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**Borrelia recurrentis** Employs a Novel Multifunctional Surface Protein with Anti-Complement, Anti-Opsonic and Invasive Potential to Escape Innate Immunity

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### Abstract

*Borrelia recurrentis*, the etiologic agent of louse-borne relapsing fever, has evolved strategies, including antigenic variation, to evade immune defence, thereby causing severe diseases with high mortality rates. Here we identify for the first time a multifunctional surface lipoprotein of *B. recurrentis*, termed HcpA, and demonstrate that it binds human complement regulators, Factor H, CFHR-1, and simultaneously, the host protease plasminogen. Cell surface bound factor H was found to retain its activity and to confer resistance to complement attack. Moreover, ectopic expression of HcpA in a *B. burgdorferi* B313 strain, deficient in Factor H binding proteins, protected the transformed spirochetes from complement-mediated killing. Furthermore, HcpA-bound plasminogen/plasmin endows *B. recurrentis* with the potential to resist opsonization and to degrade extracellular matrix components. Together, the present study underscores the high virulence potential of *B. recurrentis*. The elucidation of the molecular basis underlying the versatile strategies of *B. recurrentis* to escape innate immunity and to persist in human tissues, including the brain, may help to understand the pathological processes underlying louse-borne relapsing fever.

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### Competing Interests:

The authors have declared that no competing interests exist.

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**Introduction**

*B. recurrentis*, the causative agent of louse-borne relapsing fever (LBRF) is transmitted to humans, its reservoir host, by contamination of abraded skin with either hemolymph or feces of an infected human body louse (*Pediculus humanus humanus*) [1,2]. The last century has seen multiple epidemics of LBRF in Europe, with high mortality rates up to 40%. However, currently epidemic outbreaks of LBRF are only reported from Africa [3,4]. LBRF is a systemic inflammatory disease, characterized by one to five relapses of fever and a massive spirochetemia [5]. If treated with antibiotics, the mortality is reduced to 2–6%, however, patients often suffer from Jarish-Herxheimer reactions [6].

In order to survive in human tissues, including the blood, *B. recurrentis* has to escape innate and adaptive immune defence processes. Complement acts as an important part of host innate immunity, which is essential for recognition and elimination of microbes. However, pathogenic organisms can evade complement attack that is either mediated by the acquisition of host regulators to the surface of the pathogen or by expression of endogenous complement regulators [7]. An increasing number of pathogenic microbes utilize host complement regulators for immune evasion and for down-regulation of complement activation. In fact we and others have recently demonstrated that tick-borne pathogens, *B. henselii* and *B. burgdorferi*, are able to bind complement regulatory proteins, i.e. factor H (CFH), CFH-like protein 1 (CFHL-1) and CFH-related protein 1 (CFHR-1), via their surface lipoproteins CRASP-1, thereby conferring resistance to complement attack [8,9,10,11,12]. Surface bound CFH controls complement activation by accelerating the decay of the C3 convertase of the alternative pathway and by inactivating newly formed C3b [13,14]. CFH represents the main human fluid phase regulator of the alternative pathway of complement activation and belongs to the factor H protein family, consisting of seven structurally related proteins in humans, including Factor H-related proteins (CFHRs) [15]. All factor H protein family members are composed of short consensus repeats (SCRs) [16]. Among those, CFHR-1 was recently shown to inhibit complement activation by interfering with C5 convertase and terminal complex formation (unpublished data).

Another common strategy of pathogens to circumvent the immune system and to persist is based on their capability to exploit host plasma derived proteases, in particular human plasminogen/plasmin (PLG) in order to breach tissue barriers. Recently, we have identified and characterized a number of PLG-binding outer surface lipoproteins in related spirochetes, including *B. burgdorferi* and *B. henselii*. *B. burgdorferi* was shown to bind PLG via a multitude of lipoproteins, such as CRASP-3 (ErpP), CRASP-4 (ErpC),...
were grown at 37°C to invade host tissues. Oppsonization and simultaneously acquires an increased potential proteins confers resistance to both, complement attack and multifunctional surface lipoprotein, which by exploiting host Here we provide evidence that B. recurrentis proposed [21], but so far these proteins have not been isolated. and to persist in the host. The fact that surface-bound PLG retains its proteolytic constituents of the extracellular matrix and basement membranes [17,18]. The fact that surface-bound PLG is subsequently processed via urokinase-type plasminogen activator (uPA) to active plasmin, a broad-spectrum serine protease, capable of degrading CFHR-1 and CFHL-1 and production of recombinant proteins

Isolation and cloning of HcpA, construction of expression plasmids and production of recombinant proteins

Isolation of the CFH binding protein of B. recurrentis was carried out by co-immunoprecipitation. Protein G sepharose beads (Amersham Bioscience, Freiburg, Germany) were loaded with polyclonal anti-factor H antibody (Calbiochem, Schwabach, Germany) and purified human factor H (Calbiochem). Subsequently, beads were incubated overnight at 4°C with whole cell lysates of B. recurrentis A1. Immunoprecipitates were separated by SDS-PAGE and visualized by staining with colloidal Coomassie (Pierce/Thermofisher, Bonn, Germany). The selected protein band of approximately 17 kDa was cored from the gel and subjected to mass spectrometric analysis. The identified peptides matched an open reading frame of 325 bp of the B. recurrentis A1 genome, designated hcpA.

