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Dual Binding Specificity of a *Borrelia hermsii*-Associated Complement Regulator-Acquiring Surface Protein for Factor H and Plasminogen Discloses a Putative Virulence Factor of Relapsing Fever Spirochetes

Evelyn Rossmann,§ Peter Kraiczy,‡ Pia Herzberger,§ Christine Skerka,* Markus M. Simon,§ Peter F. Zipfel,‡ and Reinhard Wallich4*

Tick-borne relapsing fever in North America is primarily caused by the spirochete *Borrelia hermsii*. The pathogen employs multiple strategies, including the acquisition of complement regulators and antigenic variation, to escape innate and humoral immunity. In this study we identified in *B. hermsii* a novel member of the complement regulator-acquiring surface protein (CRASP) family, designated BhCRASP-1, that binds the complement regulators factor H (FH) and FH-related protein 1 (FHR-1) but not FH-like protein 1 (FHL-1). BhCRASP-1 specifically interacts with the short consensus repeat 20 of FH, thereby maintaining FH-associated cofactor activity for factor I-mediated C3b inactivation. Furthermore, ectopic expression of BhCRASP-1 converted the serum-sensitive *Borrelia burgdorferi* B313 strain into an intermediate complement-resistant strain. Finally, we report for the first time that BhCRASP-1 binds plasminogen/plasmin in addition to FH via, however, distinct nonoverlapping domains. The fact that surface-bound plasmin retains its proteolytic activity suggests that the dual binding specificity of BhCRASP-1 for FH and plasminogen/plasmin contributes to both the dissemination/invasion of *B. hermsii* and its resistance to innate immunity. *The Journal of Immunology*, 2007, 178: 7292–7301.

*B. hermsii* and *Borrelia turicatae* are the main vector-borne pathogens causing human relapsing fever, an acute infectious disorder, in the United States (1). In case of *B. hermsii*, spirochetes are transmitted to humans within minutes through the bite of infected soft ticks, in particular *Ornithodoros hermsii*. *B. hermsii* has evolved multiple strategies to escape innate and adaptive immune responses and to persist in the blood (2, 3), including multiphasic antigenic variation mediated by Vmp proteins (4–6).

A further strategy of bacteria to resist hosts’ innate immunity, which constitutes the first barriers to infection, is their potential to acquire fluid phase complement regulators, particularly those of the alternative complement pathway such as factor H (FH),7 to the spirochetal surface. Bound FH controls complement activation by accelerating the decay of the C3 convertase of the alternative pathway and by inactivating newly formed C3b (7, 8) as shown for several important human pathogens, e.g., *Candida albicans*, *Neisseria gonorrhoeae*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* (9–14). FH represents the main human fluid phase regulator of the alternative pathway of complement activation and belongs to the factor H protein family, which consists of seven structurally related proteins in humans including FH-like protein 1 (FHL-1) and the FH-related proteins (FHRs) (15). All FH protein family members are composed of short consensus repeats (SCRs) (15, 16). In contrast to FH and FHL-1, the precise function(s) of the FHR proteins is currently unknown. For *B. hermsii*, surface-bound FH was shown to participate as a cofactor for factor I-mediated cleavage of C3b (17–19). Furthermore, for the closely related spirochete *Borrelia burgdorferi*, the causal agent of Lyme disease, a strong correlation between the serum resistance of a given isolate and its expression profile of FH-binding outer surface lipoproteins, termed complement regulator-acquiring surface proteins (CRASP), was reported (20–28). Moreover, it was suggested that the dominant FH binding molecule of serum-resistant *B. burgdorferi* strains, BbCRASP-1, is necessary to resist killing by human serum (29).

Some bacteria, such as *Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, and *Clostridium perfringens*, produce their own proteolytic enzymes that digest the extracellular matrix to facilitate invasion (30). Others, like *B. burgdorferi* and *Borrelia crocidurae*, make use of the hosts’ fibrinolytic system to invade tissues (31–34).

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2 The sequence presented in this article has been submitted to EMBL/GenBank under accession number AM408562.
3 E.R. and P.K. contributed equally to this work.
4 Address correspondence and reprint requests to Dr. Reinhard Wallich, Infectious Immunology Group, Institute for Immunology, University of Heidelberg, Im Neuenheimer Feld 305, Heidelberg, Germany. E-mail address: wallich@uni-hd.de
5 Abbreviations used in this paper: FH, factor H; FHL-1, FH-like protein 1; FHR, FH-related protein; CRASP-1, complement regulator-acquiring surface protein 1; BbCRASP-1, *Borrelia burgdorferi* CRASP-1; BhCRASP-1, *Borrelia hermsii* CRASP-1; NHS, normal human serum; Osp, outer surface protein; SCR, short consensus repeat; uPA, urokinase-type plasminogen activator.

