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Complement Regulator-Acquiring Surface Protein 1 Imparts Resistance to Human Serum in *Borrelia burgdorferi*

Chad S. Brooks,2* Santosh R. Vuppala,* Amy M. Jett,* Antti Alitalo,† Seppo Meri,† and Darrin R. Akins3*

The *Borrelia burgdorferi* sensu lato complex, which includes *B. burgdorferi*, *Borrelia garinii*, and *Borrelia afzelii*, comprises pathogenic spirochetes responsible for the majority of human Lyme disease in North America and Europe (1). Interestingly, these three genospecies have been noted to exhibit distinct disease manifestations and tissue tropisms during mammalian infection (2, 3). The diversity in clinical symptoms has been suggested to result from key differences in the surface protein repertoire of each genospecies. In this regard, a collection of complement regulator-acquiring surface protein (CRASP)4 orthologs has been described recently from *B. burgdorferi* sensu lato organisms that bind human factor H (hFH) and/or FH-like protein 1 (FHL-1) (4–13). Considering the ability of pathogenic *Borrelia* spp. to survive extracellularly in the mammalian host, these spirochetes must have developed efficient strategies for circumventing the innate immune response. Along these lines, it has been suggested that CRASPs are key mediators that allow *B. burgdorferi* to evade the innate immune response and complement-mediated destruction during the acute stage of infection (14, 15).

FH and FHL-1, which is a truncated version of FH resulting from an alternative splice site in the FH transcript (16), are negative regulators of the alternative pathway of the complement system. Therefore, many bacteria bind FH and FHL-1 to inhibit activation of the alternative pathway of complement and avoid destruction (17). In addition to *B. burgdorferi*, FH binding also has been recognized as an important virulence strategy for several other pathogenic bacteria, including *Neisseria gonorrhoeae*, pneumococci, and group A and B streptococci (18, 19). Since the initial observation that *B. burgdorferi* binds FH/FHL-1 on its surface by Meri and colleagues (7), similar observations have been reported by Kraiczy et al. (20), Stevenson et al. (10), and Marconi and colleagues (11, 12). It is currently thought that the key to human serum resistance in *Borrelia* spp. is mediated by FH/FHL-1 being bound in host serum by two distinct types of CRASPs, which have been designated class 1 and class 2 (11). FH/FHL-1 being bound to the borrelial surface by these two classes of surface proteins ultimately helps to inhibit the alternative pathway of complement activation by promoting C3b inactivation on the surface of complement-resistant *B. burgdorferi* (9).

Recently, several laboratories, including our own, determined that the class 1 CRASPs correspond to outer surface lipoprotein E (OspE) and the various OspE paralogs (7, 8, 10, 12, 21). The OspE paralogs are encoded on circular plasmids (cp) and are up-regulated during tick transmission and acute infection (22, 23). The single class 2 CRASP, designated CRASP-1 (24), is a surface-exposed 27-kDa lipoprotein encoded by open reading frame (ORF) *bbA68*, which is harbored by the 54-kb linear plasmid (lp) that also
encodes the OspA/B operon (4, 6, 25). In addition to its FH/FHL-1-binding properties, CRASP-1 also was shown recently to confer protection in SCID mice challenged with *B. burgdorferi* strain ZS7 (26), indicating that CRASP-1 could be a candidate second generation vaccine for Lyme disease. Although it is likely that both the class 1 and 2 FH/FHL-1-binding proteins are important in the parasitic strategy of *B. burgdorferi*, it was suggested recently that CRASP-1 correlated with complement resistance in *B. burgdorferi*, which led these authors to speculate that CRASP-1 is the dominant FH/FHL-1-binding protein on the surface of *B. burgdorferi* (6).

It is now well recognized that serum resistance of various *Borrelia* spp. is closely correlated with their ability to bind FH/FHL-1 (11, 16, 27, 28). Consistent with this notion, it also has been shown that complement-resistant *B. burgdorferi* and *B. afzelii* strains, but not complement-sensitive *B. garinii* strains, can bind rabbit FH from growth medium (27). This finding has further implicated FH/FHL-1-binding proteins as important contributors to complement resistance in *B. burgdorferi* (27). Although it is still not entirely clear why human serum-sensitive *B. garinii* strains are susceptible to complement-mediated killing, it was recently reported that *B. garinii* does not bind FH and only weakly binds FH/FHL-1 (29). Furthermore, we recently reported that there are several key sequence differences between the *B. garinii* and *B. burgdorferi* OspE-related proteins in their FH/FHL-1 binding domains, which most likely explains why the OspE paralogs of *B. garinii* do not bind FH/FHL-1 (8). The lack of FH-binding proteins in *B. garinii* also has led to the notion that this genospecies most commonly causes chronic infections in the CNS because the concentration of cytotoxic complement in the CNS is <1% of that found in blood (3, 30).

