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

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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\textsuperscript{1,2}

Evelyn Rossmann,\textsuperscript{3,4} Peter Kraiczky,\textsuperscript{3} Pia Herzberger,\textsuperscript{3} Christine Skerka,\textsuperscript{3} Michael Kirschfink,\textsuperscript{*} Markus M. Simon,\textsuperscript{5} Peter F. Zipfel,\textsuperscript{"} and Reinhard Wallich\textsuperscript{4,4*}

Tick-borne relapsing fever in North America is primarily caused by the spirochete \textit{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 \textit{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 \textit{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 \textit{B. hermsii} and its resistance to innate immunity. \textit{The Journal of Immunology}, 2007, 178: 7292–7301.

\textbf{B}orrelia hermsii and \textit{Borrelia turicatae} are the main vector-borne pathogens causing human relapsing fever, an acute infectious disorder, in the United States (1). In case of \textit{B. hermsii}, spirochetes are transmitted to humans within minutes through the bite of infected soft ticks, in particular Ornithodoros \textit{hermsii}. \textit{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),\textsuperscript{2} 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., \textit{Candida albicans}, \textit{Neisseria gonorrhoeae}, \textit{Streptococcus pyogenes}, and \textit{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 \textit{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 \textit{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 \textit{B. burgdorferi} strains, BbCRASP-1, is necessary to resist killing by human serum (29).

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

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\item The sequence presented in this article has been submitted to EMBL/GenBank under accession number AM408562.
\item E.R. and P.K. contributed equally to this work.
\item 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
\item 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, \textit{Borrelia burgdorferi} CRASP-1; BhCRASP-1, \textit{Borrelia hermsii} CRASP-1; NHS, normal human serum; Osp, outer surface protein; SCR, short consensus repeat; uPA, urokinase-type plasminogen activator.
\end{itemize}
\end{multicols}
Accordingly, spirochetes bind the host plasminogen that is subsequently processed via urokinase-type plasminogen activator (uPA) to active plasmin, a broad-spectrum serine protease, leading to extracellular matrix degradation (31, 33, 35–37). B. burgdorferi organisms bind host plasminogen 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 plasminogen-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 plasminogen/plasmin and 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. Schwan, Rocky Mountain Laboratories) and the Lyme disease spirochete B. burgdorferi isolate B31 and mutant B313 were cultivated in Barbour-Stoenner-Kelly (BSK)-H complete medium (PAN Biotech) supplemented with 5% rabbit serum (Cell Concept) at 30°C. B313 mutant spirochetes harbor plasmids pCP2-1, pCP2-2, pCP2-3, pCP2-4, pCP2-6, and pLP7 exclusively and therefore lack expression of BhCRASP-1.

Generation of fusion proteins

BhCRASP-1 234(+) CTTTGTTAATCCATACCAAGTTTGGCAGATTTTCTCATTATC

CRASP-1 234(−) CTTTGTTAATCCATACCAAGTTTGGCAGATTTTCTCATTATC

Expression of the respective fusion proteins and affinity purification

These plasmids were used for transformation of the JM109 host cells. Expression of the respective fusion proteins and affinity purification were performed as recommended by the manufacturer (Amersham Bioscience).

Construction of a shuttle vector for transformation with BhCRASP-1

The BhCRASP-1-encoding ospA gene, including its native promoter region, was amplified by PCR amplification using plasmid pJEMb, the primers BhBam and BhR (Table I), and a Mastercycler gradient (Eppendorf). Denaturation was conducted at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 30 s, respectively. After digestion with BamHI and EcoRI, the amplified DNA fragment was ligated in-frame into 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 endoprotease thrombin cleavage of the fusion protein were performed as recommended by the manufacturer