The gene encoding HcpA was amplified by PCR using primers BrF and BrR (Table 1), cloned into pGEM-T Easy vector (Promega, Mannheim, Germany) and sequenced by using the BigDye terminator cycle sequencing kit (PE Applied Biosystems). The resulting plasmid pGEM-Br was used as template for construction of expression plasmids by PCR amplification. For recombinant full-length HcpA protein, primers BrBam and BrSal were used, for N- and C-terminal deletion mutants, these primers were applied in combination with Δ48Bam, Δ78Bam, Δ145Sal or Δ115Sal (Table 1) resulting in recombinant proteins HcpA Δ48-175, Δ78-175, Δ18-145 and Δ18-115, respectively. After digestion with restriction enzymes BamHI and SalI, PCR fragments were ligated in frame into the His6-tag encoding sequence into vector pQE-30Xa (Qiagen, Hilden, Germany). For expression of N-terminal His-tagged fusion proteins the plasmids were transformed into E.coli strain JM109 and recombinant proteins were purified as recommended by the manufacturer (Qiagen).

Expression of recombinant proteins of CFH, CFHL-1 and CFHR-1

Deletion constructs of CFH (CFHSCR1-7, CFHSCR8-20, CFHSCR13-20, CFHSCR15-19) and CFHR-1 were expressed in Spodoptera frugiperda S9 insect cells infected with a recombinant baculovirus. The cloning, expression and purification have been described previously [14,29]. The C-terminal deletion mutant CFHSCR19-20 was amplified by PCR, ligated in frame into pQE-30Xa vector and expressed as fusion protein with an N-terminal His6-tag. Expression and purification was done as recommended by the manufacturer (Qiagen).

SDS-PAGE, Ligand affinity blot and ELISA

Borrelial whole cell lysates (15 μg) or purified recombinant HcpA proteins (200 ng) were subjected to Tris/Tricine-SDS-PAGE under reducing conditions and transferred to nitrocellulose

Table 1. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>purpose of use</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrF</td>
<td>TTC AGA AGT GGA GCA ATC</td>
<td>amplification of hcpA gene</td>
</tr>
<tr>
<td>BrB</td>
<td>ACA TAA TAA AAC TTT TAA CTA TG</td>
<td>amplification of hcpA gene</td>
</tr>
<tr>
<td>BrBam</td>
<td>CAT TAT TGG ATC CGA ACT CTT AAG C</td>
<td>generation of expression plasmid</td>
</tr>
<tr>
<td>BrF1</td>
<td>AAA CAG CAG GTA TCA CTC G</td>
<td>amplification of hcpA gene</td>
</tr>
<tr>
<td>BrR1</td>
<td>TCT AGA TTA TAA TGG TGA G</td>
<td>amplification of hcpA gene</td>
</tr>
<tr>
<td>BrSal</td>
<td>ACA TAA TAA ACG TCG ACT AAT CTA TG</td>
<td>generation of expression plasmid</td>
</tr>
<tr>
<td>Δ48Bam</td>
<td>CAG GCT GGA TCC TTT TTT CAA GAA TC</td>
<td>construction of deletion mutants</td>
</tr>
<tr>
<td>Δ78Bam</td>
<td>AA GAT GGA TCC CAA AAG ACA TTA AAC</td>
<td>construction of deletion mutants</td>
</tr>
<tr>
<td>Δ115Sal</td>
<td>TA TAA GTC GAC TTA TAT AGT GTT TAT TG C</td>
<td>construction of deletion mutants</td>
</tr>
<tr>
<td>Δ145Sal</td>
<td>AA AAG GTC GAC TTA GAT AAA CTT TAT GC</td>
<td>construction of deletion mutants</td>
</tr>
<tr>
<td>BrTbam</td>
<td>TTC AGA AGT GGA TCC ATC</td>
<td>construction of pBR</td>
</tr>
<tr>
<td>BrT5ph</td>
<td>GGT AAT TAA TTA TGC ATG CAA TAA TTA ATT ATT TCA AG</td>
<td>construction of pBR</td>
</tr>
</tbody>
</table>
as previously described [24]. For ELISA using non-denatured recombinant proteins, microtiter plates (MaxiSorp, Nunc) were coated with HcpA or the deletion mutants (100 μl; 1 μg/ml) for 2 h at room temperature. The wells were washed with PBS/0.05%Tween, blocked with PBS/5% BSA and incubated with CFH (10 μg/ml), 50% normal human serum ( NHS) or PLG (20 μg/ml) for 1 h at RT. After washing, HcpA bound proteins were detected by goat anti-CFH (Calbiochem) or goat anti-PLG (Acros) antibodies or CFHR-1 specific mouse mAb JHD6 followed by peroxidase-conjugated rabbit anti-goat IgG (Dianova) or sheep anti-mouse secondary antibody (GE Healthcare), respectively. Substrate reaction was performed with o-phenyldiamine dihydrochloride (Sigma-Aldrich) and absorbance was measured at 492 nm. For competition inhibition assay, HcpA (2 μg/ml) was coated on microtiter plates. To analyze the ability of PLG to inhibit the binding of CFH to HcpA, plates were incubated simultaneously with constant amounts of CFH (3 μg/ml) and different amounts of PLG (0.001–100 μg/ml). The ability of CFH to inhibit the binding of PLG to HcpA was determined by adding constant amounts of PLG (10 μg/ml) with different amounts of CFH (0.001–100 μg/ml). HcpA bound CFH and PLG were detected as described above. For detection of purified recombinant HcpA full-length protein and deletion mutants, the anti His-tag monoclonal mouse antibody (Calbiochem) was used.

