and a 1:1,000 dilution of secondary goat anti-rabbit Alexa Fluor 546 antibody. Cells were visualized with a Leica TCS SP2 AOBs laser scanning confocal microscope equipped with an acousto-optical beam splitter (AOBS) using a physical magnification of ×100 and an 80× digital zoom, which was controlled with LCS Leica confocal software. Bacteria were also analyzed with a BD LSR-II flow cytometer utilizing fluorescein isothiocyanate (FITC) and phycoerythrin (PE) parameters and FlowJo software. The analysis of Sca1 expression in relation to normal rabbit serum (FITC) was predicted on positive detection of mouse anti-*R. conorii* (PE) fluorescence.

**TABLE 1. Sequence conservation of the *R. conorii* Sca1 protein in other *Rickettsia* spp.**

<table>
<thead>
<tr>
<th>Rickettsia strain</th>
<th>% Identity for amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. aferica ESF-5</td>
<td>94.2</td>
</tr>
<tr>
<td>R. rickettsi Sheila Smith</td>
<td>94.2</td>
</tr>
<tr>
<td>R. japonica YM</td>
<td>91.4</td>
</tr>
<tr>
<td>R. australis Phillips</td>
<td>60.7</td>
</tr>
<tr>
<td>R. typhi Wilmington</td>
<td>32.8</td>
</tr>
</tbody>
</table>

**RESULTS**

Sca1 is conserved among SFG *Rickettsia* species. *R. conorii* *sca1* is a 5,709-bp open reading frame (ORF) that encodes a predicted 212,153-Da protein. Since *sca1* is present in the genomes of the vast majority of SFG *Rickettsia* species (37, 38),

**TABLE 1. Sequence conservation of the *R. conorii* Sca1 protein in other *Rickettsia* spp.**
we wanted to ascertain the level of Sca1 protein sequence conservation among related and diverse spotted fever group (SFG) rickettsiae. As shown in Table 1, sequence analysis of Sca1 proteins from Rickettsia species dispersed throughout the world revealed considerable levels of sequence identity and similarity, with particular conservation at the N and C termini. The C-terminal conservation can be attributed in part to the chemical and structural constraints of the membrane-imbedded pore-forming β-peptide (28). However, much of the N-terminal conservation occurs outside the predicted signal sequence and in the completely uncharacterized passenger domain. The apparent limits on sequence variability in these regions imply that there is functional conservation, and these regions are an attractive target for treatment of SFG Rickettsia infections.

sca1 is transcribed and translated in R. conorii. Functional characterization of Sca1 was precluded by the need to verify transcription and translation. We first isolated total RNA from infectious R. conorii bacteria isolated from infected Vero cells and performed nonquantitative reverse transcriptase PCR (RT-PCR) amplification of sca1. We performed identical re-
actions to amplify a portion of the *ompA* ORF, which is known to produce protein in cell culture conditions (30), and reactions without RNA or reverse transcriptase as controls. As shown in Fig. 1A, the presence of a specific *sca1* product under the appropriate conditions confirmed that there was transcrip-
tion of *sca1* in *R. conorii*.

We next queried whether *R. conorii* isolated from Vero cells expressed the Sca1 protein. An *R. conorii* protein lysate was separated by SDS-PAGE, and proteins with apparent molecular masses between 130 kDa and 250 kDa were excised and analyzed by microcapillary LC-MS/MS peptide sequencing (Fig. 1B). Peptides corresponding to various proteins, including rOmpA, rOmpB, and Sca1, were identified using peptide sequencing (see Table S1 in the supplemental material). A total of 50 peptides attributed to Sca1 were detected, corresponding to 33.9% of the predicted protein.

We also created a polyclonal antisera directed against the N-terminal portion of the Sca1 passenger domain (amino acids 29 to 327). Western analysis of total *R. conorii* lysate using this anti-Sca1(29-327) serum yielded a reactive band at an apparent molecular mass of approximately 130 kDa, which was not observed using normal rabbit serum (Fig. 1C). This Sca1-reactive band likely represents a peptide processed from the full-length protein, which is predicted to have a molecular mass greater than 200 kDa. In order to confirm the specificity of this antisera, we separated the rGST-Sca1(29-327) and rOmpB(35-1334)-His proteins by SDS-PAGE, transferred the proteins to nitrocellulose, and probed for reactivity to *R. conorii* sera. Antibodies against Sca1 and rOmpB are not cross-reactive. “T7 pro” and “T7 term” refer to the transcriptional promoter and terminator sequences, respectively, present in pET-22b.

