

The Sca2 Autotransporter Protein from *Rickettsia conorii* Is Sufficient To Mediate Adherence to and Invasion of Cultured Mammalian Cells[∇]

Marissa M. Cardwell and Juan J. Martinez*

*The University of Chicago, The Department of Microbiology, 920 East 58th Street,
Cummings Life Sciences Center 707A, Chicago, Illinois 60637*

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Obligate intracellular bacteria of the genus *Rickettsia* must adhere to and invade the host endothelium in order to establish an infection. These processes require the interaction of rickettsial surface proteins with mammalian host cell receptors. A previous bioinformatic analysis of sequenced rickettsial species identified a family of at least 17 predicted “surface cell antigen” (*sca*) genes whose products resemble autotransporter proteins. Two members of this family, rOmpA and rOmpB of spotted fever group (SFG) rickettsiae have been identified as adhesion and invasion factors, respectively; however, little is known about the putative functions of the other *sca* gene products. An intact *sca2* gene is found in the majority of pathogenic SFG rickettsiae and, due to its sequence conservation among these species, we predict that Sca2 may play an important function at the rickettsial surface. Here we have shown that *sca2* is transcribed and expressed in *Rickettsia conorii* and have used a heterologous gain-of-function assay in *E. coli* to determine the putative role of Sca2. Using this system, we have demonstrated that expression of Sca2 at the outer membrane of nonadherent, noninvasive *E. coli* is sufficient to mediate adherence to and invasion of a panel of mammalian cells, including endothelial cells. Furthermore, soluble Sca2 protein is capable of diminishing *R. conorii* invasion of cultured mammalian cells. This is the first evidence that Sca2 participates in the interaction between SFG rickettsiae and host cells and suggests that in addition to other surface proteins, Sca2 may play a critical role in rickettsial pathogenesis.

The genus *Rickettsia* is composed of obligate intracellular gram-negative alphaproteobacteria, many of which are human pathogens. These pathogens are transmitted to the human host by arthropod vectors, such as ticks, which inoculate the host during a blood meal (8). Rickettsial species are divided into two groups, the typhus group (TG) and the spotted fever group (SFG) based on differences in their antigenicity to lipopolysaccharide, the presence of outer membrane proteins, and in part on the diseases that they cause (19). Members of both groups are responsible for severe human disease and two species, *Rickettsia prowazekii* (TG) and *Rickettsia rickettsii* (SFG) have been classified as select agents by the Centers for Disease Control and Prevention.

Infection by SFG rickettsiae, such as *R. conorii*, the causative agent of Mediterranean spotted fever, begins with inoculation of bacteria into the vasculature of the human host during a tick bite (8). Subsequent growth of *Rickettsia* in proximal endothelial cells can result in a localized dermal and epidermal necrosis called an eschar or tache noire (21). Further damage to the vascular endothelium and infiltration of perivascular mononuclear cells leads to increased fluid leakage into the interstitial space, ultimately resulting in a characteristic dermal rash and acute renal failure with peripheral edema (10, 21). Damage done by *R. conorii* to target endothelial cells, especially in the lungs, can lead to severe manifestations of disease including pulmonary edema and interstitial pneumonia. Although endothelial cells are the main target cell type for SFG *Rickettsia*, *R.*

conorii can attach to and invade different cell types in vitro and in vivo and spread via lymphatic vessels to the lymph nodes or via the bloodstream to various tissues (20). Initial clinical manifestations of rickettsial infection include flulike symptoms, often leading to delayed diagnosis or misdiagnosis. There is currently no vaccine available to prevent rickettsial infection but, if properly diagnosed, it can be controlled by broad-spectrum antibiotics. If left untreated, Mediterranean spotted fever and other SFG rickettsial diseases can result in severe morbidity and mortality (22).

Because *Rickettsia* cannot replicate extracellularly within the mammalian host, establishment of a successful infection relies entirely on the ability of the bacteria to invade a host cell. This process begins with adherence to the host cell through specific interactions, leading to eukaryotic downstream signaling and ultimately bacterial uptake (13). Thus far, two rickettsial surface proteins, rickettsial outer membrane protein A (rOmpA) and rickettsial outer membrane protein B (rOmpB), have been shown to participate in adhesion to mammalian cells in vitro (2, 12, 18) and in eliciting humoral and cellular protective immune responses (5, 6). In addition, rOmpB has been recently demonstrated to be sufficient in mediating bacterial invasion of mammalian cells in vitro through interaction with its mammalian receptor, Ku70 (2, 15). Interestingly, an analysis of rickettsial invasion in cells depleted of endogenous Ku70 by small interfering RNA or in Ku70^{-/-} mouse embryonic fibroblasts revealed that *R. conorii* invasion is reduced by ca. 50 to 60% (15), suggesting that factors other than Ku70 and rOmpB likely contribute to the invasion process.

rOmpA and rOmpB belong to a family of outer membrane proteins termed autotransporters found in gram-negative bacteria. This class of proteins contains an N-terminal signal se-

* Corresponding author. Mailing address: The University of Chicago, The Department of Microbiology, 920 East 58th Street, Cummings Life Sciences Center 707A, Chicago, IL 60637. Phone: (773) 834-4556. Fax: (773) 834-8150. E-mail: jmartine@bsd.uchicago.edu.