**Immunofluorescence analysis**

Spirochetes (1×10⁶) were washed with Tris buffer (30 mM Tris, 60 mM NaCl, pH 7.4) and incubated with mAb directed against HcpA (BR-1) for 1 h at RT. For detection of CFH-binding cells were treated with purified human CFH for 1 h at RT followed by incubation with CFH-specific mAb JHD6. Spirochetes were then washed with Tris buffer/0.1% BSA, spotted on coverslips and allowed to air-dry for 1 h. After methanol fixation, samples were dried for 15 min and incubated for 1 h in a humidified chamber with Cy3-labeled rabbit anti-mouse IgG (1/200, Dianova). Cells were visualized at a magnification of 1000× using a Nikon Eclipse 90i upright automatic microscope and images were obtained using a Nikon DS-1 QM sensitive black and white CCD camera at a resolution of 0.133 μm/pixel.

**Flow cytometry**

Briefly, 10⁷ B. recurrentis A1 and A17, B. henselii HS1 and B. burgdorferi B313 cells were washed twice with PBS, blocked for 15 min at RT with PBS/10%BSA, and incubated with 10 μg/ml of biotinylated CFH in FACS-buffer (PBS/1%BSA) for 1 h at RT. As a negative control, spirochetes were incubated with the same concentration of unmodified BSA. Cells were washed extensively for three times and stained with phycoerythrin (PE) labeled streptavidin (Bio-Rad). Cells were then fixed with 1% paraformaldehyde overnight and analyzed on a FACS-Calibur (BD Biosciences) and CellQuest software (BD).

**In situ protease treatment of spirochetes**

Cells of B. recurrentis strain A1 were treated with proteases using a modified, previously described method [25]. Briefly, intact borrelial cells were incubated with either protease K or trypsin and whole-cell protein preparations were separated by SDS-PAGE (13%) as described [26].

**Surface plasmon resonance analysis**

Protein-protein interactions were analyzed using surface plasmon resonance with a Biacore 3000 instrument (Biacore AB), as described elsewhere [27].

**Cofactor assay**

Cofactor activity of CFH bound to immobilized HcpA or intact B. recurrentis A1 cells was analyzed by measuring factor I-mediated conversion of C3b to inactive C3bi (C3b) [28].

**Substrate assay for plasmin bound to spirochetes and HcpA**

Intact B. recurrentis A1 cells (1×10⁶) were incubated with 10 μg PLG (Chromogenix) in the presence or absence of 100 mM tranexamnic acid for 30 min at 30°C in Eppendorf tubes. Following two washes, B. recurrentis were resuspended in 50 μl assay buffer (30 mM Tris, 60 mM NaCl, pH 7.4), transferred to microtiter plates and uPA (25 ng, Chemicon Int., Hampshire, UK) as well as plasmin substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (20 μg, S-2251, Sigma-Aldrich, Taubkirchen, Germany) was added. The absorbance change at 405 nm was monitored for up to 6 hours directly in the plates. Similarly, HcpA (0.2 μg) was coated on microtiter plates and after blocking, PLG in the presence or absence of 100 mM tranexamnic acid was added, incubated for 10 min at 37°C and experiment was carried out as described above. For plasmin-dependent degradation of fibrinogen, HcpA (0.2 μg) coated microtiter plates were incubated with PLG. Subsequently, fibrinogen (500 ng, Calbiochem) and uPA (10 ng) were added and incubated at 37°C for 0.5, 1, 2, 4 and 6 h. Reaction mixtures were then separated by SDS-PAGE, transferred to nitrocellulose and probed with rabbit anti-fibrinogen Ab (Calbiochem) and peroxidase-conjugated goat anti-rabbit secondary antibody (Dianova) for detection of fibrinogen degradation products.

**C3b deposition and degradation on borrelial surface**

Anti-opsonic properties of plasmin bound to the borrelial surface were investigated by a C3b deposition and degradation assay based on whole-cell ELISA. Intact B. recurrentis, B. burgdorferi B313 and transformed B. burgdorferi B313/pBR spirochetes (10⁷/well) were washed, resuspended in PBS and immobilized onto microtiter plates (MaxiSorp, Nunc) overnight at 4°C. After washing with PBS/0.05%Tween, wells were blocked with PBS/0.1% gelatine for 1 h at RT and incubated with 10% normal human serum (NHS) for 30 min at 37°C. B. burgdorferi B313 and B. burgdorferi B313/pBR were processed instantly, whereas B. recurrentis cells were incubated with PLG (10 μg) in the presence or absence of the lysine analogue tranexamic acid (TA); as a negative control, cells were treated with buffer. Bound PLG was activated by uPA for 3 h at 37°C. Deposited C3b was then detected by incubation with biotinylated rabbit anti-human C3c IgG (Dako) followed by peroxidase-conjugated streptavidin (Amersham Bioscience) and analyzed as described above.

**Construction of a shuttle vector for transformation of B. burgdorferi B313**

The HcpA encoding hcpA gene including its native promoter region was amplified by PCR using primers BrTBam and BrTSph. The resulting amplicon was cloned into pBSV2 yielding shuttle vector pBR [29]. Transformation of B. burgdorferi B313 and characterization of transformants was previously described [30]. Expression of HcpA of post-transformation B. burgdorferi B313 was determined by Western blot using mAb BR-1 and sheep anti mouse peroxidase-conjugated secondary Ab.