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Accordingly, spirochetes bind the host plasmidin that is subsequently processed via urokinase-type plasmidinactivator (uPA) to active plasmid, a broad-spectrum serine protease, leading to extracellular matrix degradation (31, 33, 35–37). B. burgdorferi organisms bind host plasmidin via a multitude of outer surface proteins (Osp), such as OspA and OspC, a 70-kDa protein, and several low molecular weight proteins (33, 35, 38, 39). Thus, the fact that relapsing fever spirochetes, including B. hermsii, also disseminate from the blood to many distinct organs suggests the involvement of plasmidin-binding proteins in these processes.

By screening a B. hermsii expression library we have now identified a novel 21.5 kDa outer surface lipoprotein termed BhCRASP-1. We demonstrate for the first time that BhCRASP-1 displays dual binding specificities both for members of the FH complement regulator protein family and for plasmidin/plasmidin that the two host proteins bind to distinct, nonoverlapping BhCRASP-1 domains.

Materials and Methods

Bacterial strains and growth conditions

B. hermsii (ATCC35209) strain HS1 and YOR isolates (provided by T. Stoenner-Kelly (BSK)-H complete medium (PAN Biotech) supplemented with 5% rabbit serum (Cell Concept) at 30°C. B313 mutant spirochetes were cultivated in Barbour-Schwan, Rocky Mountain Laboratories) and the Lyme disease spirochetes B. burgdorferi isolate B31 and mutant B313 were cultivated in Barbour-Schwan, also

Characterization of B. burgdorferi B313 transformants

The transformation of B. burgdorferi B313 and the characterization of transformants were previously described (27). Several clones were selected

kit (PE Applied Biosystems) in accordance with the manufacturer’s recommendation.

The gene encoding BhCRASP-1 was amplified by PCR amplification using plasmid pGEMbh, the primers BhBam and BhR (Table I), and a Mastercycler gradient (Eppendorf). Denaturation was conducted at 94°C for 30s, annealing at 50°C for 30s, and extension at 68°C for 30s, respectively. After digestion with BamH I and EcoRI, the amplified DNA fragment was ligated in-frame in the vector PGEX-2T, which included the glutathione-S-transferase gene at the N terminus of the recombinant protein. The resulting plasmid was used to transform JM109 host cells. Expression of the GST-BhCRASP-1 fusion protein in E. coli JM109, affinity purification, and endopeptidase thrombin cleavage of the fusion protein were performed as recommended by the manufacturer (Amersham Bioscience).

C-terminal and N-terminal deletion mutants of BhCRASP-1 were constructed by PCR amplification using the BH bam primer and BhR primer in combination with the EcoO-12 primer and the Δ130 Bam and Δ195 Bam primers, respectively (Table I). The amplified DNA fragments were digested with BsuRI and ligated in-frame with the His6 tag encoding sequence into the pQE30Xa vector (Qiagen), resulting in plasmid pGEMbh, the primers BhBam and BhR (Table I), and a set of primers (Table I) and performed as recommended by the manufacturer (Amersham Bioscience).

Expression of recombinant proteins of FH, FHL-1, and HFR-1

Deletions constructs of FH (FHI-2, FHI-3, FHI-4, FHI-5, FH1–6, FH8–20, FH15–20, and FH19–20), FHL-1, and HFR-1 were expressed in Spodoptera frugiperda Sf9 insect cells infected with a recombinant baculovirus. The cloning, expression, and purification of various deletion constructs have been described previously (8, 41).

Construction of a shuttle vector for transformation with BhCRASP-1

The BhCRASP-1-encoding cspA gene, including its native promotor region, was amplified by PCR using BhB and BhR primers containing the respective restriction sites. Highly purified DNA obtained from the B. hermsii strain HS1 was used as the DNA template for PCR. The resulting amplicon was digested with SacI and SphI and cloned into pKF6S1 at the corresponding restriction sites yielding the shuttle vector pBHH. The shuttle vector was transformed into E. coli JM109 and purified plasmids were subjected to nucleotide sequencing to verify that no mutations were introduced during PCR. E. coli transformants were grown in Luria-Bertani broth containing 50 μg of streptomycin (Sigma-Aldrich) per milliliter and the expression of BhCRASP-1 was checked by ligand affinity blot analysis of whole cell lysates (data not shown) as described (27).

Characterization of B. burgdorferi B313 transformants

The transformation of B. burgdorferi B313 and the characterization of transformants were previously described (27). Several clones were selected

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Used in This Study</th>
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<td>BhBam</td>
<td>ATTTAATACGACTCACTATAGT</td>
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<tr>
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<td>AACACACAGCTCCTGTTGGGCTT</td>
<td>Amplification of flaB gene</td>
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and expanded for 7 days. The analysis of genes harbored by B313 trans-
formants was determined by PCR using specific primers (Table 1). PCR
was conducted for 25 cycles using following parameters: denaturation at
94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1
min. The expression of the BchCRASP-1 of posttransformation B. burg-
dorferi B313 was determined by Western blotting using mAb BH-1.