Recent studies have raised at least two important questions regarding complement resistance in *Borrelia* spp.: 1) is CRASP-1 the dominant source of FH/FHL-1 binding and the key molecule that imparts complement resistance to *B. burgdorferi*, and 2) what role does CRASP-1 play in borrelial virulence and Lyme disease pathogenesis? To begin examining these questions, we inactivated the *CRASP-1* gene in serum-resistant *B. burgdorferi* B31cF and evaluated the resulting mutant for its sensitivity to human serum-mediated killing. We constructed a shuttle vector expressing the native *B. burgdorferi* CRASP-1, designated pKFSS-1::CRASP-1. Resulting vector served as the template for PCR to generate the final CRASP-1 inactivation construct, the *flgB::kanamycin* resistance cassette was PCR amplified from pBS2V (generously provided by P. Stewart and P. Rosa, Rocky Mountain Laboratories, Hamilton, MT) using primers incorporating *Xba*I site and the *flgB::kanamycin* resistance cassette amplicon were subsequently digested with *Xba*I and ligated together to generate a vector carrying the CRASP-1 knockout cassette. The resulting vector served as the template for PCR to generate the linear CRASP-1 inactivation construct containing the kanamycin resistance cassette. Subsequently, 10 μg of the linear inactivation construct was electroporated into B31cF. PCR using primers Kan5′X and Kan3′X. The purified pBAD vector containing the CRASP-1 fragment with the internal *Xba*I site and the flgB::kanamycin resistance cassette amplicon were digested with *Xba*I and ligated together to generate a vector expressing CRASP-1.

To complement CRASP-1 expression in the B31cF-CRASP-1 mutant and to express CRASP-1 in Bg50, the native *B. burgdorferi* B31-CRASP-1 gene plus upstream 400 bp was PCR amplified using the primers EXP-CRASP5′K and EXP-CRASP3′P (Table 1). The purified pBAD vector containing the *flgB::kanamycin* resistance cassette was PCR amplified from pBS2V (generously provided by D. Samuels, University of Montana, Missoula, MT), which contains the *aadA* gene encoding streptomycin resistance, was then digested with *Kpn*I and *Pst*I and ligated together yielding pKFSS-1::CRASP-1. Resulting *E. coli* transformants were screened by PCR and subjected to nucleotide sequencing to verify that no mutations were introduced during the amplification step. One of the pKFSS-1::CRASP-1 clones was then used to propagate the vector to generate plasmid for subsequent electroporation into B31cF-CRASP-1 and Bg50. As controls, B31cF-CRASP-1 and Bg50 also were transformed with the pKFSS-1 vector alone.

**Generation of rCRASP-1**

An amplicon corresponding to the mature CRASP-1 lipoprotein (i.e., lacking the leader peptide) was generated using primers R-CRASP5′B and R-CRASP5′X (Table 1). The amplicon was ligated into the *Bam*HI and *Pst*I site of pBAD vector, as described (34). Electroporations were performed using either 10 μg of linear DNA or 1 μg of plasmid vector, as described (34). Electroporated spirochetes were subsequently allowed to recover in 4 ml of complete BSK-II medium at 34°C. After a 16-h recovery period, spirochetes were aliquoted into microtiter plates and placed under appropriate antibiotic selection; <5% of all microtiter plate wells contained positive cultures, suggesting that each well culture was clonal in origin (35). For all borrelial selection experiments, kanamycin was used at a concentration of 400 μg/ml, and/or streptomycin was used at a concentration of 100 μg/ml.
Table I. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRASP-US5'</td>
<td>TTTACGTCACAGAAAGATTGAAA</td>
<td>Complementary to nucleotides 377–401 upstream of <em>B. burgdorferi</em> CRASP-1</td>
</tr>
<tr>
<td>CRASP-US3'/X</td>
<td>GGCCTCTAGAATGGTGGCTATTT</td>
<td>Complementary to nucleotides 99–121 downstream of <em>B. burgdorferi</em> CRASP-1 plus <em>XbaI</em> site</td>
</tr>
<tr>
<td>CRASP-DS5'/X</td>
<td>GGCCTCTAGAAGAAAATACCAAAATCCCG</td>
<td>Nucleotides 122–143 downstream of <em>B. burgdorferi</em> CRASP-1 plus <em>XbaI</em> site</td>
</tr>
<tr>
<td>CRASP-DS1'</td>
<td>ATTGCGATGAGCTGTATAGATAT</td>
<td>Complementary to nucleotides 575–599 downstream of <em>B. burgdorferi</em> CRASP-1</td>
</tr>
<tr>
<td>R-CRASP5'B</td>
<td>GCCGGATCCGGCGAATTCTCCAACTAGCCAAGCCAAGCAGA</td>
<td>Nucleotides 90–111 downstream of <em>B. burgdorferi</em> CRASP-1 plus <em>BamHI</em> site; generation of rCRASP-1</td>
</tr>
<tr>
<td>R-CRASP3'/X</td>
<td>GGCCTCTAGAGTAGAAGCTGTTTTTCAGTTAAGT</td>
<td>Complementary to nucleotides 732–756 downstream of <em>B. burgdorferi</em> CRASP-1 plus <em>XhoI</em> site; generation of rCRASP-1</td>
</tr>
<tr>
<td>EXP-CRASP5'K</td>
<td>GCCGGATCCGGCGAATTCTCCAACTAGCCAAGCCAAGCAGA</td>
<td>Nucleotides 377–401 upstream of <em>B. burgdorferi</em> CRASP-1 plus <em>KpnI</em> site; generation of CRASP-1 expression cassette</td>
</tr>
<tr>
<td>EXP-CRASP3'P</td>
<td>GCCGGATCCGGCGAATTCTCCAACTAGCCAAGCCAAGCAGA</td>
<td>Complementary to nucleotides 732–756 downstream of <em>B. burgdorferi</em> CRASP-1 plus <em>PstI</em> site; generation of CRASP-1 expression cassette</td>
</tr>
<tr>
<td>Kan5'X</td>
<td>GCCCTCTAGAGTAGAAGCTGTTTTTCAGTTAAGT</td>
<td>Nucleotides 394–410 upstream of kanamycin resistance gene plus <em>XbaI</em> site; generation of kanamycin resistance cassette</td>
</tr>
<tr>
<td>Kan3'X</td>
<td>GCCCTCTAGAGTAGAAGCTGTTTTTCAGTTAAGT</td>
<td>Complementary to nucleotides 897–918 downstream of kanamycin resistance gene plus <em>XbaI</em> site; generation of kanamycin resistance cassette</td>
</tr>
<tr>
<td>Kan5'S</td>
<td>GCCGGATCCGGCGAATTCTCCAACTAGCCAAGCCAAGCAGA</td>
<td>Complementary to nucleotides 1–23 upstream of kanamycin resistance gene plus <em>SalI</em> site</td>
</tr>
<tr>
<td>OspA5'</td>
<td>ATGAAAAAATATTTTTAGGAGAATAG</td>
<td>Nucleotides 1–24 of <em>B. burgdorferi</em> ospA</td>
</tr>
<tr>
<td>OspA3'</td>
<td>TTTATCGATCTTTTATTTTATTTTATTTTATTTTATTTTATTTT</td>
<td>Complementary to nucleotides 794–819 of <em>B. burgdorferi</em> ospA</td>
</tr>
</tbody>
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XhoI sites of the GST protein expression vector pGEX-4T-3 (Amersham Biosciences). Resulting clones were screened by PCR and verified not to contain errors by nucleotide sequence analysis. rCRASP-1 was purified and cleaved free of the GST moiety using procedures previously described (36, 37).