![Diagram of the sca1-containing pET-22b plasmid variant pSca1-200, showing the relevant 5' and 3' features. This vector encodes a recombinant protein fusion containing an N-terminal *E. coli* PelB signal sequence, *R. conorii* Sca1, and a C-terminal His6 tag. Due to the N-terminal PelB signal sequence and the inherent ability of autotransporter proteins to direct their own translocation across the bacterial outer membrane, Sca1 is predicted to be anchored in the bacterial outer membrane and primarily exposed to the extracellular environment. RBS, ribosome binding site. (B) Western blot analysis of outer membrane preparations of *E. coli* harboring the empty vector pET22b, pSca1-200, or *ompB*-containing pYC9 with anti-Sca1 (amino acids 144 to 157) or polyclonal anti-rOmpB. The presence of pYC9 has previously been demonstrated to permit rOmpB expression. Antibodies against Sca1 and rOmpB are not cross-reactive. “T7 pro” and “T7 term” refer to the transcriptional promoter and terminator sequences, respectively, present in pET-22b.](http://www.journals.ashp.org/doi/figure-pdf/10.14537/jmr.2010.2.1.1.3899)

**FIG. 2.** Expression of *R. conorii* Sca1 on the surface of *E. coli*. (A) Diagram of the *sca1*-containing pET-22b plasmid variant pSca1-200, showing the relevant 5’ and 3’ features. This vector encodes a recombinant protein fusion containing an N-terminal *E. coli* PelB signal sequence, *R. conorii* Sca1, and a C-terminal His6 tag. Due to the N-terminal PelB signal sequence and the inherent ability of autotransporter proteins to direct their own translocation across the bacterial outer membrane, Sca1 is predicted to be anchored in the bacterial outer membrane and primarily exposed to the extracellular environment. RBS, ribosome binding site. (B) Western blot analysis of outer membrane preparations of *E. coli* harboring the empty vector pET22b, pSca1-200, or *ompB*-containing pYC9 with anti-Sca1 (amino acids 144 to 157) or polyclonal anti-rOmpB. The presence of pYC9 has previously been demonstrated to permit rOmpB expression. Antibodies against Sca1 and rOmpB are not cross-reactive. “T7 pro” and “T7 term” refer to the transcriptional promoter and terminator sequences, respectively, present in pET-22b.

*E. coli* expression system to analyze the function of Sca1 when it was exposed to the extracellular environment. The entire *R. conorii sca1* open reading frame was cloned into the IPTG-inducible pET-22b vector to produce pSca1-200 (Fig. 2A) and then transformed into the *E. coli* BL21(DE3) strain. Induction of protein expression in *E. coli* harboring pSca1-200 but not pET22b or pYC9 (*ompB*) resulted in the presence of a high-molecular-weight anti-Sca1 reactive product in isolated outer membrane protein preparations (Fig. 2B).

Other rickettsial autotransporter proteins have previously been determined to mediate adherence and invasion of host cells (4, 5, 34, 54). We therefore examined the ability of Sca1-expressing *E. coli* to adhere to cultured mammalian cells. Sca1-expressing *E. coli* was applied to confluent monolayers of mammalian cells, and contact with epithelial (HeLa and Vero) and endothelial (EAhy.926) cells was induced by centrifugation. After incubation, cells were extensively washed to remove nonadherent bacteria, fixed, and, for analysis of immunofluorescence, treated with rabbit anti-*E. coli* and anti-rabbit Alexa Fluor 488 to stain *E. coli* (green), with DAPI to stain nuclei (blue), and with Phalloidin-TR to stain actin (red). The immunofluorescence analysis revealed that there was an increase in the number of adherent *E. coli* cells when Sca1 was expressed (Fig. 3A). The Sca1-mediated adhesion was verified by removal of adherent bacteria from live mammalian monolayers and enumeration using a CFU-based quantification assay. This
assay confirmed that significant increases in the adherence to both epithelial and endothelial cell lines were mediated by Sca1 expression (Fig. 3B to D). The observed percentages of adherence are comparable to those for other defined adherence proteins (3–5, 19, 33). Therefore, the expression of R. conorii Sca1 on the outer surface of E. coli cells is sufficient to mediate adherence of these bacteria to mammalian cells. Sca1-expressing E. coli does not invade host cells. A previous study demonstrated that the expression of R. conorii rOmpB (Sca5) in E. coli was sufficient to mediate adherence to and entry into mammalian cells (5, 54). We therefore sought to determine if R. conorii Sca1 expression is also sufficient to mediate invasion of host cells. E. coli cells expressing either Sca1 (pSca1-200) or rOmpB (pYC9) or E. coli cells with an empty vector (pET22b) were applied to cultured monolayers of Vero, HeLa, or EAhy.926 cells, and internalization was quantified by performing a gentamicin protection assay. As shown in Fig. 4, Sca1-expressing E. coli did not invade nonphagocytic mammalian cells, whereas E. coli expressing rOmpB (pYC9) did invade these cells. These results demonstrate that unlike other R. conorii Sca proteins, Sca1 is not sufficient to mediate invasion of either epithelial or endothelial cells.