**Serum susceptibility testing for Borrelia strains**

The serum susceptibility of B. hermsii HS1 mutants B313 and B313 con-
taining shuttle vector pBH was assessed using a growth inhibition assay
for the mAb B22 directed against the SCR5 of FH and FHL-1, the mAb VIG8
was quantified by ELISA applying a neoepitope-specific mouse monoclonal
specific mAb 10-V-1. Following four washes with PBS and blocked by incubation with
PBS plus 0.1% gelatin. FH (1 µg/ml) was added to the wells and after a 2-h incubation and three washes with PBS
was incubated with 25% NHS or 25% heat-inactivated NHS. Barbour-
Stoenner-Kelly medium containing 240 µg/ml phenol red was
incubated with 100 µl of 0.1 M glycine-HCl (pH 2.0) for 15 min. Borrellial cells were removed
by centrifugation at 5000 × g for 10 min, washed, and resuspended in 100 µl of
veronal-buffered saline supplemented with 1 mM Mg²⁺, 150 mM NaCl, and
0.1% gelatin (pH 7.4). To inhibit complement activation, NHS was
incubated with 0.34 M EDTA for 15 min at room temperature. The cell
temperature was then incubated in 1.5 ml NHS-EDTA for 1 h at room
temperature with gentle agitation. After three washes with PBSA (0.15 M
NaCl, 0.03 M phosphate, and 0.02% sodium azide, pH 7.2) containing
0.05% Tween 20, the proteins bound to the cells were eluted by incubation with
0.1 M glycine-HCl (pH 2.0) for 15 min. Borrelial cells were removed
by centrifugation at 14,000 × g for 20 min at 4°C and the supernatant was
analyzed by Western blotting and probed with mAb VIG8 for FH and
FHL-1 or 10-V-1 (Calbiochem) for plasminogen.

**Immunofluorescence analysis**

Spirochetes were grown to mid-log phase, harvested by centrifugation at
5000 × g for 10 min, washed, and resuspended in 300 µl of 30 mM Tris,
60 mM NaCl (pH 7.4). Cells (2 × 10⁸) were incubated for 1 h with a mAb
directed either against BchCRASP-1 (BH-1) or the periplasmic flagellin
protein (LA21). After incubation with the Abs, spirochetes were gently
washed three times in Tris buffer containing 0.2% BSA and collected by
centrifugation at 5000 × g for 10 min. Pellets were then resuspended in
100 µl of PBSA, centrifuging 0.2% BSA, and aliquots (10 µl) were spotted
on coverslips and allowed to air dry for 3 h. After fixation with acetone,
samples were dried for 15 min at room temperature and incubated for 60
min in a humidified chamber with a 1/200 dilution of Cy3-conjugated
rabbit anti-IgG (Dianova) and a 1/1000 dilution of the DNA-binding
dye 4',6'-diamidino-2-phenylindole (Roche) for counterstaining. Slides
were then washes four times with 0.2% BSA in Tris buffer before being
sealed with Mowiol mounting medium (Calbiochem) and covered with
glass slides. Organisms were visualized at a magnification of ×1000 using a
Nikon Eclipse 90i microscope.

**SDS-PAGE, ligand affinity blot, Western and slot blot analyses, and ELISA**

Borrelial whole cell lysates (15 µg) or purified recombinant proteins (500
µg) were subjected to 10% Tris/Tricine SDS-PAGE under reducing con-
tions and transferred to nitrocellulose as previously described (24).
Al-
ternatively, recombinant proteins (1 µg/lane) were transferred onto nitro-
cellulose membranes using the Bio-Rad mini Protean blotting apparatus (Bio-Rad).
After the transfer of proteins onto nitrocellulose, nonspecific binding sites
were blocked using 5% (w/v) dried milk in TBS (50 mM Tris-HCl 200 mM
NaCl, and 0.1% Tween 20) (pH 7.4), for 6 h at room temperature. Subse-
quent, membranes were rinsed four times in TBS and incubated at 4°C
overnight with NHS, recombinant proteins, or human plasminogen (Cell
Systems). After four washings with 50 mM Tris·HCl 150 mM NaCl, and
0.2% Tween 20 (TBST) (pH 7.5), membranes were incubated for 3 h with
a polyclonal rabbit antisem recognizing the N terminus (anti-SCR1–4),
the mAb B22 directed against the SCR5 of FH and FHL-1, the mAb VIG8
directed against the C terminus of FH and FHR-1, or the plasminogen-
specific mAb 10-V-1. Following four washes with TBST, blot strips were incubated with a secondary peroxidase-conju-
gated anti-mouse IgG Ab (DakoCytomation) for 60 min at room temperature.
Detection of bound Abs was performed using 3,3′,5,5′-tetramethylbenzini-
dine as the substrate.