**Generation of Abs**

Fifty micrograms of rCRASP-1 protein in 250 μl of PBS (pH 7.4) was mixed with 250 μl of CFA (Difco Laboratories) and injected i.p. into Sprague Dawley rats (Harlan). Two and 4 wk following the primary immunization, i.p. booster immunizations were performed with 50 μg of recombinant protein in IFA (Difco Laboratories). Rats were exsanguinated 2 wk after the second boost to collect serum containing specific anti-CRASP-1 Abs. *B. burgdorferi* B31 FlaB protein was purified (38), and polyclonal Abs were generated in rabbits, as described (39). Generation of rat anti-OspE Abs has previously been described (22).

**Proteinase K (PK) accessibility experiments**

Spirochetes (2 × 10⁸) were gently washed three times in 1 ml of PBS (pH 7.4) and collected by centrifugation at 4,000 × g for 4 min. Washed spirochetes were then gently resuspended in 1 ml of PBS and split into two equal 500-μl volumes. One aliquot received 200 μg of PK (Sigma-Aldrich), while the other aliquot received an equal volume of PBS without PK. Both aliquots were incubated for 1 h at room temperature before addition of 10 μl of PMSF (Sigma-Aldrich) to stop PK activity. Spirochete suspensions were subsequently pelleted by centrifugation at 10,000 × g for 10 min and resuspended in PBS for immunoblot analysis.

**SDS-PAGE, immunoblotting, and FH ligand affinity blot analysis**

Whole cell lysates of spirochetes were boiled for 10 min in final sample buffer (62.5 μM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-ME, 5% SDS, 0.001% bromphenol blue) before electrophoresis through 2.4% stacking and 12.5% separating gels. Gels were transferred electrophoretically to nitrocellulose (Schleicher & Schuell Microscience) for enhanced chemiluminescent immunoblot analysis. Immunoblots were first blocked for 45 min in PBS (pH 7.4) plus 0.2% Tween 20 (PBS-T), followed by blocking overnight in PBS-T plus 10% FCS (Difco Laboratories). Membranes were then washed three times in PBS-T for 10 min before being incubated for 45 min with a 1/2000 dilution of primary rat anti-CRASP-1, rabbit anti-FhB, or rat anti-OspE polyclonal Ab diluted in Blotto HD (2% IGEPLAL, 0.2% SDS, 28 mM Tris-HCl, 22 mM Tris base, 1.5 mM calcium chloride, and 80 mM sodium chloride). After incubation with the primary Ab, membranes were washed three times in Blotto HD plus 0.2% BSA. Subsequently, a 1/5000 dilution of HRP-conjugated goat anti-rat or goat anti-rabbit Ab in Blotto HD plus 0.2% BSA was added and incubated with the membranes for 45 min. Membranes were then washed three times for 10 min with PBS before being developed with the ECL plus reagent, according to manufacturer’s directions (Amersham Biosciences). hFH ligand affinity blot analyses were performed, as previously described by Marconi and colleagues (11, 12). Briefly, membranes were first incubated with 25 μg of hFH (Calbiochem) for 1 h and washed three times in Blotto HD plus 0.2% BSA. Proteins binding hFH were then identified by adding a primary goat anti-hFH (Calbiochem) Ab at a final dilution of 1/1000 for 1 h before three washes in Blotto HD plus 0.2% BSA. This was followed by 45-min incubation with a secondary HRP-conjugated rabbit anti-goat Ab (Zymed Laboratories) at a dilution of 1/4000 before three washes in PBS (pH 7.4) and subsequent development by ECL (Amersham Biosciences).