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gation, resuspended in Tris-buffered saline (50 mM Tris [pH 8.0] plus 150 mM NaCl) containing protease inhibitor cocktail, and lysed by sonication (40% amplitude, 5 s on and 10 s off for 20 min; Fisher Scientific sonic dismembrator model 500). Lysates were cleared by centrifugation at $13,000 \times g$ for 30 min at 4°C and applied to a GST-TRAP FF column (GE Healthcare, Piscataway, NJ) by fast protein liquid chromatography on a Äkta fast protein liquid chromatography. Protein was eluted with 30 mM reduced glutathione, and fractions were dialyzed into Tris-buffered saline before storage at -80°C. Protein to be utilized as antigen for antibody production was cleaved with thrombin to remove the GST tag. Thrombin and GST were purified away from Sca₂₃₄₋₇₉₄ by collection of the flowthrough after passage over a benzamide Sepharose column (GE Healthcare) and a GST-TRAP FF column.

Cell association and invasion assays. Cell association and invasion assays were performed as described previously (2, 14). Briefly, mammalian cells were seeded into 24-well plates at 1.2×10^5 cells per well 24 h prior to infection. Cells were washed with serum-free DMEM, infected with 50 μ l of induced *E. coli* (OD₆₀₀ = 1.0) resuspended in PBS, centrifuged to induce bacterial contact at 200 *g* for 5 min, and then incubated at 37°C and 5% CO₂ for 20 or 60 min to assess the cell association or invasion, respectively. In some experiments, mammalian cells were preincubated with 1 μ M GST or GST-Sca₂₃₄₋₇₉₄ for 30 min at 37°C and 5% CO₂ prior to the addition of bacteria. In cell association assays, cells were washed extensively with PBS, lysed with 0.1% Triton X-100 in double-distilled H₂O, and plated onto LB agar plates for determination of the CFU. Adherence frequencies were calculated as the percentage of cell-associated bacteria recovered after the PBS washes out of the total bacteria in each well after the 20 min of incubation. To determine bacterial invasion, cells were washed with PBS and then incubated in complete DMEM for an additional 2 h with 100 μ g of gentamicin sulfate (MP Biomedicals)/ml. Thereafter, the cells were washed with PBS, lysed with 0.1% Triton X-100 in double-distilled H₂O, and plated onto LB agar plates. Invasion frequencies were determined as the number of bacteria surviving the gentamicin challenge out of the total bacterial input after the initial 1 h of incubation. *P* values were determined by using a two-tailed Student *t* test.

For *R. conorii* invasion assays, Vero cells were seeded at 1.5×10^4 cells/well in 96-well plates 24 h prior to infection with *R. conorii*. Prior to infection, the medium was aspirated from the cells, 50 μ l of purified GST or GST-Sca₂₃₄₋₇₉₄ diluted in cold serum-free DMEM was added, and then Vero cells were incubated for 30 min at 37°C and 5% CO₂. *R. conorii* were diluted to a concentration of 1.6×10^5 bacteria/ml in room temperature serum-free DMEM, and 50 μ l was added to each well of Vero cells. The plate was centrifuged at $300 \times g$ for 5 min at room temperature to induce contact, incubated for 30 min at 37°C and 5% CO₂, washed with PBS, and fixed in 4% paraformaldehyde (PFA) for 20 min. The infected monolayers were then processed for immunofluorescence and differentially stained to distinguish between extracellular and intracellular *R. conorii*. Briefly, extracellular *R. conorii* were stained with Rc7 antiserum (1:1,000), followed by Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:1,000), prior to permeabilization of the mammalian cells with 0.1% Triton X-100. The total *R. conorii* cells were then stained with Rc7, followed by Alex Fluor 499-conjugated goat anti-rabbit IgG. Images were captured as described for immunofluorescence assays, and the numbers of nuclei and extracellular and total bacteria were enumerated by using the ImageJ software "Analyze Particles" function.

Immunofluorescence. Cell association assays were performed as described above for the Sca2-expressing *E. coli*. After the nonadherent bacteria were washed away from the mammalian monolayer with PBS, the cells were fixed in 4% PFA for 20 min at room temperature, washed with PBS, permeabilized with PBS plus 0.1% Triton X-100 for 3 min, and washed again with PBS to removal all Triton X-100. PBS plus 2% bovine serum albumin was used to block the fixed cells, as well as for the antibody dilutions. To assess the association of *E. coli* with mammalian cells, cells were stained with anti-*E. coli* antisera (1:1,000), followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000), Texas Red-phalloidin (1:200), and DAPI (1:10,000). Images were digitally captured on a Nikon Eclipse TE2000-u microscope coupled to a charge-coupled device camera using $\times 200$ magnification and processed using Adobe Photoshop.

Electron microscopy. For scanning electron microscopy, HeLa cells grown on 12-mm glass coverslips in 24-well plates were infected with 100 μ l of induced bacteria (OD₆₀₀ = 1.0) for 20 min and then processed as described previously (2). The samples were visualized by using Fei NovaNano SEM200 at a distance of 5 mm. For transmission electron microscopy, HeLa cells grown in six-well plates (3×10^5 cells per well) were infected with 2 ml of induced bacteria (OD₆₀₀ = 2.0) for 2 h, washed, and then bathed in fixative (2% glutaraldehyde, 4% PFA, 0.1 M sodium cacodylate buffer.) Cells were carefully scraped from the plate and allowed to fix overnight. The next day, cells were pelleted at $500 \times g$

TABLE 1. Comparison of Sca2 amino acid sequence among rickettsial species

Rickettsial species	Size (amino acids)	<i>R. conorii</i>	
		% Identity	% Similarity
<i>R. rickettsii</i> Sheila Smith	1,821	90	92
<i>R. japonica</i>	1,813	89	92
<i>R. sibirica</i>	1,812	94	95
<i>R. australis</i>	1,804	83	90
<i>R. typhi</i>	1,483	25	38

for 5 min and subsequently processed as previously described (2). The samples were imaged on the FEI Tecnai F30 with a Gatan charge-coupled device digital micrograph.