For Western blot analysis, membranes were incubated for 60 min at room
temperature with either mAb or immune sera. Following four washes with
TBST, membranes were incubated with a secondary peroxidase-conju-
gated anti-mouse IgG Ab (DakoCytomation) for 60 min at room tem-
perature and bound Abs were detected using 3,3′,5,5′-tetramethylbenzidine as substrate.

For ELISA using nonadened recombinant proteins, the wells of mi-
cro-titer plates (Maxisorp; Nunc) were coated for 2 h at room temperature
with BchCRASP-1 or the deletion mutants thereof (100 µl; 1 µg/ml). The
wells were washed three times with PBS and blocked by incubation with
PBS plus 0.1% gelatin. FH (1 µg/ml) or plasminogen (10 µg/ml) was added
to the wells and after a 2-h incubation and three washes with PBS
was incubated with 25% NHS or 25% heat-inactivated NHS. Barbour-
Stoenner-Kelly medium instead of human serum was included in all
assays as control. Growth of spirochetes was monitored by measuring the
indicator color shift of the medium at 562/630 nm in an ELISA reader
(PowerWave 200; Bio-Tek Instruments). For calculation of the growth
curves, the MikroWin version 3.0 software (Mikrotek) was used.

**Serum adsorptions assays using intact borreliell cell**

To determine whether B. hermsii HS1 can bind FH, FHL-1, and plasmin-
ogen, a whole cell absorption assay was performed as previously described.
Borreliae (2 × 10⁸ cells) were grown to mid-log phase, harvested by cen-
trifugation (5000 × g for 30 min at 4°C) and resuspended in 100 µl of
veronal-buffered saline supplemented with 1 mM Mg²⁺, 0.15 mM Ca²⁺,
and 0.1% gelatin (pH 7.4). To inhibit complement activation, NHS was
incubated with 0.34 M EDTA for 15 min at room temperature. The cell
temperature was then incubated in 1.5 ml NHS-EDTA for 1 h at room
temperature with gentle agitation. After three washes with PBSA (0.15 M
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by centrifugation at 14,000 × g for 20 min at 4°C and the supernatant was
analyzed by Western blotting and probed with mAb VIG8 for FH and
FHL-1 or 10-V-1 (Calbiochem) for plasminogen.

**Surface plasmon resonance analysis**

Protein-protein interactions were analyzed by surface plasmon resonance
technology using a Biacore 3000 instrument at 25°C. The immobilized
B. hermsii strain HS1 were treated with proteases by mod-
fication of a method described previously (43). Briefly, freshly harvested
spirochetes were washed twice with PBSA, centrifuging 0.2% BSA and
sonication five times using a Branson B-12 sonifier (Heinemann). Whole
protein preparations (10 µl) were separated by using Tris/Tricine
SDS-PAGE via 4% stacking and 10% separating gels as described pre-
viously (23).

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protein preparations (10 µl) were separated by using Tris/Tricine
SDS-PAGE via 4% stacking and 10% separating gels as described pre-
viously (23).

**Functional assay for cofactor activity of FH**

The cofactor activity of FH was analyzed on immobilized recombinant
BchCRASP-1 by measuring the factor I-mediated conversion of C3b to
C3b. Briefly, recombinant BchCRASP-1 (20 µg/ml) immobilized on a mi-
nority substrate was incubated in 0.5 ml of purified FH (20 µg/ml) for 15 min
at 37°C. After washing, purified C3b (Calbiochem) and purified factor I (Sigma-Aldrich)
were added and the mixture was incubated for 15 min at 37°C. C3b generation
was quantified by ELISA applying a neoeptope-specific mouse monoclonal
anti-iC3b IgG (Quidel) as the capture Ab and biotinylated rabbit anti-C3c IgG (DakoCytomation) as the detector Ab. The reaction was visualized by the addition of streptavidin-peroxidase followed by o-phenylenediamine with H$_2$O$_2$ as the substrate. Purified iC3b (Calbiochem) was used as a standard. Control experiments included BhCRASP-1, BhCRASP-3, or buffer instead of BhCRASP-1 as well as soluble and immobilized FH, respectively, in the identical system.