**Scal peptide competitively inhibits R. conorii-host cell association.** We next determined if Sca1 could competitively inhibit R. conorii-host cell interactions. We preincubated mammalian monolayers with soluble rGST-Sca1(29-327) or rGST-His peptides and then assessed the ability of R. conorii to associate with the cells. As shown in Fig. 5A to C, preexposure of cells to rGST-Sca1(29-327) inhibited R. conorii-host cell association, as assessed by immunofluorescence and subsequent determination of the ratio of R. conorii cells to host cells. This observation demonstrates that preincubation with excess rGST-Sca1(29-327) can competitively inhibit association of the full-length Sca1 protein with the cognate mammalian ligand and that the observed adherence phenotype is, in fact, a consequence of Sca1 expression. The competitive inhibition further demonstrated that Sca1 mediates adherence of R. conorii to host cells.

**DISCUSSION**

Adherence and invasion are absolutely critical events in the life cycle of SFG rickettsiae, and these processes are predicted to be mediated by specific ligand-receptor interactions. Bioinformatic analysis of sequenced SFG *Rickettsia* genomes revealed a family of *sca* (surface cell antigen) genes that encode proteins that are predicted to be exposed to the extracellular environment (2, 28). Most of these genes, including *sca3* and *sca6* to *sca16*, are not well conserved across the spotted fever group, and many residual open reading frames do not encode complete proteins. In contrast, *sca1*, *sca0* (*ompA*), *sca2*, *sca4*, and *sca5* are nearly universally present in SFG rickettsiae. We
demonstrated that like other sca genes, sca1 is actively transcribed and expressed in R. conorii isolated from infected mammalian cells. Furthermore, using immunofluorescence microscopy, we demonstrated that Sca1 is localized on the R. conorii cell surface. To our knowledge, this is the first observation of the presence of Sca1 at the outer membrane of any SFG rickettsial species.

Interestingly, the predicted amino acid sequence of R. conorii Sca1 shares high levels of sequence identity and similarity with Sca1 proteins present in geographically diverse SFG Rick-

FIG. 4. Sca1-expressing E. coli cells do not invade mammalian cells. Sca1-expressing E. coli cells were incubated with the HeLa (A), Vero (B), or EAhy.926 (C) mammalian cell lines, washed with PBS to remove nonadherent bacteria, and then treated with gentamicin to kill extracellular bacteria. The remaining bacteria were enumerated. All experiments were conducted with rOmpB-expressing E. coli(pYC9) as a positive control for invasion of mammalian cells. The results are expressed as percentages of the input CFU and are typical of the results of three separate experiments.

FIG. 5. Incubation with a recombinant GST-Sca1 peptide blocks interaction with mammalian cells. Preincubation of Vero (A and C) or EAhy.926 (B) cells with rGST-Sca1(29-327), but not preincubation with rGST-His blocks association of R. conorii with host cells. After incubation of the peptides with the host cells, R. conorii cells were added to the mixtures, and contact was induced by centrifugation. After incubation, the cells were extensively washed and stained for immunofluorescent analysis with anti-R. conorii and DAPI to visualize the host nuclei (C). The remaining R. conorii cells were enumerated by immunofluorescence, and the results are expressed as the ratios of R. conorii cells to host cells (nuclei) (A and B). The data are representative of the data from at least three independent experiments. *, P < 0.01. Scale bars = 50 mm.
protein is unlikely to be responsible for host intracellular signaling. Sca1 expression does not mediate invasion of host cells, this mediated interaction with host cells (5, 32). However, since of host signaling has been identified for the rickettsial rOmpB-bacterium-host interactions that result in invasion. Modulation of adherence is a vital function and likely aids in the subsequent completely prevent the infection by SFG rickettsia species. The limited diversity implies that there is a conserved function that is either necessary or advantageous for the bacteria. The N and C termini of Sca1 are particularly well conserved. While we observed the expected conservation in regions with defined functions (namely, the signal sequence and β-peptide), the N-terminal portion of the Sca1 passenger peptide also appears to have limited sequence diversity, suggesting that the N-terminal portion of the Sca1 passenger peptide has a conserved function. We hypothesize that the Sca1-mediated adherence function is associated with the N terminus of the passenger domain, likely including the region encompassing amino acids 29 to 327.