High-passage, non-infectious B. burgdorferi strain B313 were grown in 100 ml BSK medium and harvested at mid exponential phase (10⁸ cells/ml). Electroporant cells were prepared as described previously [30] with slight modifications. Briefly, 50 μl
aliquots of competent *B. burgdorferi* strain B313 cells were electroporated at 12.5 kV/cm in 2-mm cuvettes with 10 μg of plasmid DNA. For control purpose *B. burgdorferi* strain B313 cells also were transformed with pBSV2 vector alone. Cells were immediately diluted into 10 ml BSK medium and incubated without antibiotic selection at 30°C for 48 to 72 h. Bacteria were then diluted into 100 ml BSK medium containing kanamycin (30 μg/ml) and 200 μl aliquots were plated into 96-well cell culture plates (Corning) for selection of transformants. After three weeks, wells were evaluated for positive growth by color change of the medium, confirmed by dark-field microscopy for the presence of motile spirochetes.

The *hcpA* genes of transformed *B. burgdorferi* B313 strain was detected by PCR using primers BrF1 and BrR1 (Table 1) and expression of the HcpA was determined by Western blot and ligand affinity blot analysis.

Serum susceptibility testing of Borrelia strains

The serum susceptibility of *B. recurrentis* A1, *B. burgdorferi* B313 and transformed *B. burgdorferi* B313 was assessed using a survival assay. Cells grown to mid-logarithmic phase were harvested, washed and 3 x 10⁷ spirochetes were resuspended in BSK-H medium (PAN) supplemented with either 50% or 25% NHS or heat inactivated serum (hiNHS) as indicated. Cells were incubated in Eppendorf tubes at 30°C for 3 days. At day 0, 1, 2 and 3, cells were washed in 0.85% NaCl, transferred to microtiter plates and incubated with SYTO9 (Molecular Probes, Invitrogen) as recommended by the manufacturer. Subsequently, relative growth of spirochetes was determined measuring the fluorescence intensity at 485 nm/530 nm on a microtiter plate reader (Victor plate reader, Perkin Elmer).

Production of monoclonal antibodies

Monoclonal antibodies directed against HcpA were generated by immunization of Balb/c mice with the respective purified recombinant protein according to a method as described elsewhere [31]. For specific detection of CFH-1, mAb JHD8 and for specific detection of His-tagged CFH deletion mutant SCR19-20, mAb JHD7 were generated accordingly.

Nucleotide sequence deposition

The *hcpA* gene sequence encoding HcpA has been deposited in the EMBL/GenBank databases under the following accession numbers: FM946025.

Statistical analysis

Statistics were analyzed with the unpaired Student’s t-test, P values less than 0.01 were considered significant.

Results

*B. recurrentis* spirochetes acquire complement regulators CFH and CFHR-1

In light of previous experience that *B. hermsii* and *B. burgdorferi* can specifically bind CFH via their outer surface lipoproteins [8,11,32], *B. recurrentis* spirochetes were incubated with biotinylated human CFH and analysed by flow cytometry. As seen in Figure 1A, both, *B. recurrentis* strains (A1 and A17) and *B. hermsii* HS1, but not *B. burgdorferi* B313, which lacks the CFH-binding lipoprotein, were able to bind CFH. In addition, biotinylated control protein, BSA, did not bind to any of the borrelial strains (Fig. 1A). Binding of CFH to intact and viable *B. recurrentis*

![Figure 1](https://example.com/fig1.png)

**Figure 1. Binding of CFH and CFHR-1 to the spirochetal surface.** Binding of CFH to intact *B. recurrentis* cells was analyzed by flow cytometry and immunofluorescence microscopy. (A) Spirochetes were incubated with biotinylated purified human CFH (bold lines) or as a negative control with biotinylated BSA followed by PE-labeled streptavidin. *B. hermsii* HS1 and *B. burgdorferi* B313 were included as controls. (B) Cells were incubated with purified human CFH followed by the CFH-specific mAb JHD7 and a Cy3-conjugated anti-mouse IgG. Images were obtained employing epifluorescence microscopy. On the right panel the corresponding differential interference contrast image (DIC) is depicted. (C) Whole cell lysates of *B. recurrentis* strain A1 and A17 (*B.r. A1 and B.r. A17*) were separated by Tris/Tricine SDS-PAGE, transferred to nitrocellulose membrane and incubated with normal human serum. CFH binding was detected employing CFH specific mAb JHD7 and binding of CFHR-1 was analyzed using specific mAb JHD8. For control, cell lysates of *B. hermsii* HS1 (*B.h HS1*) and *B. burgdorferi* B313 (*B.b B313*) were included.

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spirochetes was confirmed by microscopy, using a CFH-specific mAb, JHD7 (Fig. 1B). By applying ligand affinity blot analysis for detection of CFH- and CFHR-1 binding molecules, a protein of approximately 17 kDa was detected in B. recurrentis but not in B. burgdorferi B313 (Fig. 1C), termed human complement regulator(s) and plasminogen binding protein A (HcpA) [8,9].

HcpA is a CFH, CFHR-1 and PLG binding protein

To isolate and further characterize HcpA, whole cell lysates of B. recurrentis A1 were incubated with CFH and subsequently treated with a goat anti-CFH immune serum. A resulting co-precipitated protein of 17 kDa was analyzed by mass spectrometry and peptides generated matched an open reading frame of 525 bp in the genome of B. recurrentis A1 (unpublished data), designated hcpA. Due to the presence of a spirochetal lipobox at its N-terminus HcpA represents a putative outer surface lipoprotein [33]. The deduced amino acid sequence exhibits 54% similarity with the recently identified BhCRASP-1 of B. hermsii HS1 (Fig. 2) [8]. A BLAST search detected another protein with significant homology in the genome of B. turicatae, indicating that this protein is to be found in other Borelia species. To further elucidate the binding properties of HcpA for complement regulators CFH and CFHR-1, various N- and C-terminal deletion mutants of HcpA were generated. Variants of the encoding hcpA gene lacking the hydrophobic leader peptide and the indicated N- or C-terminal regions were cloned and expressed as His-tagged fusion proteins in E. coli (Fig. 3). Expression of each protein was confirmed by immunoblot analysis using anti-His antiserum (Fig. 3A).