**Immunofluorescence**

Spirochetes were gently washed twice in complete BSK-II medium, collected by centrifugation at 4000 × g for 4 min, and subsequently resuspended in 100 μl of complete BSK-II. Cell suspensions were enumerated and brought up to a final concentration of 1 × 10⁹ bacteria/ml in complete BSK-II. Subsequently, a 1/100 dilution of rat polyclonal CRASP-1 Ab was incubated for 45 min with intact spirochetes. Additionally, a 1/100 dilution of rabbit polyclonal Ab directed against the periplasmic flagellin protein (rabbit anti-FlaB) was added as a control to verify that the spirochetal outer membrane was intact, as described (32, 33). After incubation with the Abs, spirochetes were gently washed three times in sterile PBS and collected by centrifugation at 4000 × g for 4 min. Pellets were then resuspended in PBS, and 10-μl aliquots were allowed to air dry on glass slides. Dried samples were then fixed for 10 min with acetone before being blocked with 1% BSA in PBS (pH 7.4) for 30 min. Samples were subsequently incubated for 45 min at room temperature in a humidified chamber with a 1/1000 dilution of Alexa 488-conjugated goat anti-rat Ab (Molecular Probes) and a 1/1000 dilution of Alexa 568-conjugated rat anti-rabbit Ab (Molecular Probes). Slides were then washed three times with 1% BSA in PBS (pH 7.4) and mounted in buffered glycerol containing the permeable DNA-binding fluorophore 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Vector Laboratories) before being sealed with coverslips. Slides were visualized using an Olympus BX-60 fluorescence microscope (Olympus).

**Serum sensitivity assay**

Serum sensitivity assays were performed essentially as described (27). Briefly, triplicate samples of spirochetes were seeded into 1-ml tubes at a final concentration of 5 × 10⁶ bacteria/ml. Spirochetes were subsequently incubated for different times with 40% normal human serum or 40% heat-inactivated human serum. At 1, 2, 4, and 16 h after addition of serum,
samples were extracted and spirochete viability was examined using dark-field microscopy. Human serum was acquired from healthy, anonymous donors with no prior history of *Borrelia* spp. infection and no reactivity to *B. burgdorferi* or *B. garinii* after chemiluminescent immunoblot analyses.

**Computer analysis**

Primer design and sequence analyses were performed using the MacVector version 6.5.3 software package (Oxford Molecular Group). Student’s *t* test was used to analyze data resulting from the serum sensitivity assays.

**Results**

*Inactivation of CRASP-1 in B. burgdorferi B31cF*

CRASP-1 was first identified by Kraiczy et al. (20) as a 27-kDa protein that binds hFH/FHL-1. In this same study, CRASP-1 also was determined to be expressed by serum-resistant *B. afzelii* isolates, but not by serum-sensitive *B. garinii* isolates (20). Since this initial discovery, at least three other important observations have been made regarding CRASP-1: 1) CRASP-1 is a surface-exposed lipoprotein encoded by ORF *bbaA68* on lp54 of the *B. burgdorferi* genome (6); 2) CRASP-1 is the major FH/FHL-1-binding protein of *B. burgdorferi* and correlates with serum resistance in all *B. burgdorferi* sensu lato isolates (24); and 3) CRASP-1 forms a homodimer with a novel fold consisting almost entirely of α-helices (40). However, even with this recent attention placed on CRASP-1, it is still unclear what role CRASP-1 plays in human serum resistance. To directly examine this issue, we first inactivated the CRASP-1 gene using allelic exchange in a clonal, serum-resistant isolate of *B. burgdorferi* B31cF (B31cF) (41). The B31cF strain was chosen because it is not only serum resistant, but it also is highly transformable. Before generating a mutant in this clonal isolate, however, we first confirmed its plasmid content using plasmid-specific primers (42), which revealed that it contains lp54, lp17, cp26, cp32-8, cp-32-1, cp32-4, and cp32-3 (data not shown).