Numerous reports have implicated a “zipper-like” mechanism in entry of SFG Rickettsia into host cells (20, 21, 25, 32, 49). This invasion strategy normally involves many receptor-ligand interactions at the interacting surfaces, followed by induction of host intracellular signaling to modulate the local host cytoskeletal environment and endocytic machinery at the site of interaction (8). Since Sca1 is able to mediate adherence but not invasion, this protein is likely to be a participant in the initial interaction between the bacterium and the host. In fact, Sca1 is likely to be a single component of overall rickettsial adherence because competitive inhibition with Sca1 peptides did not eliminate all R. conorii cell association and other rickettsial proteins have been demonstrated to be involved in association with host cells (Fig. 6) (4, 5, 34, 54). Thus, disruption of any single receptor-ligand interaction is unlikely to completely prevent R. conorii interaction with the host cells. Initial adherence is a vital function and likely aids in the subsequent bacterium-host interactions that result in invasion. Modulation of host signaling has been identified for the rickettsial rOmpB-mediated interaction with host cells (5, 32). However, since Sca1 expression does not mediate invasion of host cells, this protein is unlikely to be responsible for host intracellular signaling. It is possible that Sca1 augments signaling events mediated by other rickettsial proteins through induced adhesion and an intimate interaction with the host.

Innate immune responses are thought to limit the growth and spread of rickettsiae before establishment of specific anti-Rickettsia antibody responses. Several studies using different animal models of infection have identified critical components of the host defense response, including natural killer (NK) cells (1), CD8+ T lymphocytes (13–15, 22, 59), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) (14, 15). Importantly, passively transferred polyclonal antibodies to rOmpA or rOmpB protect SCID mice from lethal R. conorii infection in the absence of any other adaptive immune response (17). Also, specific Fc-dependent adherence and invasion of opsonized bacteria are not productive, because the bacteria cannot escape from the phagosome (16, 18). It is therefore important to note that an antibody-dependent immune response is able to efficiently capture and kill extracellular bacteria. Furthermore, antibody-bound bacteria are strong activators of natural antirickettsial innate immunity. Natural killer (NK) cells are thought to be vital mediators of early control of SFG Rickettsia infection (1), and NK cells are acutely responsive to pathogen-bound antibodies through the actions of their many FC receptors (48, 52). It is therefore likely that circulating Sca antibodies amplify and expedite the normal innate immune response to SFG Rickettsia, including NK cell-dependent activation of endothelial cells (15, 16, 18, 59).

Our phenotypic observations of Sca1-mediated adherence do not preclude other functions for Sca1. Currently, we are not able to perform genetic manipulation of the sca1 gene in R. conorii; therefore, our ability to query whether there are other Sca1-mediated functions is partially limited. The surface-exposed Sca1 passenger peptide is quite large (∼130 kDa), and
Sca1 appears to be one of the few rickettsial proteins exposed to the environment. Therefore, it is likely that there are other rickettsial functions that are mediated by Sca1. We predict that Sca1-mediated adherence is a universal function in SFG rickettsiae. The current definition of Sca1-mediated adherence emphasizes the need to identify the interacting Sca1 ligand in order to expand our targets for disruptive therapies for severe Rickettsia infections.

In conclusion, we demonstrated that the Sca1 protein is produced in R. conorii. When this protein is expressed on the surface of E. coli cells, it can allow these cells to adhere to host cells, and the presence of a recombinant Sca1 peptide can disrupt association of R. conorii with mammalian host cells. This work identified an attractive target for development of preventive therapies or treatments for severe infections with spotted fever group Rickettsia species.

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