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 I. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Used in This Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>BhBam</td>
<td>ATTATTAAGCCCTGGCATCGGA</td>
<td>Generation of fusion proteins</td>
</tr>
<tr>
<td>Δ130Bam</td>
<td>GCCTCCTCGACTTTTGAACCATCCAAG</td>
<td>Generation of fusion proteins</td>
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<tr>
<td>Δ195Bam</td>
<td>AGTTATTGGAGTGCGTTCCCAAAAGTCCTCCTGG</td>
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<tr>
<td>Ecoα12</td>
<td>CATTATAAGGATCCCAAAAAATTCCGCGAGTTGC</td>
<td>Generation of fusion proteins</td>
</tr>
<tr>
<td>BhR</td>
<td>CATTCAATTGTTATTTAGGATCATAC</td>
<td>Amplification of ospA gene of B. hermsii</td>
</tr>
<tr>
<td>BhF</td>
<td>ACCAGCCACGAAATGGGGAATGGTTATAATTTC</td>
<td>Amplification of ospA gene of B. hermsii</td>
</tr>
<tr>
<td>CRASP-1 57(+)</td>
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<td>Amplification of ospA gene of B. burgdorferi</td>
</tr>
<tr>
<td>CRASP-1 234(+)</td>
<td>CTTTTGTTAATCCATACCAAGTTTGGCAGATTTTCTCATTATC</td>
<td>Amplification of ospA gene of B. burgdorferi</td>
</tr>
<tr>
<td>CRASP-1 234(−)</td>
<td>CTTTGTTAATCCATACCAAGTTTGGCAGATTTTCTCATTATC</td>
<td>Amplification of ospA gene of B. burgdorferi</td>
</tr>
<tr>
<td>CSPZ-1</td>
<td>TATGAGCAATTTTATTTTTTTTGATTTTCTCATTATC</td>
<td>Amplification of ospZ gene</td>
</tr>
<tr>
<td>CSPZ-2</td>
<td>TATGAGCAATTTTATTTTTTTTGATTTTCTCATTATC</td>
<td>Amplification of ospZ gene</td>
</tr>
<tr>
<td>BbCRASP-3 79(+)</td>
<td>GATGAGCAAAGTAGTGGTGAGATAAACC</td>
<td>Amplification of erpP gene</td>
</tr>
<tr>
<td>BbCRASP-3 530(−)</td>
<td>CTTTTATTTTTTTTGATTTTCTCATTATC</td>
<td>Amplification of erpP gene</td>
</tr>
<tr>
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<td>Amplification of erpP gene</td>
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<tr>
<td>OspA3n(−)</td>
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<tr>
<td>OspA1</td>
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<td>Amplification of ospA gene</td>
</tr>
<tr>
<td>OspA2</td>
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<td>Amplification of ospA gene</td>
</tr>
<tr>
<td>Fla6</td>
<td>AACACACACACGTCTGGTTCAGTTTGTTTTTCTCATTATC</td>
<td>Amplification of flaB gene</td>
</tr>
<tr>
<td>Fla7</td>
<td>TATGAGCAATTTTATTTTTTTTGATTTTCTCATTATC</td>
<td>Amplification of flaB gene</td>
</tr>
</tbody>
</table>
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 BcrCRASP-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 
with mAb B22 directed against the SCR5 of FH and FHL-1, the mAb VIG8 
Systems). After four washings with 50 mM Tris-HCl 150 mM NaCl, and 
overnight with NHS, recombinant proteins, or human plasminogen (Cell 
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 washings with PBS-magnesium, resuspended in 20 
mg/ml) was mixed with different amounts of plasminogen (0.001–100 
μg/ml) and these mixtures were added to the wells coated with BcrCRASP-1. Bound FH was detected as de-
scribed above. The ability of different amounts of FH (0.001–100 
μg/ml) to inhibit the binding of plasminogen (5 μg/ml) to immobilized 
BcrCRASP-1 was analyzed accordingly.

**In situ protease treatment of spirochetes**

Whole cells of B. hermsii strain HS1 were treated with proteases by mod-
fication of a method described previously (43). Briefly, freshly harvested 
were washed twice with PBS-MgCl and, after centrifugation at 5000 
rpm for 10 min, the sedimented spirochetes were resuspended in 100 μl of 
this buffer. To 5 × 10^6 intact borrelial cells (final volume of 0.5 ml), 
proteinase K in distilled water (Sigma-Aldrich) was added to a final con-
centration of 12.5–100 μg/ml. Following incubation for 1 or 2 h at room 
temperature, proteinase K was inhibited by adding 5 μl of PMSF (Sigma-
Aldrich) (50 mg/ml in isopropanol). The cells were then washed twice with PBS (pH 7.2) and 
syringe five times using a Branson B-12 sonifier (Heinemann). Whole 
cell protein preparations (10 μl) were separated by using Tris/Tricine 
SDS-PAGE via 4% stacking and 10% separating gels as described pre-
viously (23).