As shown in Fig. 1, a CRASP-1 gene inactivation construct was produced by generating a fragment of CRASP-1 that corresponds to a region 401 bp upstream of the start codon to 599 bp downstream of the start codon with a novel *XhoI* restriction inserted in the middle. Subsequently, a kanamycin resistance cassette driven by the *flgB* promoter was inserted into the unique *XhoI* restriction site, as described in *Materials and Methods*. Ten micrograms of the gene inactivation construct was electroporated into B31cF, and homologous recombination resulted in a CRASP-1 gene truncated at position 121 (aa 19 of the processed lipoprotein). Several kanamycin-resistant isolates were identified, and one, designated B31cF-CRASP-1, which maintained the full complement of plasmids identified in B31cF, was chosen for further analyses. To confirm that homologous recombination had occurred in the CRASP-1 locus as expected, total DNA from B31cF-CRASP-1 and wild-type B31cF was subjected to PCR using primers R-CRASP3′/X and Kan5′/X, which were specific to the 3′ end of the CRASP-1 gene and was situated outside of the inactivation construct, while the Kan5′ primer was complementary to a region found only on the kanamycin resistance cassette. As shown in Fig. 2A, only the B31cF-CRASP-1 mutant produced the expected amplicon product of 1046 bp (Fig. 2A, lane 1). Primers specific for *ospA*, which also is encoded on lp54, were included in this experiment as a control to confirm that the wild-type B31cF strain and mutant strain still contained the lp54 plasmid.

To verify that the B31cF-CRASP-1 mutant did not express CRASP-1, immunoblot analysis was performed with CRASP-1-specific Abs. As shown in Fig. 2B, when lysates of the B31cF-CRASP-1 mutant and B31cF were subjected to immunoblot with rat anti-CRASP-1 Abs, only the wild-type B31cF expressed a reactive 27-kDa protein corresponding to CRASP-1. Additionally, lysates from the wild-type and mutant strains also were immunoblotted with rat anti-OspE-specific Abs. This was done because it is well recognized that OspE can bind FH/FHL-1 and it was important to ensure that OspE expression was not altered in the B31cF-CRASP-1 mutant strain generated. As a control for possible sample loading differences, an identical amount of the same cell lysates also was immunoblotted with rabbit anti-FlaB Abs, which are specific for the constitutively expressed flagellin protein.

**B31cF and Bg50 hFH ligand affinity blot analyses**

To examine the ability of wild-type B31cF and Bg50 to bind hFH, ligand affinity blots were performed, as previously described (11, 12). As shown in Fig. 3, when hFH was incubated with transferred lysates of B31cF and Bg50, only B31cF expressed a reactive 27-kDa protein corresponding to CRASP-1 (+FH panel). This reactivity was not observed when hFH was omitted from the ligand-blotting procedure (−FH panel), indicating that the reactivity of B31cF for hFH was specific. To ensure that Bg50 did not express a FH/FHL-1-binding protein(s) only under specific conditions, the Bg50 isolate also was examined for hFH-binding proteins after: 1) cultivation at 23°C, which mimics the tick environment; 2) temperature shifting from 23° to 37°C, which mimics the tick feeding and
transmission process; and 3) being mammalian host adapted in dialysis membrane chambers within the peritoneal cavities of rats, which mimics animal infection. These different laboratory conditions are often used in the laboratory because they approximate the complete borrelial enzootic cycle in nature (22, 33, 43, 44). Under none of these conditions was a FH/FHL-1-binding protein observed. Although the B31cF-CRASP-1 mutant and Bg50 transformed with pKFSS-1::CRASP-1 were both observed to express a CRASP-1 protein that could bind hFH, the above analyses did not determine whether the CRASP-1 expressed by these strains was translocated specific for the constitutively expressed FlaB protein (FlaB panel).

Complementation of the B31cF-CRASP-1 mutant and expression of CRASP-1 in Bg50

We next complemented the B31cF-CRASP-1 mutant with the B. burgdorferi pKFSS-1 shuttle vector expressing CRASP-1, designated pKFSS-1::CRASP-1. We also electroporated the pKFSS-1::CRASP-1 expression plasmid into Bg50. As shown in Fig. 4, the complemented B31cF-CRASP-1 mutant and the Bg50 strain transformed with CRASP-1 were reactive with CRASP-1 Abs (CRASP-1 panel). As expected, the B31cF-CRASP-1 mutant, the B31cF-CRASP-1 mutant containing the pKFSS-1 vector alone, and Bg50 containing the pKFSS-1 vector alone did not express proteins recognized by the CRASP-1 Abs. When hFH ligand affinity blots were performed on these isolates, only the complemented B31cF-CRASP-1 mutant and Bg50 isolate expressing CRASP-1 from the pKFSS-1::CRASP-1 expression plasmid were positive for hFH binding (+FH panel). As expected, no reactivity was observed when hFH was omitted from the ligand affinity assay (−FH panel). As above, to control for loading of the various cell lysates, identical numbers of spirochetes were immunoblotted with Abs specific for the constitutively expressed FlaB protein (FlaB panel).