RESULTS

Conservation of *sca2* among spotted fever group rickettsia.

Previous bioinformatic annotations of several rickettsia genomes have demonstrated that a complete *sca2* gene is present among most SFG rickettsiae (1, 16). To determine the degree of conservation within the predicted Sca2 protein sequence, an amino acid alignment was performed comparing the sequence of Sca2 from five geographically diverse SFG *Rickettsia* spp. (*R. conorii*, *R. rickettsii* Sheila Smith, *R. japonica*, *R. sibirica*, and *R. australis*) and one TG *Rickettsia* sp. (*R. typhi*). As shown in Table 1, the percent identity of Sca2 sequences observed among different SFG rickettsial species is substantial, varying from 83% in *R. australis* to 94% in *R. sibirica* to the Sca2 sequence from *R. conorii* Malish 7. Furthermore, the percent similarity is more striking, ranging from 90 to 95% among the SFG *Rickettsia* species analyzed. In contrast, the predicted Sca2 amino acid sequence appears to be less conserved among *R. typhi* possibly due to the degeneration of the *sca2* gene within this class of *Rickettsia*. The high degree of Sca2 sequence identity and conservation among SFG *Rickettsia* suggests that Sca2 may play a critical function during SFG rickettsial infections.

Detection of endogenous *sca2* protein and expression of recombinant Sca2 protein in *E. coli*. We first determined whether *sca2* is actively transcribed in *R. conorii* Malish 7 during association with mammalian cells. We purified mRNA from *R. conorii* Malish 7, freshly isolated from a Vero cell infection, and removed any contaminating rickettsial or Vero genomic DNA by DNase I treatment. The DNase I-treated rickettsial RNA was then reverse transcribed by using random primers to yield a cDNA/RNA hybrid. The reverse transcription products were used as templates for PCRs amplifying one of four rickettsial *sca* genes using the indicated primer pairs (Table 2). As shown in Fig. 1A, agarose gel electrophoresis analysis of PCR products reveals the amplification of the rickettsial gene *sca2* from *R. conorii* cDNA, indicating that *sca2* mRNA transcript was present prior to reverse transcription. PCR product was also detectable for the genes *ompA*, *ompB*, and *sca4*, which are expressed in *R. conorii* (data not shown). The PCR products were cloned and sequence verified to confirm the amplification of the correct gene fragment. PCR products were not observed in the control reactions lacking RNA template or reverse transcriptase, indicating that amplification

TABLE 2. RT-PCR primers

<i>R. conorii</i> gene	Primer sequence (5'-3')	
	Forward	Reverse
<i>ompA</i>	AATGACACCGCTACAG GAAGCAGA	AAACGGGCTTATCCA CGCACCAA
<i>ompB</i>	CAAACAGGCGTTGTTG ATGCGAGT	ATGCGCGAATTGTAC TGCACCGTT
<i>sca2</i>	TGGTGCTGATAAAGGT GAGGCAAC	TTGAGAGCGTCCGGT AGATGGTTT
<i>sca4</i>	AGTTCTCAGTCCGGCA CAACAACA	AGCTTCAAGCGAGCT TCCTGCAAT

was due solely to the presence of mRNA and not due to contaminating genomic DNA. This result demonstrates that mRNA transcript of *sca2* is present in *R. conorii* isolated from infected Vero cells.

The *sca2* gene is predicted to encode a protein with a molecular mass of ~200 kDa. To identify endogenous Sca2 protein in *R. conorii*, Sca2 antiserum was produced by the immunization of a New Zealand White rabbit with a soluble fragment of the predicted *R. conorii* Sca2 passenger domain encompassing amino acids 34 to 794 (Sca2₃₄₋₇₉₄). Western immunoblotting of *R. conorii* whole-cell lysate reveals the presence of an ~150-kDa species recognized by anti-Sca2₃₄₋₇₉₄ antiserum and not preimmune rabbit sera (Fig. 1B). This result indicates that Sca2 protein is present in *R. conorii* isolated from a Vero cell infection and suggests, that similar to other rickettsial autotransporter proteins, Sca2 may be processed in vivo.

Previous studies have successfully utilized an *E. coli*-based heterologous protein expression system to demonstrate that a conserved rickettsial surface cell antigen, rOmpB, expressed at the outer membrane of *E. coli* is sufficient to mediate adher-

ence and invasion of cultured mammalian cells (2, 18). To study the putative function of Sca2 as a bacterial surface protein, we adapted this system to express Sca2 at the outer membrane of *E. coli*. The full-length *R. conorii sca2* open reading frame was cloned into the *E. coli* IPTG-inducible expression vector, pET-22b, resulting in plasmid pSca2-200 (Fig. 1C). The plasmid pSca2-200 was transformed into the *E. coli* expression strain, BL21(DE3), induced for protein expression, and then biochemically fractionated to isolate *E. coli* outer membrane proteins. As shown in Fig. 1D, increasing concentrations of IPTG leads to the appearance of a high-molecular-weight His₆ reactive species as analyzed by Western immunoblotting (left panel) with 0.5 mM IPTG induction, yielding the best protein expression. The identity of this induced His₆ reactive protein was confirmed as Sca2 by using anti-Sca2₃₄₋₇₉₄ antiserum (right panel). Taken together, these results demonstrate that Sca2 is present at the outer membrane of induced *E. coli* in this heterologous expression system.