**Chromogenic substrate assays for plasmin and plasminogen activators**

Intact *B. hermsii* spirochetes were incubated with 10 μl of plasminogen (1 mg/ml; Chromogenix) with or without 50 mM tranexam acid for 30 min at 34°C in Eppendorf tubes if not otherwise indicated. Following two washes, *B. hermsii* was resuspended in 50 μl of assay buffer (30 mM Tris, 60 mM NaCl (pH 7.4)) and transferred to microtiter plates, and 50 μl of uPA (2.5 μg/ml; Chemicon International) as well as 50 μl of the plasmin substrate n-Val-Leu-Lys 4-nitroanilide dihydrochloride (S-2251; Sigma-Aldrich) was added (0.4 mg/ml). Control reactions without *B. hermsii* consisted of buffer alone (followed by uPA) and a sham preparation to control for possible residual unbound plasminogen not subsequently removed by washing (this reaction received plasminogen in buffer at the same concentration as that used in tubes with *B. hermsii*, followed by uPA). Control reactions with *B. hermsii* consisted of plasminogen alone (no uPA) and uPA alone (no previous plasminogen incubation) at the same concentrations as in the experimental reaction mixture. All samples received the chromogenic substrate S-2251 and were subjected to the same manipulations. The absorbance change at 405 nm was followed for several hours directly in the plates and the background activity of OD$_{450}$ = 0.1 (*B. hermsii* plus substrate) was subtracted. Similarly, BhCRASP-1 (0.2 μg/ml) was coated to microtiter plates and, after blocking, 10 μl of plasminogen (1 mg/ml) with or without 50 mM tranexam acid was added and incubated for 10 min at 37°C. Following three washes with 200 μl of buffer, 50 μl of uPA (2.5 μg/ml) and 50 μl of substrate S-2251 were added (0.4 μg/ml). The absorbance change at 405 nm was followed as indicated above.

**Nucleotide sequence deposition**

The cspA gene sequence reported in this paper has been deposited in the EMBL/GenBank databases under the accession number AM408562.

**Statistical analysis**

To determine the statistical significance of the observed absorbance values, BIAS version 8.1 software was used. Values of *p* < 0.05 were considered to be statistically significant.

**Results**

**Cloning and characterization of BhCRASP-1**

To identify the FH binding proteins of *B. hermsii*, a genomic DNA expression library derived from *B. hermsii* strain HS1 was screened for FH binding clones. The sequence of one clone that strongly bound FH revealed an open reading frame of 555 bp encoding a putative lipoprotein with a calculated molecular mass of 21.5 kDa. The encoding gene was designated cspA. Pulse-field gel electrophoresis and hybridization analysis revealed that the cspA gene encoding BhCRASP-1 represents a single genetic locus that maps to a plasmid of ~200 kb. Hybridization analyses using a cspA PCR-generated probe with *Hae*III- and *Bam*HI-digested DNA yielded fragments of ~3 and 8 kb, respectively (data not shown). After cleavage of the leader peptide, the predicted molecular mass of BhCRASP-1 is 19.5 kDa. The N terminus of BhCRASP-1 shows significant homology to the signal peptides of other bacterial lipoproteins (45, 46). This motif includes two lysine residues near the N terminus, a hydrophobic region, and a sequence with significant similarity to the consensus signal peptide II cleavage sequence Leu(Ala, Ser)→Leu(Val, Phe, Ile),Gly→Ile(Val, Gly)→Ala(Ser, Gly)→Cys,1. Using LipoP for prediction of the lipoproteins of Gram-negative bacteria (47), a unique cleavage side for signal peptide II was found between aa 19 and 20, suggesting lipidation at cysteine residue 20 of BhCRASP-1. The amino acid sequence exhibited 83% identity with the recently identified FHBP19/FhBα protein of *B. hermsii* YOR (18). A mAb, BH-1, with specificity for BhCRASP-1 was shown to be nonreactive with FHBP-19/FhBα and with the deletion mutant BhCRASP-1Δ66, suggesting that the specific epitope recognized by mAb BH-1 includes amino acids residing in the N-terminal domain of BhCRASP-1 (data not shown).

**Surface exposure and protease sensitivity of BhCRASP-1**

To determine whether BhCRASP-1 is surface exposed, an immunofluorescence assay was performed using the mAb BH-1, specific for BhCRASP-1. *B. hermsii* was incubated sequentially with mAb BH-1 and the rabbit anti-mouse Cy3-conjugated IgG. The images were obtained by epifluorescence microscopy using a Nikon Eclipse 90i upright automated microscope and a Nikon DS-1 QM sensitive black and white charge-coupled device camera at a resolution of 0.133 μm/pixel (right); for counterstaining, the DNA-binding 4',6-diamidino-2-phenylindole was used (middle), and a differential interference contrast image is also shown (left). Proteinase K treatment affects the surface expression of native BhCRASP-1. *B. hermsii* cells were incubated with the indicated concentration of proteinase K, lysed by sonication, immunoblotted, and screened with anti-BhCRASP-1 (BH-1) and anti-FlaB (LA21) mAbs.
Interaction of BhCRASP-1 with serum proteins