CRASP-1 expressed from the pKFSS-1 shuttle vector is translocated to the cell surface in both B31cF-CRASP-1 and Bg50

Although the B31cF-CRASP-1 mutant and Bg50 transformed with pKFSS-1::CRASP-1 were both observed to express a CRASP-1 protein that could bind hFH, the above analyses did not determine whether the CRASP-1 expressed by these strains was translocated

FIGURE 2. Characterization of the B31cF-CRASP-1 mutant. A. Genomic DNA from the mutant B31cF-CRASP-1 (lanes 1 and 3) and wild-type B31cF (lanes 2 and 4) were subjected to PCR using primers Kan5′S and R-CRASP3′X (lanes 1 and 2). The 1046-bp amplicon was observed only for the B31cF-CRASP-1 mutant, confirming the orientation of the kanamycin cassette insert in the B31cF-CRASP-1 mutant (lane 1). Primers OspA5′ and OspA3′ also were used (lanes 3 and 4) to confirm that the lp54 plasmid was maintained by both the CRASP-1 mutant (lane 3) and wild-type strains (lane 4). Molecular mass sizes, in kb, are shown at left. B. Rat anti-CRASP-1 Abs were used to immunoblot whole cell lysates from 1 × 108 wild-type B31cF and mutant B31cF-CRASP-1; only the wild-type strain retained expression of CRASP-1 (upper panel). Rat anti-OspE Abs were used to immunoblot the same amount of whole cell lysate (1 × 108) from each strain to confirm that expression of the OspE-related FH/FHL-1-binding proteins was similar in both strains (middle panel). Identical amounts of whole cell lysate (1 × 108) also were subjected to immunoblot with rabbit anti-FlaB Abs to confirm that sample loading was equal.

FIGURE 3. Human serum-sensitive Bg50 does not express hFH-binding proteins. To analyze hFH binding to borrelial proteins, multiple gels containing 100 ng of rCRASP-1 and cell lysates from 1 × 108 serum-resistant B. burgdorferi strain B31cF, serum-sensitive Bg50 organisms cultivated at 23°C, temperature shifted from 23°C to 37°C (TS), or mammalian host adapted (HA) were transferred to nitrocellulose membranes for analysis. After hFH was incubated with the transferred membranes, binding was only observed for B31cF and the rCRASP-1; no binding was observed for Bg50 under any condition (+FH panel). When hFH was omitted from the ligand blot assay, reactivity was abolished for B31cF (−FH panel). Consistent with the hFH ligand blot experiment, reactivity with rat anti-CRASP-1 Ab was only observed for B31cF and rCRASP-1 (CRASP-1 panel). The protein identified by CRASP-1 Abs in B31cF was the same size (~27 kDa) as the hFH-reactive protein in the upper panel, indicating that the hFH-reactive protein was indeed CRASP-1. No reactivity for anti-CRASP-1 Abs was observed for Bg50. Abs specific for the constitutively expressed flagellin protein, FlaB, were included to show equal loading of all cell lysates (FlaB panel).
CRASP-1 IMPARTS SERUM RESISTANCE TO B. burgdorferi

To directly examine the role of CRASP-1 in resistance to human serum, serum sensitivity assays were performed, as previously described by Meri and colleagues (27). In triplicate, the wild-type B31cF and mutant B31cF- CRASP-1, B31cF- CRASP-1 and Bg50 transformed with the pKFSS-1::CRASP-1 expression vector bind hFH. Cell lysates from 1 x 10^7 mutant B31cF- CRASP-1, B31cF- CRASP-1 and Bg50 transformed with the pKFSS-1 vector alone, and B31cF- CRASP-1 and Bg50 transformed with the CRASP-1 expression vector pKFSS-1::CRASP-1 were subjected to immunoblot analysis with CRASP-1 (CRASP-1 panel) and hFH affinity ligand blot analysis with (+FH panel) or without (−FH panel) hFH preincubation. Neither the mutant B31cF- CRASP-1, the B31cF- CRASP-1 mutant transformed with the pKFSS-1 vector alone, nor Bg50 transformed with the pKFSS-1 vector alone reacted with CRASP-1-specific Abs or bound hFH. In contrast, both the B31cF- CRASP-1 and Bg50 strains that were transformed with the CRASP-1 expression vector pKFSS-1::CRASP-1 expressed a ~27-kDa protein that was recognized by the CRASP-1 Abs and also bound hFH. Abs specific for the constitutively expressed flagellin protein, FlaB, were used to show equal loading of the cell lysates (FlaB panel).

to the cell surface. Therefore, we next performed surface immunofluorescence assays. As shown in Fig. 5 (left panels), CRASP-1 was expressed on the surface of wild-type B31cF containing the pKFSS-1 vector alone and both the B31cF- CRASP-1 mutant and Bg50 transformed with pKFSS-1::CRASP-1. As expected, the B31cF- CRASP-1 mutant and wild-type Bg50 transformed with the pKFSS-1 vector alone were not observed to express CRASP-1. Rabbit Abs directed against the periplasmic flagellin protein were co-incubated with the rat anti-CRASP-1 Abs in these experiments as an internal control to confirm that the fragile spirochetal outer membranes were not damaged (center panels). DAPI, a nonspecific DNA-binding dye, also was included so that all spirochetes within a given microscopic field could be identified (right panels). As an independent method to confirm that CRASP-1 was localized to the borrelian outer membrane, PK accessibility experiments also were performed. Consistent with the immunofluorescence experiments, CRASP-1 could be degraded from the surface of the wild-type B31cF and both the B31cF- CRASP-1 mutant and Bg50 strains expressing CRASP-1 from the pKFSS-1::CRASP-1 expression vector (data not shown). The combined surface-localization experiments confirmed that CRASP-1 expressed by pKFSS-1::CRASP-1 was competent for export to the surface of both B. burgdorferi and B. garinii.