Sca2 expression in *E. coli* is sufficient to mediate association to epithelial and endothelial cells. To study the putative contribution of Sca2 in mediating interactions with host cells, *E. coli* expressing Sca2 at the outer membrane was assessed for the ability to associate with cultured mammalian cells in vitro by immunofluorescence and CFU-based adherence assays (see Materials and Methods). As shown in Fig. 2A, expression of Sca2 was sufficient to mediate cell association of *E. coli* to cultured Vero cells compared to *E. coli* harboring the empty vector, pET-22b. A CFU-based assay used to quantify cell association of *E. coli* expressing Sca2 to epithelial cell lines (HeLa and Vero), transformed human umbilical vein endothelial cells (EAHY 926), and HLMV cells confirmed these results (Fig. 2B). These data illustrate that

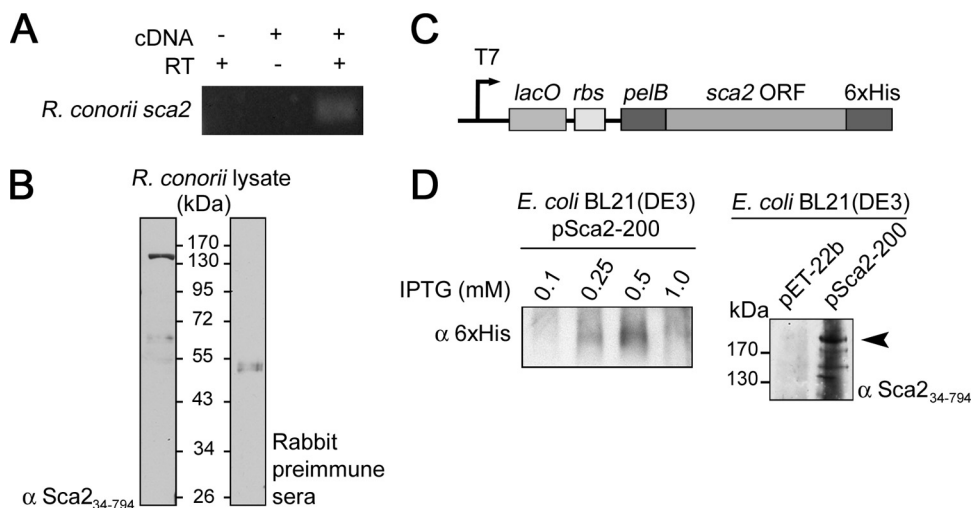


FIG. 1. Detection of endogenous Sca2 protein in *R. conorii* and expression of recombinant Sca2 protein in *E. coli*. (A) Agarose gel electrophoresis analysis of PCR products resulting from amplification of *R. conorii* cDNA using a *sca2*-specific primer pair (Table 2). PCR product is 240 bp in size. A no-template control (-cDNA) and no-reverse-transcriptase control (-RT) are included to control for environmental DNA contamination and genomic DNA contamination, respectively. (B) Western immunoblot analysis of *R. conorii* whole-cell lysate probed with rabbit anti-Sca2₃₄₋₇₉₄ hyperimmune sera (left panel) and rabbit preimmune sera (right panel). (C) Schematic of the pET-22b expression vector with the inserted *sca2* open reading frame. Relevant features of the 5' and 3' ends of pSca2-200, including the in-frame N-terminal *pelB* leader sequence and the C-terminal His₆ tag are shown. (D) Western immunoblot analysis of outer membrane fractions of *E. coli* BL21(DE3) expressing the empty vector (pET-22b) or full-length *R. conorii* Sca2 (pSca2-200) probed with anti-His₆ (left panel) and anti-Sca2₃₄₋₇₉₄ antibodies (right panel). Maximal expression of recombinant Sca2 is observed with 0.5 mM IPTG induction (left panel), resulting in a protein product larger than 170 kDa (arrow).