To assess binding of recombinant HcpA for CFH and CFHR-1, ligand affinity blotting techniques and ELISA were employed in combination with intact HcpA and various deletions thereof. CFH and CFHR-1 only bound to full-length HcpA, but not to any of the deletion mutants. This indicates that long-range intramolecular interactions are involved in the formation of the CFH and CFHR-1 binding site rather than linear peptide sequences (Fig. 3A). Furthermore, full-length and mutant HcpA proteins were analyzed for binding of human PLG using ELISA. Full-length HcpA (residues 18 to 175) as well as the truncated versions bound PLG (Fig. 3B), thus indicating that the binding site for PLG is localized to the central domain of HcpA. Together, these data suggest that CFH and PLG bind to distinct, non-overlapping domains of the HcpA molecule (Fig. 3C). To verify this assumption, competition assays were performed using immobilized HcpA and increasing amounts of PLG or CFH (up to 100 μg/ml) in combination with constant amounts of CFH (2 μg/ml) or PLG (10 μg/ml), respectively. As shown in Fig. 3D, PLG did not compete with CFH for binding to HcpA even at high concentrations (100 μg/ml). Vice versa, using up to 100 μg/ml of CFH no inhibition of PLG binding to HcpA could be observed.

To map the binding domain of CFH that interacts with HcpA, recombinant deletion constructs of CFH representing SCRs 8-10, SCRs 15-19, and SCRs 1-7/FHL-1 were employed. HcpA showed strong binding to CFH and deletion constructs CFHSCR8-20, CFHSCR15-20, and CFHSCR19-20, as well as CFHR-1. Construct CFHL-1 (CFHSCR1-7) did not bind to HcpA, indicating that the most C-terminal domains (SCR19-20) of CFH are involved in binding (Fig. 4A). We next conducted surface plasmon resonance analyses, a more physiological assay system, to further define the CFH domain interacting with HcpA. CFH and deletion constructs CFHSCR8-20, CFHSCR15-20, and CFHSCR19-20 bound to immobilized HcpA with similar intensity (Fig. 4B). However, in the absence of SCR20, represented by the deletion construct CFHSCR15-19, binding to HcpA was completely abrogated (Fig. 4B). As indicated by the schematic representation, domain SCR20 of CFH displays 97% sequence similarity to the SCR5 of CFHR-1 (Fig. 4C) [7]. Therefore it is assumed that the binding region of CFHR-1 for HcpA is located in the C-terminus, accordingly.

HcpA is exposed on the outer surface of B. recurrentis

To determine whether HcpA is exposed on the outer surface of B. recurrentis immunofluorescence microscopy was performed. Intact B. recurrentis spirochetes were incubated with the HcpA specific mAb BR-1 followed by a Cy3-conjugated secondary antibody (Fig. 5A, left panels). B. recurrentis expressed HcpA on its outer surface and the staining showed a patchy distribution. Mouse mAb LA21 directed against the periplasmic flagellin was used as an internal control to confirm that the fragile spirochetal outer membrane was intact (right panels). To further verify surface localization of HcpA, B. recurrentis spirochetes were pre-treated with either proteinase K or trypsin, lysed, separated by SDS-PAGE and assayed by Western blotting. Fig. 5B demonstrates a significant reduction of HcpA after 2 h of incubation with trypsin at concentrations ≥12.5 μg/ml, whereas treatment with proteinase K at low concentrations (≥3.125 μg/ml) resulted in complete degradation. Signal intensity observed for flagellin remained unchanged, indicating that periplasmic flagella were not affected by proteolytic digestion. These data strongly suggest that HcpA is exposed on the outer surface of B. recurrentis.

Figure 2. HcpA exhibits 54% amino acid sequence similarity to BhCRASP of B. hermsii HS1.

doi:10.1371/journal.pone.0004858.g002
Figure 3. Binding of CFH, CFHR-1 and PLG to HcpA. (A) Purified recombinant HcpA protein and various deletion mutants were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with a monoclonal anti-His-tag mAb (upper panel). CFH and CFHR-1 binding capabilities were analyzed by ligand affinity blotting utilizing normal human serum and goat anti-CFH immune serum (middle panel) or the CFHR-1 specific mAb JH88 (lower panel). (B) Microtiter plates were coated with full-length recombinant HcpA and the indicated deletion mutants, respectively, and incubated with CFH, normal human serum (as source for CFHR-1) or PLG. Binding was detected using goat anti-CFH, the CFHR-1 specific mAb JH88 or goat anti-PLG immune serum followed by the respective peroxidase-conjugated IgGs. (C) Diagrammatic representation of native and expressed recombinant HcpA proteins and their binding characteristics for serum proteins CFH, CFHR-1 and PLG as determined by ligand affinity blot analysis and ELISA. Numbers refer to amino acid residues. (D) Dose dependent binding of CFH and PLG by HcpA. A competition inhibition assay was performed, adding different amounts of PLG or CFH to inhibit the binding of 2 μg/ml CFH (left panel) or 10 μg/ml PLG (right panel) to immobilized HcpA. Bound CFH and PLG was detected using goat anti-CFH (dashed line) and goat anti-PLG Ab (solid line), respectively, followed by peroxidase-conjugated secondary antibody.