CRASP-1 is required for resistance to human serum

FIGURE 4. Wild-type Bg50 and mutant B31cF- CRASP-1 transformed with the pKFSS-1::CRASP-1 expression vector bind hFH. Cell lysates from 1 x 10^7 mutant B31cF- CRASP-1, B31cF- CRASP-1 and Bg50 transformed with the pKFSS-1 vector alone, and B31cF- CRASP-1 and Bg50 transformed with the CRASP-1 expression vector pKFSS-1::CRASP-1 were subjected to immunoblot analysis with CRASP-1 (CRASP-1 panel) and hFH affinity ligand blot analysis with (+FH panel) or without (−FH panel) hFH preincubation. Neither the mutant B31cF- CRASP-1, the B31cF- CRASP-1 mutant transformed with the pKFSS-1 vector alone, nor Bg50 transformed with the pKFSS-1 vector alone reacted with CRASP-1-specific Abs or bound hFH. In contrast, both the B31cF- CRASP-1 and Bg50 strains that were transformed with the CRASP-1 expression vector pKFSS-1::CRASP-1 expressed a ~27-kDa protein that was recognized by the CRASP-1 Abs and also bound hFH. Abs specific for the constitutively expressed flagellin protein, FlaB, were used to show equal loading of the cell lysates (FlaB panel).

FIGURE 5. CRASP-1 expressed from the pKFSS-1 shuttle vector is surface exposed in Bg50 and B31cF- CRASP-1. To examine surface localization of CRASP-1, B31cF and Bg50 transformed with the pKFSS-1 vector alone, the B31cF- CRASP-1 mutant, and the B31cF- CRASP-1 mutant and Bg50 strain transformed with the CRASP-1 expression vector pKFSS-1::CRASP-1 were subjected to indirect immunofluorescence in solution before fixation. Samples were probed with rat anti-CRASP-1 (left panels) and rabbit anti-FlaB (middle panels) at the same time. FlaB Abs, directed at the periplasmic flagellin protein, were included to ensure that spirochetes positive for CRASP-1 were negative for FlaB, which confirmed that their fragile outer membranes were intact. For all experiments, DAPI, a permeable DNA-binding dye, was used to identify all spirochetes within a given field (right panels). The B31cF- CRASP-1 mutant and Bg50 transformed with the CRASP-1 expression plasmid both expressed CRASP-1 on their surface. As expected, the wild-type B31cF strain transformed with the pKFSS-1 vector alone also expressed the native CRASP-1 on its surface. Organisms were visualized by fluorescence microscopy at a magnification of ×1000.
strains with the pKFSS-1 vector alone had no impact on serum resistance (Fig. 6, B and C). When these experiments were repeated using temperature-shifted organisms, no differences in serum resistance or sensitivity were observed for any of the isolates tested (data not shown). The combined serum sensitivity assays strongly suggest that CRASP-1 is required for resistance to human serum and is the dominant source of serum resistance in *B. burgdorferi*.

**Discussion**

FH in humans is essential for protection of host cells and tissue from destruction by complement activation (30). FH accelerates decay of the C3 convertase (C3bBb) and also increases factor I-mediated inactivation of C3b (30). Although the activity of FH is important in protecting host cells and tissues from complement-mediated damage, FH/FHL-1 has also been exploited by several different bacterial pathogens to help them evade complement-mediated destruction during infection. For example, *Streptococcus pyogenes* (45), *Streptococcus pneumoniae* (46), and *Neisseria gonorrhoeae* (47) all bind FH on their surface as a means of evading complement-mediated destruction. Similarly, it was determined that two distinct classes of proteins in human serum-resistant strains of *B. burgdorferi sensu lato* bind hFH and/or FHL-1 (6, 7, 9–12, 20). The single class 2 FH/FHL-1 protein corresponds to CRASP-1, while the class 1 proteins (designated CRASP-2 through CRASP-5) are all thought to be related to OspE and the various OspE-related lipoproteins (4). Interestingly, there are multiple copies of CRASP-2–5 in the borrelial genome, and they are all up-regulated by *B. burgdorferi* during tick transmission and/or during mammalian infection (22, 32, 33, 48). In contrast, CRASP-1 appears to be expressed only in the tick midgut and during early infection (26). To begin a direct examination of the role played by these two distinct classes of FH/FHL-1-binding proteins, we inactivated the single copy CRASP-1 gene in a serum-resistant *B. burgdorferi* B31cF isolate. Inactivation of this gene revealed that CRASP-1 alone can impart resistance to human serum. Additionally, when we exogenously expressed CRASP-1 in a human serum-sensitive isolate *B. garinii* 50, serum resistance was imparted on this strain. The combined analyses presented in this study strongly suggest that CRASP-1 is a key molecule required for human serum resistance in *B. burgdorferi sensu lato*.