**Surface plasmon resonance analysis**

Protein-protein interactions were analyzed by surface plasmon resonance 
technique using a Biacore 1000 instrument of purified FH (after washing) 
B. burgdorferi. Otherwise, recombinant protein BcrCRASP-1 (20 μg/ml) was 
dialyzed against 10 mM acetate buffer (pH 5.5) was coupled via a standard amine-
coupling procedure to the flow cell of a sensor chip (CM5; Biacore) until 
a level of resonance units >4000 was reached. A control cell was prepared in 
the same way but without injecting a protein. FH, FHL-1, and the deletion 
construct FH–6 were dialyzed against running buffer (75 mM PBS (pH 7.4)). 
Each protein (FH, 333 nM; FHL-1, 0.1 μM; FHL-1, FH–20, and FHL–20.1), 
were injected separately into the flow cell coupled with BcrCRASP-1 or the deletion 
mutants and into a control cell using a flow rate of 5 μl/min at 25°C. Each 
interaction was analyzed at least three times.

The binding kinetics were determined by using a lower density of the 
immunobilized ligand (<1000 resonance units) at 22°C in 75 mM PBS (pH 
7.4) and by using a natural logarithmic Langmuir 1:1 binding model and 
the simultaneous K_d/K_o fitting routine of the BIAevaluation 3.1 software 
(Biacore). The equilibrium constants were calculated from the rate 
constants.

**Functional assay for cofactor activity of FH**

The cofactor activity of FH was analyzed on immobilized recombinant 
BcrCRASP-1 by measuring the factor I-mediated conversion of C3b to 
C3b. Briefly, recombinant BcrCRASP-1 (20 μg/ml) immobilized on a mi-
croplate was incubated with 25% NHS or 25% heat-inactivated NHS. Barbour-
Stoenner-Kelly medium containing human plasmogen (0.1 μM; Dialyzed) 
immobilized on a microplate was incubated with an excess of purified FH. 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 neopetide-specific mouse monoclonal

**Immunofluorescence analysis**

Proteins were grown to mid-log phase, harvested by centrifugation at 
5000 × g for 30 min at 4°C and resuspended in 100 μl of 
veronal-buffered saline supplemented with 1 mM Mg^2+ and 0.15 mM Ca^2+, and 
0.1% gelatin (pH 7.4). To inhibit complement activatio, NHS was 
incubated with 0.34 M EDTA for 15 min at room temperature. The cell 
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 10 min, washed, and resuspended in 300 
l of the same buffer, and lysed by sonication five times using a Branson 
sonicator. 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 
secondary peroxidase-conjugated mAb 10-V-1. Following four washes with TBST, blot strips were 
incubated with a 5000-fold dilution of goat anti-FH (Calbiochem) or a 3000-fold di-

**Serum adsorptions assays using intact borrelial cells**

To determine whether B. hermsii HS1 can bind FH, FHL-1, and plasmino-
gen, a whole cell absorption assay was performed as previously described. 
Borreliae (2 × 10^9 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 Mg^2+ and 0.15 mM Ca^2+, and 
0.1% gelatin (pH 7.4). To inhibit complement activatio, NHS was 
incubated with 0.34 M EDTA for 15 min at room temperature. The cell 
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.
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 H₂O₂ 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 nM trancexam 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-nitroaniline 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 measured for several hours directly in the plates and the background activity of OD₅₅₀ = 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 nM trancexam 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 mg/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 for FHBP19/FhbA protein of B. hermsii YOR (18). A mAb, BH-1, with specificity for BhCRASP-1 was shown to be nonreactive with FHB9/FhbA and with the deletion mutant BhCRASP-1, 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-Q1 sensitive black and white charge-coupled device camera.

To further define the surface localization of BhCRASP-1, B. hermsii organisms were treated with proteinase K and subjected to Western blot analysis. As shown in Fig. 1B, a significant reduction was observed for BhCRASP-1 after 2 h of incubation with proteinase K at concentrations ≥12.5 μg/ml. The band intensity observed for FlAB was not changed, indicating that periplasmic flagella are not affected by proteolytic digestion. Thus, the susceptibility of BhCRASP-1 to proteolytic digestion indicates that this protein is exposed at the outer surface of B. hermsii.
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 borrelial 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 HS1 does not bind FHL-1 on its surface. In addition, plasminogen was also present in the eluate fractions of B. hermsii (Fig. 2C).