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CFH retains cofactor activity when bound to HcpA

To assess whether HcpA-bound CFH maintains its complement regulatory function, cofactor activity of the attached CFH was analyzed by measuring factor I-mediated conversion of C3b to iC3b. To this end, CFH was attached to immobilized HcpA and incubated with C3b and factor I. As shown in Figure 6A, HcpA-bound CFH efficiently mediated C3b conversion as indicated by the appearance of C3b cleavage products (68, 46 and 43 kDa α'-chain). Incubation of immobilized HcpA alone had no effect on C3b conversion under similar conditions. Next we determined whether CFH also retains its cofactor activity after previous binding to B. recurrentis. Accordingly, spirochetes were pre-treated with CFH and after intensive washings, incubated with factor I and C3b. Lysates were separated by SDS-PAGE and C3b cleavage products were detected by Western blotting. Surface-bound CFH retained cofactor activity as indicated by the presence of representative C3b inactivation products (Fig. 6B). B. recurrentis spirochetes alone did not promote cleavage of C3b demonstrating that B. recurrentis lack endogenous C3b cleaving activity and cofactor activity for cleavage. Thus, binding of CFH to the surface of B. recurrentis renders them resistant to complement attack.

Surface bound PLG is processed by host-derived plasminogen activators to plasmin, cleaves fibrinogen and exhibits anti-opsonic activities

To assess whether B. recurrentis-attached PLG can be processed to active plasmin, spirochetes were pre-treated with PLG and subsequently incubated with the exogenous human plasminogen activator uPA. As shown in Figure 7A, B. recurrentis-attached PLG was readily processed by exogenous uPA. In contrast, only marginal plasmin activity was observed in the presence of tranexamic acid, a competitive inhibitor of the lysine-binding site of PLG. These data demonstrate that spirochetal surface-bound PLG is accessible for uPA, and that processed plasmin retains its proteolytic activity. No plasmin was generated from surface-bound PLG in the absence of uPA, indicating that spirochetes do not express endogenous plasminogen activators. Similar findings were observed when recombinant HcpA was coated onto microtiter plates supporting the notion that HcpA-bound PLG can be processed to stable functional active plasmin (Fig. 7B).

The proteolytic activity of HcpA-bound plasmin was further analyzed by its ability to cleave fibrinogen. When incubated with HcpA-bound plasmin, fibrinogen was degraded to low molecular mass fragments, as assessed by Western blotting (Fig. 7C). In the absence of either PLG or uPA, no fibrinogen degradation was observed. Furthermore, the addition of the plasminogen inhibitor tranexamic acid, a competitive inhibitor of the lysine-binding site of fibrinogen, blocked the respective proteolytic activity (data not shown).

Upon activation of complement by bacterial pathogens C3b is covalently attached to the target surfaces and together with the cleavage products, such as iC3b, the C3b molecules opsonize the pathogenic organisms for phagocytosis. To assess whether plasmin bound to HcpA is able to degrade C3b deposited on the cell surface of B. recurrentis a C3b deposition and degradation assay based on whole-cell ELISA was performed. Spirochetes were immobilized onto microtiter plates and treated with human serum and PLG. After extensive washing B. recurrentis-attached PLG was activated by uPA and deposition of C3b molecules on the spirochetal cell surface was monitored. B. recurrentis-attached active plasmin led to a dramatic decrease of C3b molecules on the spirochetal surface when compared to control spirochetes cultured either with buffer alone, or those pre-treated with PLG in the absence of uPA, indicating that spirochetes do not express endogenous plasminogen activators. Similar findings were observed when recombinant HcpA was coated onto microtiter plates supporting the notion that HcpA-bound PLG can be processed to stable functional active plasmin (Fig. 7B).

HcpA confers resistance to complement-mediated killing

To further verify the significance of HcpA of B. recurrentis for complement resistance and removal of C3b from the bacterial surface the serum-sensitive B. burgdorferi B313 strain, lacking CFH and CFH1L-1 binding proteins was transformed with the shuttle vector pBR containing the complete hcpA gene (B. burgdorferi B313/
Expression of HcpA was determined by Western blot analysis using an HcpA-specific mAb, BR-1. B. recurrentis A1 and the transformed B313/pBR isolate, but not the CFH and CFH L-1-deficient B. burgdorferi B315 mutant, expressed HcpA (Fig. 9A, upper panel). HcpA expression on the cell surface of transformed B. burgdorferi B313 strains was also detected by whole cell ELISA (Fig. 4B). Moreover, the expressed HcpA protein bound CFH as confirmed by ligand affinity blotting (Fig. 9A, middle panel). In addition, we demonstrate that HcpA expressed on the surface of transformed B. burgdorferi B313/pBR cells promotes degradation of deposited C3b (Fig. 9C). To compare the susceptibility of B. recurrentis A1, B313 and B313/pBR to complement-mediated killing, the three strains were subjected to a human serum sensitivity assay. Accordingly, B. burgdorferi B313/pBR, B. recurrentis A1, and B. burgdorferi B313 were incubated in NHS or heat-inactivated serum for up to three days. Spirochetal growth was monitored by uptake of a nucleic acid dye. As shown in Figure 9D, B. recurrentis A1 readily multiplied during the 72 h time interval in normal human serum, demonstrating the pronounced resistance to human serum of louse-borne relapsing fever spirochetes. Serum-sensitive B. burgdorferi B313 as well as B313 spirochetes containing the shuttle vector alone (data not shown) did not grow under similar conditions, suggesting their susceptibility to complement-mediated lysis (Fig. 9E). In contrast, B313/pBR expressing HcpA survived and multiplied in human serum. B. burgdorferi B313 and the transformed B313/pBR isolate showed similar growth rates when cultured in heat-inactivated human serum. These findings strongly suggest that HcpA is required for resistance of B. recurrentis to complement-mediated killing.