Using an in vitro ELISA capture assay system, it was recently shown that both FH and FHL-1 maintain their cofactor activity for factor I-mediated inactivation of C3b to iC3b after binding to a recombinant form of CRASP-1 (6). This observation led the authors to speculate that CRASP-1 is the dominant protein imparting complement resistance to *B. burgdorferi* (6). However, because it has been shown by several laboratories, including our own, that all three OspE-related proteins (i.e., ErpA, ErpN, and ErpP) of *B. burgdorferi* also bind hFH (7–10, 12, 27, 28), it is presently unclear what role CRASP-1 and/or the OspE-related proteins play in complement resistance. The combined results of this analysis have provided the first empirical evidence that CRASP-1 is, in fact, a dominant borrelial surface lipoprotein providing *B. burgdorferi* with resistance to human serum.

Although it is possible that the B31cF-CRASP-1 mutant used for the present study had an artificially high serum-sensitive phenotype because it only contains two of the three known OspE-related FH/FHL-1-binding proteins (i.e., ErpA and ErpN, but not ErpP),
CRASP-1 expressed from the B. burgdorferi shuttle vector pKFSS-1 (56) in B. garinii was determined to be expressed at high levels and to be exported to the outer surface. This has two important implications. First, this is the initial report showing that a B. burgdorferi shuttle vector is not only competent, but can also be used to express foreign genes in a borrelial genspecies other than B. burgdorferi. The close genetic relationship between all three borrelial genspecies would suggest that B. afzelii could be genetically manipulated using B. burgdorferi shuttle vectors. Given that pKFSS-1 is a derivative of the original B. burgdorferi shuttle vector pBSV-2 (56, 57), it would also imply that pBSV-2 and pBSV-2-derived selection vectors could be used for genetic studies in all three genspecies. Second, the B. burgdorferi CRASP-1 gene was not only expressed and translated in B. garinii, but it also was transported to the surface, which would strongly suggest that protein secretion and lipid modification pathways are similar among B. burgdorferi sensu lato isolates. Although we did not attempt to inactivate OspE because we would have had to inactivate two genes in the same isolate, the finding that pKFSS-1 is operational in B. garinii suggests that we can now examine the role played by OspE-related proteins, individually or in tandem, by exogenously expressing them in a serum-sensitive B. garinii isolate using B. burgdorferi shuttle vectors.

The evidence presented in this study details the apparent necessity of CRASP-1 expression for the survival of B. burgdorferi in the presence of human serum. This presents two obvious paradoxes with regard to serum-sensitive strains of B. burgdorferi sensu lato that cause human disease, such as B. garinii. First, how do pathogenic B. garinii spirochetes avoid complement-mediated death during human infection, and second, how are they perpetuated in nature if they are sensitive to mammalian serum? When considering how B. garinii causes disease without resistance to human serum, it is noteworthy that B. garinii organisms appear to consistently traffic to the CNS. The CNS contains <1% of the amount of cytotoxic complement of whole blood (30). Therefore, residing in the protected niche of the human CNS has obvious benefits for complement-sensitive B. garinii. It also was recently shown that a CRASP-1 ortholog found in B. garinii does not bind hFH, but can weakly bind FHL-1 (29). This weak FHL-1 activity in B. garinii may provide a small amount of serum resistance and could allow some organisms to evade complement in the blood as they disseminate to the CNS. The lack of a high affinity hFH/FHL-1-binding protein in B. garinii may help to explain why the median infectious dose (ID$_{50}$) of B. burgdorferi is ~100 spirochetes (32), while the ID$_{50}$ of B. garinii is >10,000 spirochetes for mouse infection (58). Consistent with this notion, Norris and colleagues (58) have shown that the ID$_{50}$ of B. garinii is almost 6-fold lower in mice that are deficient in complement component C3.

The second paradox brought up by the present study is how serum-sensitive strains of B. garinii can be propagated in nature if they are killed so readily by mouse serum (58). This is most likely explained by recent evidence indicating that B. garinii isolates sensitive to mammalian serum are propagated by birds (59, 60), which led to the conclusion that birds are an important reservoir in nature for B. garinii. A recent analysis of B. garinii strain ZQ1 identified two CRASP-1 orthologs that are encoded on lp54 (29). Although neither protein could bind hFH, both were found to weakly bind human FHL-1. Although these newly identified proteins do not provide resistance to mouse serum (59), inactivation of one or both of these genes in future experiments could be used to determine their role in resistance to bird serum. At the same time, however, it also is recognized that some B. garinii isolates have increased resistance to mammalian serum (61). It will be interesting to determine whether these specific strains of B. garinii that are
more resistant to mammalian serum also express hFH/HL-1-binding proteins. Because an FH-binding analysis of 16 different B. garinii isolates was recently performed by Marconi and colleagues (11), which identified five different isolates that bind hFH, these strains would be prime candidates to further examine the relationship between host specificity and expression of FH/HL-1-binding proteins in B. garinii. Future studies using virulent B. burgdorferi, B. afzelii, and B. garinii strains with inactivated or exogenously expressed CRASP proteins will be necessary to fully examine the complex interplay between borrelial organisms, serum sensitivity, and the role of the various CRASPs in the ecology and clinical manifestations caused by different genospecies.

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References

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