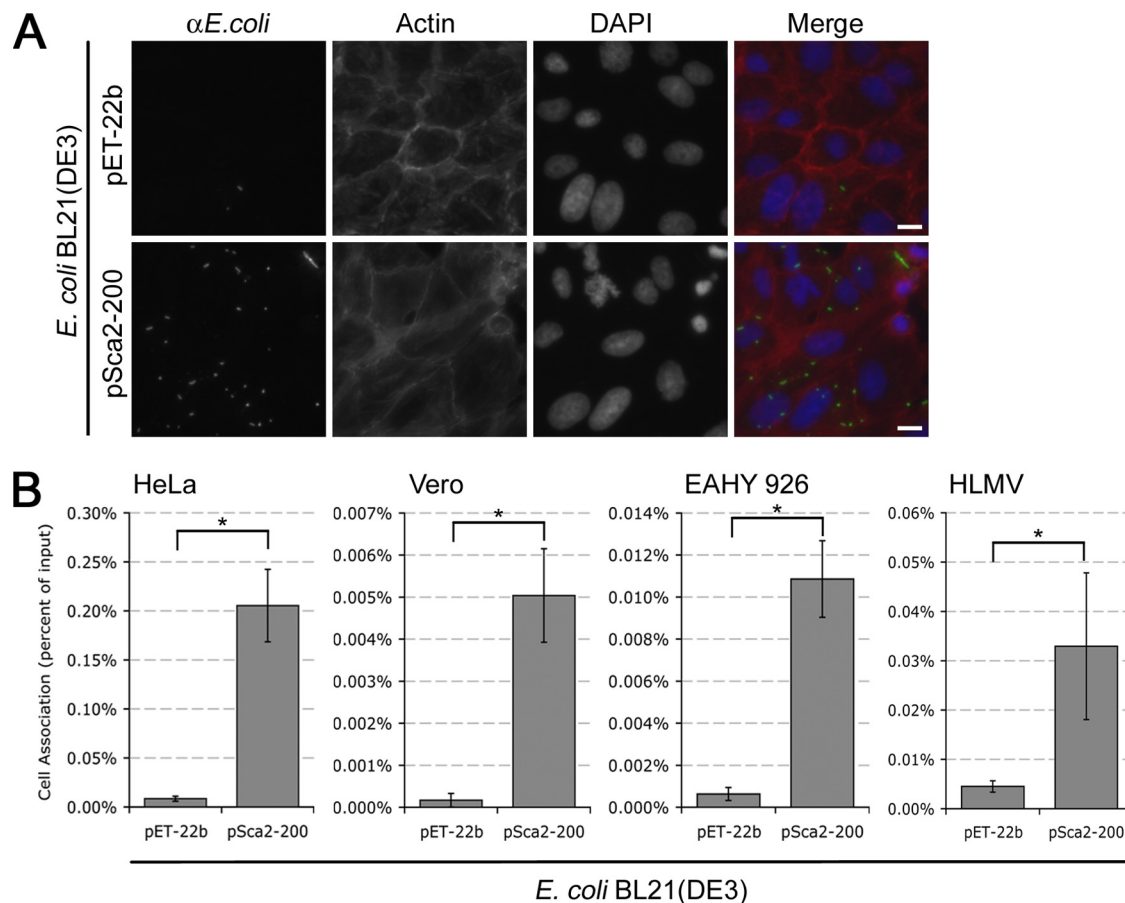


FIG. 2. Expression of Sca2 in *E. coli* is sufficient to mediate adherence to mammalian cells. (A) Fluorescence micrographs of monolayers of Vero cells infected with *E. coli* BL21(DE3) expressing the empty vector (pET-22b) or the full-length Sca2 protein from *R. conorii* (pSca2-200). Confluent monolayers of Vero cells were infected for 20 min at 37°C, washed repeatedly with PBS, and then processed for immunofluorescence. Scale bars, 10 μ m. (B) CFU-based quantification of bacterial adherence to mammalian host cells. HeLa, Vero, EAHY 926, and HLMV cells were infected with induced cultures as in panel A. Confluent monolayers were infected with induced bacteria for 20 min at 37°C and washed repeatedly with PBS, and then cell-associated bacteria were extracted from host cells by detergent lysis, and plated for CFU quantification. Association was determined as the % CFU of cell-associated bacteria from the initial bacterial inoculums. Actual percentages varied from assay to assay (ranging from 0.001 to 0.2%) depending on the passage number of cells used and the expression of Sca2 at the *E. coli* outer membrane. *, $P < 0.05$. The data presented are representative of at least three independent assays for each cell line. Error bars represent the standard deviation of each data set.

expression of Sca2 at the *E. coli* outer membrane is sufficient to mediate adherence to a variety of mammalian cell types.

Sca2 expression in *E. coli* is sufficient to mediate invasion of epithelial and endothelial cells. An ultrastructural analysis of Sca2-mediated *E. coli* association to HeLa cells by scanning electron microscopy (SEM) revealed the induction of subtle membrane perturbations and close contacts between bacteria and host cell (Fig. 3A), a finding reminiscent of those observed during rOmpB-mediated internalization (2, 18). To investigate the ability of Sca2 to mediate invasion of host epithelial and endothelial cells, we utilized a standard gentamicin protection assay. Confluent monolayers of mammalian cells were infected for 60 min with induced *E. coli* BL21(DE3) harboring either the empty vector, pET-22b, or the Sca2 expression construct, pSca2-200. Extracellular bacteria were killed by gentamicin, and internalized bacteria were plated for CFU enumeration. As shown in Fig. 3B, in the absence of other rickettsial factors, Sca2 is sufficient to mediate uptake by

both epithelial and endothelial mammalian cells. These results were confirmed by transmission electron microscopy, demonstrating that Sca2-expressing *E. coli* induces changes in the plasma membrane of HeLa cells, ultimately leading to internalization (Fig. 3C).