Discussion

The present results demonstrate for the first time that the etiologic agent of louse-borne relapsing fever, B. recurrentis, expresses a novel multifunctional surface lipoprotein, termed HcpA. By exploiting host proteins, HcpA simultaneously confers resistance to complement attack and opsonization, and in addition, imparts an increased potential to invade host tissues. We show that B. recurrentis can bind via its surface expressed HcpA molecule to human complement regulators, i.e. CFH as well as CFHR-1, and in parallel to plasminogen/plasmin. The finding that host-derived factors retain their functional activities, when simultaneously bound to the surfaces of the pathogen underscores the high virulence potential of B. recurrentis and makes HcpA a promising target for therapeutic treatment of severe louse-borne relapsing fever [21,34,35,36,37,38].

Our data extend previous findings on a related CFH receptor expressed by tick-borne relapsing fever B. hermsii spirochetes, including the observation that surface bound CFH facilitates factor I-mediated cleavage of C3b and is critical for immune evasion of these and other pathogens [8,9]. In fact, CFH binding has also been reported for a number of human pathogens, such as S. pyogenes (group A streptococcus) [39], Neisseria gonorrhoeae [40], S. pneumoniae [41,42], Borrelia burgdorferi [24,43], Candida albicans [44] and other relapsing fever spirochetes [9,43,46]. Recombinant HcpA specifically binds CFH and/or CFHR-1 and in addition plasminogen, however via different binding domains. The CFH binding site of HcpA was determined by using mutants with either N-terminal (HcpA48-175, HcpA78-175) or C-terminal (HcpA18-145, HcpA18-115) truncations. All mutant of HcpA caused complete abrogation of CFH binding. These data suggest that the determinants required for CFH binding are defined by conformation rather than contiguous linear elements. This finding is reminiscent of the observed CFH binding capabilities for B. burgdorferi BbCRASP-1 and BbCRASP-3 [11,32,46,47]. In contrast, binding of PLG to HcpA was not affected by any of the indicated truncations of the HcpA protein, demonstrating that PLG and CFH interact with distinct HcpA domains and suggest that both host proteins can bind simultaneously to HcpA. This assumption is supported by our findings that PLG and CFH bind independently and coordinately to immobilized HcpA and do not

![Figure 5. Surface localization of HcpA.](image)

(A) Immunofluorescence analysis of B. recurrentis A1 after incubation with a mAb specific for HcpA (BR-1) (left panels) or a flagellin-specific mAb (LA21, right panels) followed by rabbit anti-mouse Cy3-conjugated IgG. Corresponding differential interference contrast images are shown in the lower panels. The images were obtained as described above. (B) Proteinase K and trypsin treatment affects surface expression of native HcpA. B. recurrentis bound to HcpA coated microtiter plates (A) or to the surface of analyzed by measuring factor I-mediated conversion of C3b to iC3b. doi:10.1371/journal.pone.0004858.g005

![Figure 6. Cofactor activity of CFH bound to either HcpA or intact B. recurrentis spirochetes.](image)

Functional activity of CFH was analyzed by measuring factor I-mediated conversion of C3b to iC3b. CFH bound to HcpA coated microtiter plates (A) or to the surface of intact B. recurrentis spirochetes (B) was incubated with C3b and factor I. Reaction mixtures were separated by SDS-PAGE and transferred to nitrocellulose membrane. C3b degradation products were evaluated by detection of α-chain cleavage fragments of 68, 46 and 43 kDa using biotinylated rabbit anti-C3c IgG followed by peroxidase-conjugated streptavidin. Purified iC3b was included as control. doi:10.1371/journal.pone.0004858.g006
compete with each other. Due to the efficient concurrent binding of CFH and PLG to immobilized HcpA it is to be expected that both host proteins similarly bind to HcpA expressed on the outer surface of *B. recurrentis*. Further studies are needed to elucidate the possibility that binding of CFH and PLG to the surface of *B. recurrentis* may be involved in certain pathologies of the central nervous system.

Plasma adsorption experiments and surface plasmon resonance analyses clearly demonstrate that binding of CFH to HcpA is exclusively associated with its C-terminal domain, SCR20. This finding is further substantiated by the fact that HcpA bound CFHR-1, another member of the factor H family that exhibits an almost identical (97%) C-terminal short consensus repeat domain [7]. Thus, the ability of the pathogen to coordinately bind CFH and/or CFHR-1 to HcpA most probably adds to the virulence of *B. recurrentis* by establishing its resistance to complement attack, even under conditions when the human complement regulators are differentially regulated.

HcpA coated microtiter plates were incubated with PLG, subsequently fibrinogen and uPA were added. The reaction mixtures were separated by SDS-PAGE, transferred to nitrocellulose and probed with rabbit anti-fibrinogen followed by peroxidase-conjugated IgG for detection of α, β and γ chains (67, 57 and 47 kDa) and the small-size degradation products of fibrinogen.