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 construct 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 Kd 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 BhCRASP-3 (21, 22). Incubation of immobilized proteins in the

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

<table>
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<th>Equilibrium Association Constant (M–1)</th>
<th>Equilibrium Dissociation Constant (M)</th>
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<tr>
<td>FH</td>
<td>1.7 × 107</td>
<td>1.7 × 10–8</td>
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* The equilibrium constants were calculated from the association and dissociation rate constants.
Western blotting with the specific mAb (BH-1). As shown in Fig. 9B, *B. hermsii* HS1 and the transformant B313/pBH, but not the mutant strain B313, showed a specific band. Furthermore, a growth inhibition assay (42) was used to compare the susceptibility of *B. hermsii* HS1, B313, and B313/pBH to human serum. Resistance to complement-mediated killing was indicated by a continuous growth of spirochetes in the presence of human serum and a subsequent reduction of A562/A630 ratios, whereas the inhibition of sensitive cells was indicated by lack of changes in absorbance. As shown in Fig. 9C, the growth of *B. burgdorferi* mutant B313 was significantly inhibited as compared with growth of B313/pBH in the presence of 25% NHS (p < 0.05). B313 containing the shuttle vector alone showed similar growth properties as the nontransformed *B. burgdorferi* mutant B313 (data not shown). This finding indicates that complement resistance can be increased when BhCRASP-1 is expressed in a heterologous *B. burgdorferi* strain. Heat inactivation of human serum before assaying the borrelial cells did not influence the growth of any strain (data not shown).

**Discussion**

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 absence of FH served as negative controls and had no effect on C3b conversion (data not shown).

**BhCRASP-1 increases resistance to complement-mediated killing**

Preliminary experiments indicated that the *B. hermsii* strain HS1 was not suitable for genetic manipulation. We thus transformed the serum-sensitive *B. burgdorferi* mutant strain B313 lacking the FH/FHL-1 binding proteins BhCRASP-1 to BhCRASP-4 with the shuttle vector pBH containing the entire cspa gene to assess the role of BhCRASP-1 for complement resistance. Transformants were selected by limiting dilution and characterized for the presence of the cspa gene by PCR analysis (Fig. 9A). B313/pBH, but not parental strain B313, showed the expected amplicon product. Lack of plasmids lp54, lp28-3, and cp32-9 harboring ospA and cspa (BbCRASP-1), cspZ (BbCRASP-2), and erpP (BbCRASP-3) in B313 was confirmed by PCR, respectively (Fig. 9A). Next, the expression of BhCRASP-1 was determined by

![FIGURE 9. Characterization and serum susceptibility analysis of *B. hermsii* and *B. burgdorferi* strains. A, *B. burgdorferi* B31, mutant B313, and B313/pBH were characterized by PCR amplification of the cspa, cspZ, erpP, erpA, ospA, and flaB genes using the primers listed in Table I. B, Expression of *B. hermsii* BhCRASP-1 by recombinant *B. burgdorferi* B313 was assessed using Western blot. Whole cell lysates of the indicated borreliae (1 × 10⁸) were separated by SDS-PAGE and transferred to nitrocellulose. BhCRASP-1 was detected using mAb BH-1. M, Marker proteins; lane 1, *B. hermsii*; lane 2, B313; lane 3, B313/pBH. C, Growth inhibition assay. *B. burgdorferi* B313 and B313/pBH cells were examined for sensitivity to human serum. Spirochetes were seeded in microtiter plates and incubated in NHS over a cultivation period of 3 days at 33°C. Data are shown as mean ± SD of three independent experiments. Color changes were monitored by measurement of the absorbance at 562/630 nm.]
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 \textit{B. burgdorferi} strain as an amenable host to express a heterologous outer surface lipoprotein for studying complement resistance. In fact, \textit{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 borrelial 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 \textit{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 \textit{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 \textit{B. burgdorferi} BhCRASP-1 and BhCRASP-3 (21, 22). However, Hovis and colleagues proposed that although determinants of the C-terminal domain of FhbA are important in FH/FHL-1 binding, the 10-aa C-terminal tail is dispensable (19). In this context the recent identification of two genetically distinct groups of \textit{B. hermsii} is of interest (56). Together with the proposition of two clusters of FH binding proteins found in the \textit{B. hermsii} genome, the combined studies thus suggest a differential association of FhbA and BhCRASP-1 with the two subgroups (57). However, further studies are required to settle this issue.

Several independent investigations have proposed that the binding of plasminogen to the surface of bacteria is of importance for the invasive capacity of a number of \textit{Borrelia} species (32, 33, 35, 58–60). We have shown now that \textit{B. hermsii} binds plasminogen to its outer surface and that bound plasminogen can, in turn, be converted to enzymatically active plasmin in the presence of exogenous human uPA as measured by the cleavage of the chromogenic plasmin substrate S-2251. The interaction of plasminogen with \textit{B. hermsii} was shown previously to successfully display \textit{B. hermsii} in humans. Our findings may have broad implications for the invasive potential of human pathogens and support the concept of their exploitation of host factors as a suitable survival strategy.

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**Disclosures**

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