Soluble Sca2 protein inhibits rickettsial invasion. In order to confirm the Sca2-mediated invasive phenotype observed in our heterologous assay, we used purified Sca2 protein to competitively inhibit *E. coli* BL21(DE3) invasion with mammalian cells when expressing Sca2 at the bacterial surface. We cloned the first half of the Sca2 passenger domain encompassing amino acids 34 to 794 into the *E. coli* expression vector pGEX-2TKP in order to produce and affinity purify a soluble N-terminal GST tagged Sca2 fragment (GST-Sca2₃₄₋₇₉₄). Attempts to express and purify larger soluble portions of Sca2 were unsuccessful due to protein instability. The addition of GST-Sca2₃₄₋₇₉₄ to a HeLa cell monolayer prior to infection with *E. coli* BL21(DE3) expressing full-length *R. conorii* Sca2

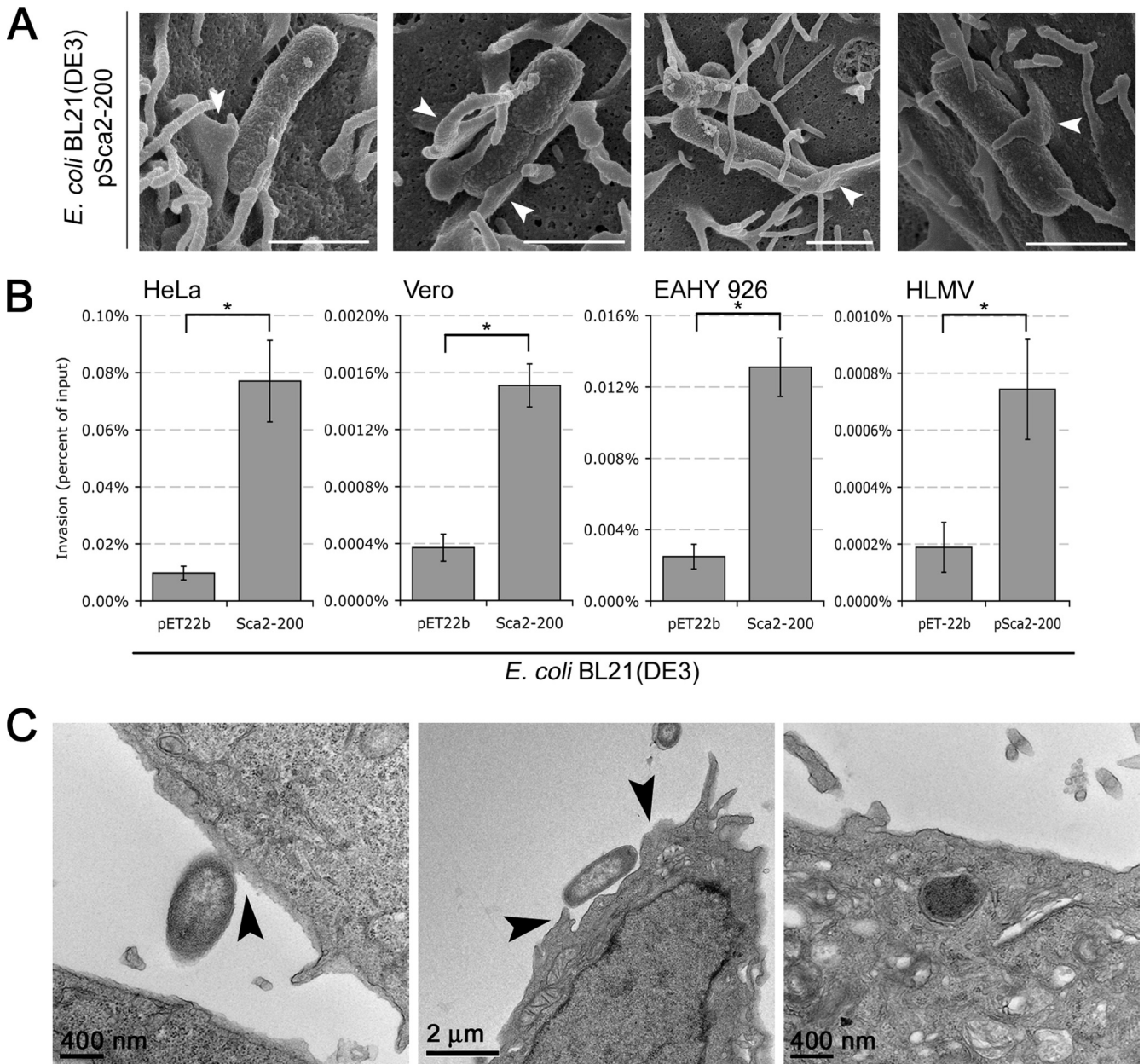


FIG. 3. Sca2 mediates invasion of mammalian cells. (A) Scanning electron micrographs examining the surface interaction of Sca2-expressing *E. coli* with HeLa cells. HeLa cell monolayers on glass coverslips were infected with induced bacteria for 20 min and then processed for SEM. White arrowheads highlight possible Sca2-mediated cellular membrane rearrangements. Scale bars, 1 μ m. (B) CFU-based quantification of bacterial invasion of HeLa, Vero, EAHY 926, and HLMV cells. Confluent cell monolayers were infected for 1 h with *E. coli* BL21(DE3) expressing the empty vector (pET-22b) or Sca2 from *R. conorii* (pSca2-200) and assessed for invasion by gentamicin protection assay. Invasion is presented as the percentage of bacteria recovered after the gentamicin challenge out of the inoculums. Actual percentages varied from assay to assay (ranging from 0.0007 to 0.07%), depending on the passage number of the cells used and the expression of Sca2 at the *E. coli* outer membrane. *, $P < 0.05$. The data presented are representative of at least two independent experiments for each individual cell line. Error bars represent the standard deviation of each data set. (C) Transmission electron micrographs of HeLa cells infected with Sca2-expressing *E. coli* demonstrate different steps of the uptake process, including the initial attachment event (left panel), the induction of changes in the plasma membrane (arrows in middle panel), and the presence of bacteria within membrane bound vacuoles (right panel). Scale bars are indicated in each panel.

at the surface reduced the ability of these bacteria to invade host cells by 50% (Fig. 4A). This result confirms that bacterial invasion of the host cell in our heterologous system is specifically mediated by Sca2. To assess the role of Sca2 during a rickettsial infection, we similarly used GST-Sca2₃₄₋₇₉₄ to com-

petitively inhibit *R. conorii* invasion of Vero cells. As shown in Fig. 4B, preincubation of Vero cells with GST-Sca2₃₄₋₇₉₄ reduced the ability of *R. conorii* to invade host cells by ca. 30%, suggesting that Sca2 is involved in mediating the rickettsial invasion of host cells.