To test the binding of recombinant BhCRASP-1 to the serum proteins FH, FHL-1, and FHR-1 or to plasminogen, slot blot analysis was used. Of the three members of the factor H family analyzed, FH and FHR-1 bound to BhCRASP-1 whereas no binding was observed for FHL-1 (Fig. 2A). Using BhCRASP-1 derived from B. burgdorferi as a control, binding to FHL-1 and FH but not to FHR-1 could be detected. OspA, OspB, and BSA did not bind to any of the three proteins. Furthermore, plasminogen bound to recombinant BhCRASP-1 and OspA, whereas no binding was observed for the control proteins OspB and BSA (Fig. 2B). To assess the binding of serum proteins to the surface of Borrelia cells in a more physiologic assay, intact spirochetes were incubated with NHS, a natural source for FH, FHL-1, FHR-1, and plasminogen that was supplemented with EDTA to prevent complement activation. Serum proteins were adsorbed to spirochetes and subsequently eluted by using a pH shift assay. The eluted fractions were separated by SDS-PAGE and tested for FH, FHL-1, FHR-1, and plasminogen by Western blotting. FH and FHR-1 were detected in the eluted fractions of B. hermsii. In contrast, FHL-1 was not found in the eluate of B. hermsii, indicating that the B. hermsii strain H51 does not bind FHL-1 on its surface. In addition, plasminogen was also present in the eluate fractions of B. hermsii (Fig. 2C).

Localization of the FH/FHR-1 and the plasminogen-binding domains of BhCRASP-1

To localize the binding sites for FH/FHR-1 and plasminogen on BhCRASP-1, a number of BhCRASP-1 deletion mutants with N- and C-terminal truncations were constructed (Fig. 3). Protein expression was confirmed by using Coomassie blue staining, and all of the recombinant proteins exhibited the predicted size and reacted with the BhCRASP-1 immune serum (data not shown). Screening for FH/FHR-1 binding, using ELISA revealed that, of the protein preparations tested, only the full-length form of BhCRASP-1 bound to FH and FHR-1 (Fig. 4A). No binding to FH was detected with any of the other deletion mutants of BhCRASP-1. Thus, the binding of FH/FHR-1 required determinants located in both the C- and N-terminal domains of BhCRASP-1, suggesting that long-range intramolecular interactions are involved in the formation and presentation of the FH/FHR-1 binding pocket.

The different BhCRASP-1 mutants were also analyzed for the ability to bind plasminogen. Full-length BhCRASP-1 (residues 21 to 185) and the truncated versions retained plasminogen binding activity (Fig. 4B), indicating that the binding site for plasminogen is localized to the central domain of BhCRASP-1. Assuming that BhCRASP-1 contains one unique plasminogen binding site, the increased binding capacity of the truncated mutants vs the complete BhCRASP-1 for plasminogen correlates with the relative molar amounts of the respective proteins used in this assay. Together, these data suggest that FH and plasminogen bind to distinct, non-overlapping domains of the BhCRASP-1 molecule. To test this assumption, increasing amounts of plasminogen or FH (up to 100 μg/ml) together with constant amounts of FH (0.1 μg/ml) or plasminogen (5 μg/ml), respectively, were added to immobilized BhCRASP-1. As seen in Fig. 4, plasminogen did not compete with the binding of FH to BhCRASP-1 even at a 1000-fold excess and, vice versa, high amounts of FH did not inhibit the binding of plasminogen to BhCRASP-1.

Activation of bound plasminogen by host-derived plasminogen activators

To determine whether plasminogen bound to the outer surface of B. hermsii was converted to its enzymatically active form, plasmin,
by either endogenously or exogenously supplied plasminogen activator(s), B. hermsii spirochetes were incubated with plasminogen. After the transfer of extensively washed spirochetes to microtiter plates, human uPA and the chromogenic plasmin substrate S-2251 were added. As shown in Fig. 5A, degradation of the chromogenic substrate demonstrates that plasminogen bound to the surface of B. hermsii is converted to enzymatically active plasmin in the presence of exogenous uPA. No or only marginal plasmin activity was seen in the presence of tranexamic acid, indicating that the previous binding of plasminogen to the spirochete is a prerequisite for optimal cleavage by plasminogen activators. Spirochetes treated with plasminogen alone (without subsequent activation with uPA) or with uPA alone (without previous incubation with plasminogen) showed only marginal, if any, degradation of S-2251. No plasmin was formed in the absence of plasminogen activators, indicating that spirochetes do not express endogenous plasminogen activators. Similar findings were observed using BhCRASP-1-coated microtiter plates. In contrast to intact spirochetes, plasmin activity bound to BhCRASP-1 was reduced by ~50% in the presence of tranexamic acid (Fig. 5B).