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Figure 7. Activation and proteolytic activity of HcpA- and *B. recurrentis*-bound plasminogen. Intact *B. recurrentis* organisms (A) or recombinant HcpA (B) were incubated with PLG. Bound PLG was converted into plasmin by uPA addition and plasmin activity was measured using the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (S-2251). uPA mediated PLG activation was inhibited by tranexamic acid (TA). Substrate cleaving was monitored by measurement of the absorbance at 405 nm for up to 6 hrs. Mean of triplicates ± SEM is shown. (C) Degradation of fibrinogen by HcpA-bound plasmin.

*Figure 8. Degradation of deposited C3b by acquiring plasmin on the surface of *B. recurrentis*. Spirochetes were immobilized onto microtiter plates and incubated with 10% NHS as source for C3b. Washed bacteria were treated with PLG in the presence or absence of tranexamic acid (TA) and bound PLG was activated by uPA. Deposited C3b was detected using biotinylated anti-C3c IgG followed by peroxidase-conjugated streptavidin. C3b deposition is expressed as the mean absorbance at 492 nm of triplicates. Error bars indicate ± SEM. *, P < 0.0001.

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Most recently, it was demonstrated that CFHR-1 is an inhibitor of the alternative complement pathway that binds to C3b, inhibits the C5 convertase activity and interferes with C5b surface deposition and membrane attack complex formation (Heinen et al., unpublished). In this context it is of interest that HcpA is also capable of binding another member of the factor H family, i.e. CFHR-2, whose function is yet to be disclosed [7]. The present data show that HcpA-bound CFH retains its regulatory capacity and controls both C3b deposition and C3-convertase activity, resulting in enhanced complement regulatory activity. This process is expected to increase resistance of louse-borne relapsing fever spirochetes to complement attack, an assumption supported by our previous findings with B. hemis. There is ample evidence suggesting that binding of PLG to bacterial surfaces, including spirochetes, is critical for their invasive potential and persistance [9,17,48]. B. recurrentis binds PLG and upon processing to enzymatically active plasmin by human uPA the surface bound protease is shown to degrade the physiological substrate fibrinogen. Together with the finding that PLG-coated B. recurrentis also bind to the PLG receptors on endothelium cells, our results suggest that spirochetes exploit their increased proteolytic capacity to breach tight junctions of endothelium, cross basement membranes, and to initiate patho-physiological processes in the affected organs [48]. The finding that in accordance to the related Lyme disease spirochetes, B. recurrentis bind PLG and can disseminate from the blood to many distant organs.
organs, including the brain, supports the assumption that similar mechanisms could be involved [47].

It was recently shown that *Staphylococcus aureus* employs host plasmin to degrade both, surface-bound IgG as well as C3b, and thereby confers resistance to innate and adaptive immune defense processes [49]. Corresponding finding that HcpA bound plasmin leads to a decrease of C3b molecules on the surface of *B. recurrentis* may represent a novel mechanism, by which spirochetes interfere with C3b deposition on their cell surface and moreover could escape phagocytosis. In line with this assumption, transformed *B. burgdorferi* B313/pBR cells exhibit a significant reduction of C3b molecules deposited on their surface as compared to the parental *B. burgdorferi* B313 strain. Depleted C3b at the bacterial surface correlates with lowering phagocytic activity of human neutrophils [49]. Modulation of opsonic molecules such as C3b seems an ideal strategy for bacterial survival and may account for the extraordinary virulence of *B. recurrentis*. Furthermore, it was recently demonstrated that *B. recurrentis* acquire C4b-binding protein, another regulator of the classical pathway of complement activation on their surface [21]. However, in preliminary experiments binding of HcpA to C4b-binding protein could not be observed. In contrast, a novel 45 kDa protein was shown to strongly interact with C4b-binding protein suggesting that in *B. recurrentis* at least two different receptors for binding of the two complement regulators, CFH and C4b-binding protein, are expressed (unpublished).

Although the biological significance of HcpA expression by *B. recurrentis* has still to be elucidated, the present finding that the *B. burgdorferi* B313 mutant, deficient in CFH receptors, resists complement-mediated killing and following transfection with hcpA strongly suggests the involvement of HcpA in immune evasion of *B. recurrentis*. Recently, ectopic expression of the *B. burgdorferi* BbCRASP-1 lipoprotein on the surface of a serum-sensitive Borrelia strain imparts resistance of the transformed isolate to human serum [12,30,50]. In the murine model of Lyme disease the precise role of another complement binding protein, BbCRASP-2, was explored and the results of this study suggested that BbCRASP-2 function is dispensable for infectivity [51]. However, studies of the biology of *B. recurrentis* and louse-borne relapsing fever have been hampered by the lack of an animal model. Further investigations are needed to fully examine the complex interplay between *B. recurrentis*, serum sensitivity, and the role of HcpA in the pathogenesis of the disease.

In summary, this is the first study showing the simultaneous and non-competitive binding of CFH and PLG to the outer surface protein HcpA of *B. recurrentis*, the agent of louse-borne relapsing fever. Our finding that HcpA is a multifunctional virulence factor with the potential to simultaneously mediate innate immune evasion and degradation of extracellular matrix components significantly adds to our understanding of the pathological processes underlying louse-borne relapsing fever. Moreover, the presented data support the concept of exploitation of host factors as suitable survival strategies.

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**Author Contributions**

Conceived and designed the experiments: SG RW. Performed the experiments: SG MS CB PFZ. Analyzed the data: SG CB PFZ RW. Contributed reagents/materials/analysis tools: SJC PK PFZ MMS. Wrote the paper: SG SJC MMS RW.

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