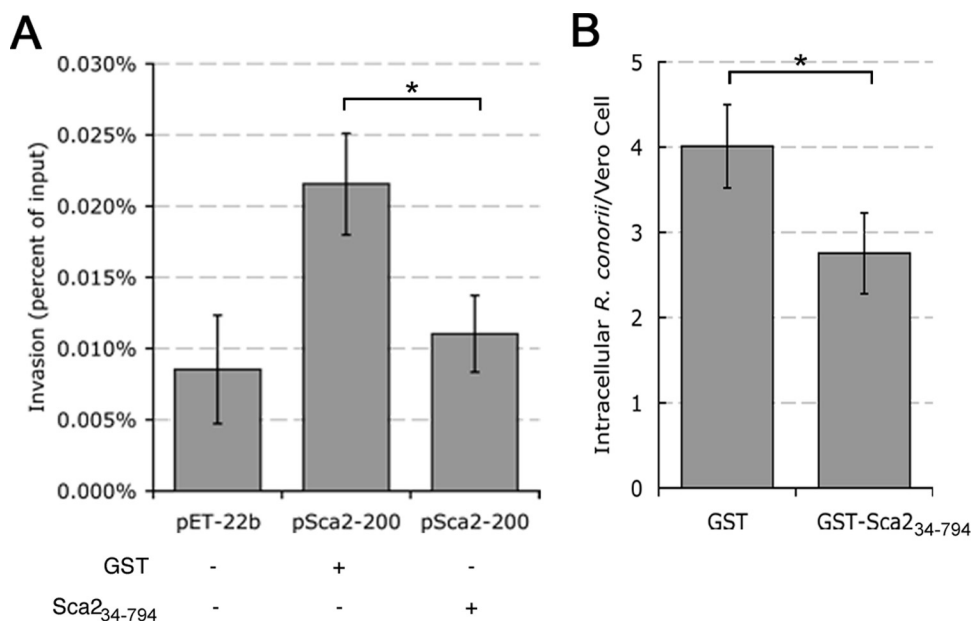


FIG. 4. Soluble Sca2 protein inhibits rickettsial invasion. (A) Monolayers of HeLa cells were incubated with 1 μ M soluble GST or GST-Sca2₃₄₋₇₉₄ protein for 20 min at 37°C and 5%CO₂ prior to infection with *E. coli* BL21(DE3) expressing pSca2-200. BL21(DE3) harboring pET-22b was used as a negative control. Bacterial invasion was assessed as described in Fig. 3B. (B) Vero cell monolayers seeded at 90% confluence were incubated with 8 μ M GST or GST-Sca2₃₄₋₇₉₄ protein prior to infection with *R. conorii* for 30 min. Total rickettsial association and intracellular bacteria were enumerated by differential immunofluorescence microscopy. *, $P < 0.05$. The data presented are representative of at least two independent experiments. The error bars represent the standard deviation from the average in these experiments.

DISCUSSION

Pathogenesis of a rickettsial infection relies on the ability of the bacterium to adhere to and invade host cells. These interactions are predicted to be governed by rickettsial outer membrane proteins and cognate host cell receptors, whose interactions induce cellular signal transduction events leading to bacterial internalization. A search for genes encoding putative outer membrane proteins in several rickettsia genomes revealed the presence of a family of at least 17 genes, termed *sca*, whose predicted sequences exhibit similarity to a family of modular proteins, termed autotransporters, found in gram-negative bacteria (1). Overall, the *sca* gene family exhibits a high degree of degeneration since several genes, including *sca6* through *sca16*, are fragmented, split, or otherwise completely deleted from the genomes of different SFG rickettsia species (1). In contrast, several genes, including *sca2*, are present in the genomes of a variety of geographically distinct SFG *Rickettsia* species. The predicted Sca2 amino acid sequence of *R. conorii* and other SFG *Rickettsia* spp. exhibits a high degree of identity and similarity among geographically dispersed SFG rickettsial species, including *R. japonica* (found in Japan and East Asia), *R. australis* (Australia), and *R. rickettsii* (North and South America), suggesting that the function of this protein may be conserved. To our knowledge, this is the first demonstration of endogenous *sca2* mRNA and Sca2 protein in *R. conorii* Malish 7 isolated from infected mammalian cells.