Identification of the short consensus repeat(s) of FH that bind to BhCRASP-1

To precisely map the binding domain of FH that binds to the recombinant BhCRASP-1 of B. hermsii, various deletion constructs of FH and FHL-1 were used for ligand affinity assays. As shown in Fig. 6A, BhCRASP-1 strongly bound to FH (lane 7 from left) as well as to the deletion constructs FH8–20 (lane 8), FH15–20 (lane 9), and FH19–20 (lane 10), but not to the deletion constructs SCR1–2, SCR1–3, SCR1–4, SCR1–5, SCR1–6, FHL-1 (SCR1–7) (lanes 1–6), and deletion constructs FH15–19 (lane 11). These data indicate that SCR20 of FH is critical for interaction with BhCRASP-1. In addition, FHR-1 but not FHL-1 bound to immobilized BhCRASP-1 using ligand affinity blotting (Fig. 2A), supporting the notion that the SCR20 of FH is primarily involved in binding BhCRASP-1. As indicated in the schematic representation of FH, FHL-1, and FHR-1 (Fig. 6B), domain...
SCR20 of FH displays 97% sequence similarity to the SCR5 of FHR-1 (48).

Applying surface plasmon resonance analyses, a more physiological assay system, FH and the deletion constructs FH8–20 and FH15–20 bound to immobilized BhCRASP-1 with similar high affinities. Furthermore, quantitative analysis revealed a high binding affinity of FH to BhCRASP-1 as demonstrated by a calculated K\textsubscript{D} value of 17 nM (Table II). However, FHL-1, consisting of SCR1–7, failed to bind BhCRASP-1 indicating that the C-terminal domain of FH is required for BhCRASP-1 binding (Fig. 7A). This assumption was verified by showing that the mAb C18 (49) directed against the most C-terminal domain SCR20, completely blocked the interaction of FH with BhCRASP-1 (Fig. 7B).

FH retains cofactor activity when bound to BhCRASP-1

The cofactor activity of FH was analyzed on immobilized recombinant BhCRASP-1 protein by measuring factor I-mediated conversion of C3b to iC3b (21). Recombinant BhCRASP-1 immobilized on a microtiter plate was incubated with excess of purified FH or buffer alone. As controls, functional activity of FH bound to B. burgdorferi BhCRASP-1 or BhCRASP-3 was tested for C3b-inactivating capacity (21, 22). BhCRASP-1-bound FH was more efficient in mediating C3b conversion than FH bound to either BbCRASP-1 or BbCRASP-3 under similar conditions (Fig. 8). As previously shown, FH bound to BhCRASP-1 is up to 10-fold more efficient in factor I-mediated C3b conversion as compared with BbCRASP-3 (21, 22).

Table II. Quantitative analysis of the interaction between FH and immobilized BhCRASP-1 protein

<table>
<thead>
<tr>
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<th>Equilibrium Association Constant (M\textsuperscript{-1})</th>
<th>Equilibrium Dissociation Constant (M)</th>
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<tbody>
<tr>
<td>FH</td>
<td>1.7 \times 10\textsuperscript{7}</td>
<td>1.7 \times 10\textsuperscript{-8}</td>
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*The equilibrium constants were calculated from the association and dissociation rate constants.

FIGURE 6. Complement-regulatory functions and binding domains of FH, FHL-1 and FHR-1. A, Purified recombinant BhCRASP-1 proteins (lanes 1–11, counting from the left) were separated by 10% Tris/Tricine SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with either recombinant FHL-1 (FH1–7) or several deletion constructs of FH (FH1–2, FH1–3, FH1–4, FH1–5, FH1–6, FH8–20, FH15–20, FH19–20 and FH19–20), or with human serum (FH). Bound proteins were visualized using antisera specific for SCR1–7 (αSCR1–4), SCR19–20 (αFH), and the mAb (VIG8). B, Schematic representation of the FH, FHL-1, and FHR-1 proteins. The complement regulatory domains are localized to the N-terminal four domains SCR1–4 (shaded). The interaction domains for other microbial surface proteins are mainly localized to SCR6–7 and SCR19–20 (gray). SCR domains are aligned vertically according to their observed amino acid sequence similarities (%).

FIGURE 7. Analysis of BhCRASP-1 for binding to FH and deletion mutants by surface plasmon resonance. A, FH, FHL-1, or the various FH mutants in the fluid phase were injected into a flow cell precoated with BhCRASP-1 and to a control flow cell without protein (PBS). The control was subtracted from the displayed binding curves. Binding of FH, FH8–20, FH15–20, and FHL-1 to BhCRASP-1 was measured. As compared with the intact FH, the binding of FH15–20 was slightly increased whereas the binding of FH8–20 was reduced and no binding was observed with FHL-1. B, Using mAb C18 directed against SCR20 completely abolished the binding of FH15–20 to BhCRASP-1.