Efficient genetic tools to target specific genes in obligate intracellular pathogens have not yet been fully developed. Therefore, to study Sca2 function, we adapted an *E. coli* heterologous expression system that has been successfully used to characterize rOmpB function of *R. conorii* and *R. japonica* (2,

18). By expressing Sca2 at the *E. coli* outer membrane, we demonstrated that in the absence of other rickettsial virulence factors, Sca2 is sufficient to mediate adherence to and invasion of cultured mammalian epithelial and endothelial cells. Inhibition of these phenotypes with purified GST-Sca2 peptide confirms that invasion of host mammalian cells is specifically mediated by Sca2. Interestingly, the endogenous, native form of Sca2 in isolated *R. conorii* migrates at a smaller molecular mass (~150 kDa) than the predicted full-length protein (~200 kDa) observed in Sca2-expressing *E. coli*. This suggests that, as has been previously observed for a related Sca protein, rOmpB, (9), Sca2 may also be processed in vivo to release the passenger domain from the translocon domain. The processing machinery is likely absent in *E. coli*; however, as has been previously demonstrated for rOmpB (2), the processing of a heterologously expressed rickettsial autotransporter on the surface of *E. coli* is not required to mediate adhesion and invasion of mammalian cells.

Scanning and transmission electron micrographs of HeLa cells infected with Sca2-expressing *E. coli* revealed the induction of localized changes in the host cell plasma membrane that are likely snapshots of the events leading to invasion. These images revealed cellular membrane protrusions that are reminiscent of those observed in the actin-dependent rOmpB-mediated invasion process (2). Whether Sca2-mediated uptake is mechanistically similar to that mediated by rOmpB is currently under investigation. This is the first demonstration that a *Rickettsia* gene product other than rOmpA and rOmpB is sufficient to mediate surface interactions with target host cells in vitro.

The ability of a pathogen to induce internalization into

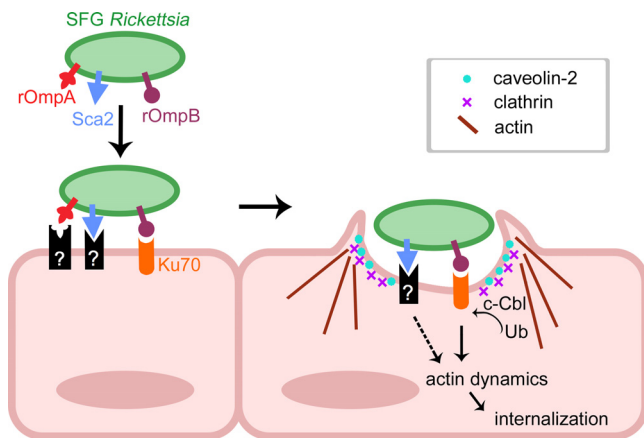


FIG. 5. Model of SFG *Rickettsia* interactions with mammalian cells. Interactions of SFG *Rickettsia* with host cells are likely governed by coordinated interactions between rOmpB-Ku70 and Sca2 with an unknown mammalian receptor (black box). Scanning and transmission electron microscopy analyses revealed similarities between rOmpB and Sca2-mediated uptake of mammalian cells, suggesting that while signals may be triggered by different receptors at the plasma membrane, signals ultimately converge to recruit localized actin filaments and components of the endocytic machinery (clathrin, caveolin-2, and c-Cbl) to entry foci.

mammalian cells is likely governed by multiple adhesin-receptor interactions that are coupled to drive changes in the host cell plasma membrane. In the hostile extracellular environment of the host where microorganisms encounter both the innate and adaptive immune systems, it is to the pathogens' advantage to have as many tools as possible to successfully adhere to and in some cases invade host cells. Other invasive pathogens such as *Listeria monocytogenes*, *Yersinia pseudotuberculosis*, and uropathogenic *E. coli* utilize multiple nonfimbrial and fimbrial adhesins that are necessary and sufficient to mediate uptake in nonphagocytic mammalian cells (3, 14). Previous studies have identified rOmpA (Sca0) and rOmpB (Sca5) as proteins involved in adhesion and invasion, respectively, of cultured mammalian cells (2, 12, 18). Whereas rOmpA receptors have yet to be identified, recent studies have identified plasma membrane associated Ku70 as a receptor for rOmpB (2, 15). Since SFG *Rickettsia* are to an extent still able to invade mammalian cells depleted of Ku70, factors other than rOmpB and Ku70 are likely important. We have demonstrated that recombinant Sca2 protein reduces *R. conorii* invasion of mammalian cells (~30%), suggesting that Sca2 plays an important role in *R. conorii* infection process and may complement invasion mediated by other rickettsial virulence factors (i.e., rOmpB). It is possible that Sca2 may work in concert with other rickettsial proteins to interact with target mammalian cells, especially endothelial cells during the infection process (Fig. 5). The interactions of Sca2 with its cognate receptor(s) may be coordinated with other receptor-ligand pairs to trigger previously observed downstream signaling events, involving the recruitment of host endocytic machinery components (c-Cbl, caveolin-2, and clathrin), and the activation of protein tyrosine and phosphatidylinositol 3-kinases (2, 13) that ultimately lead to localized actin recruitment required for entry. Interestingly, a biochemical

analysis of *R. conorii* proteins that interact with Ku70 identified rOmpB, but not other putative Sca proteins, as a ligand (15), suggesting that Sca2-mediated adherence and entry would be independent of Ku70. The identification of mammalian receptors involved in Sca2-mediated uptake of mammalian cells is of utmost importance and remains the focus of ongoing studies.

Clearly, all SFG *Rickettsia* proteins involved in adherence and invasion of host cells have not been identified. Further characterization of the roles of conserved Sca proteins in vitro and in vivo is crucial to fully understanding the complex host pathogen interactions during SFG rickettsial infections in humans and will hopefully expand our targets for the development of more efficacious therapies against rickettsial diseases.

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