FIGURE 8. Analysis of cofactor activity of FH bound to BhCRASP-1. Recombinant BhCRASP-1 immobilized to microtiter plates was used to capture FH. After sequential addition of C3b and factor I, bound FH enabled factor I-mediated cleavage of C3b to iC3b. iC3b was quantified by ELISA using a neoeptope-specific anti-iC3b IgG. BhCRASP-1 and BhCRASP-3 derived from B. burgdorferi strain ZS7 served as controls. Data are given as mean ± SD of three independent experiments.
In this study we have identified and characterized BhCRASP-1, a novel member of the CRASP family in B. hermsii. BhCRASP-1 binds human FH, FHR-1, and, in addition, plasminogen/plasmin via distinct, nonoverlapping domains. Both FH and plasmin retain their biological activities when bound to B. hermsii or BhCRASP-1, suggesting that BhCRASP-1 is a critical virulence factor of the pathogen.

The previous findings that the relapsing fever spirochete B. hermsii expresses a receptor for FH, FhbA, and that surface-bound FH facilitates factor I-mediated cleavage of C3b suggest a suitable strategy of the pathogen to evade the first-line host defense via the complement system (18). FH binding has been reported for a number of bacterial species such as S. pyogenes (group A streptococcus) (50), Neisseria gonorrhoeae (10, 51), S. pneumoniae (11, 13, 52), B. burgdorferi (24), Borrelia afzelii (25), Borrelia recurrentis (53), Borrelia duttonii (53), Borrelia parkeri (17), and B. hermsii (18, 19). Moreover, for B. hermsii YOR it was found that it specifically binds both FH and FHL-1 via FhbA and that FH and FHL-1 interact with FhbA through the SCR domains 1–7 and SCR16–20 (19). In contrast, the presented plasma adsorption experiments and surface plasmon resonance analyses clearly showed that FH binding to BhCRASP-1 of B. hermsii HS1 is exclusively associated with SCR20. This is further substantiated by the fact that BhCRASP-1 also bound FHR-1, another member of the factor H family, exhibiting a C-terminal domain that is almost identical to the C terminus of FH but different to FHL-1, which consists of SCR1–7 (48). Thus, BhCRASP-1 and FhbA clearly express distinct biological activities in that both show similar binding potential for FH but different capacities to interact with FHL-1 and FHR-1. Although the actual function of FHR-1 is yet to be disclosed, it was suggested to be involved in the adhesion processes of the pathogen to neutrophils (54). The interaction of BhCRASP-1 with FH may be an important mechanism by which B. hermsii spirochetes control C3b deposition on their surface and escape opsonophagocytosis. In addition, FH may function in adherence due to its binding to surface glycosaminoglycans and host cell membrane receptors. In this context it is noteworthy that the C-terminal part of FH has previously been implicated in the binding to other bacterial surface structures, e.g., the sialylated lipooligosaccharide of N. gonorrhoeae and several lipoproteins of B. burgdorferi, including CRASP-3, -4, and -5 (10, 21, 22, 44).

Our results show that FH associated with BhCRASP-1 maintains its regulatory activity and controls C3b deposition and C3-convertase activity. Thus, the acquisition of FH molecules to surface-exposed
BhCRASP-1 results in enhanced complement-regulatory activity, a process that would allow relapsing fever spirochetes to evade clearance by the innate immune system. The biological significance of BhCRASP-1 interaction with FH was further examined by using a B. burgdorferi strain as an amenable host to express a heterologous outer surface lipoprotein for studying complement resistance. In fact, B. burgdorferi was shown previously to successfully display surface-exposed lipoproteins, e.g., Vsp1 and Vsp2 of relapsing fever borreliae (55). In addition, the complementation of BhCRASP-1 or BhCRASP-2 expression in serum-sensitive borrelian cells imparts resistance to human serum (27, 29). Here we demonstrate that expression of the BhCRASP-1 of relapsing fever borreliae in the serum-sensitive Lyme disease spirochete B. burgdorferi B313 results in an increased resistance of the mutant strain to complement-mediated killing, suggesting the involvement of BhCRASP-1 in the immune evasion of B. hermsii.

To localize the peptide binding domain(s) of BhCRASP-1 for FH binding, BhCRASP-1 proteins with N- and C-terminal truncations were generated and used for functional analyses. Deletions of either the N terminus (fragment spanning residues 51–185) or the C terminus (fragment spanning residues 21–173) portion of BhCRASP-1 completely abrogated FH binding, suggesting that the FH binding site of BhCRASP-1 consists of a conformational rather than a contiguous linear peptide structure. Similar features have been previously reported for B. burgdorferi BhCRASP-1 and BhCRASP-3 (21, 22). However, Hovis and colleagues proposed that although determinants of the C-terminal do-