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Complement regulator-acquiring surface proteins of *Borrelia burgdorferi*: Structure, function and regulation of gene expression

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Abstract

Borrelia burgdorferi, the etiological agent of Lyme disease, exploits an array of strategies to establish infection and to overcome host innate and adaptive immune responses. One key borrelial immune escape mechanism involves the inactivation of host complement attack through acquisition of human immune regulators factor H (CFH), factor H-like protein 1 (FHL1), factor H-related protein 1 (CFHR1), CFHR2, and/or CFHR5. Binding of these host proteins is primarily mediated by bacterial surface-exposed proteins that have been collectively referred to as complement regulator-acquiring surface proteins, or CRASPs. Different strains of *B. burgdorferi* produce as many as 5 different CRASP molecules that comprise 3 distinct, genetically unrelated groups. Depending on bacterial genetic composition, different combinations of these proteins can be found on the borrelial outer surface. The 3 groups differ in their gene location, gene regulatory mechanisms, expression patterns during the tick-mammal infection cycle, protein sequence and structure as well as binding affinity for complement regulators and other serum proteins. These attributes influence the proteins' abilities to contribute to complement resistance of this emerging human pathogen. In this review, we focus on the current knowledge on structure, function, and gene regulation of these *B. burgdorferi* infection-associated proteins.

Keywords

Borrelia burgdorferi; Spirochetes; Immune evasion; Complement; Complement regulators

Introduction

Lyme borreliosis/Lyme disease is the most commonly reported vector-borne infectious disease in Eurasia and the United States. It is caused by species of the *Borrelia burgdorferi* sensu lato complex, which includes *B. burgdorferi* sensu stricto (s.s.) (hereafter referred to as *B. burgdorferi*), *B. garinii*, *B. afzelii*, *B. spielmanii*, and *B. bavariensis* (Margos et al., 2009; Richter et al., 2004; Stanek and Reiter, 2011; Steere, 1989; Wang et al., 1999). The ability of Lyme disease spirochetes to perpetuate their natural vertebrate-tick infectious cycle requires an array of strategies to survive in different host environments and necessitates mechanisms to evade innate and adaptive immune responses of their reservoir hosts. Most Lyme disease spirochetes associated with human infection, members of the

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species *B. burgdorferi*, *B. afzelii*, *B. spielmanii*, and *B. bavariensis*, are resistant to killing by human complement (Bhide et al., 2005; Brade et al., 1992; Breitner-Ruddock et al., 1997; Herzberger et al., 2007; Kraiczy et al., 2000; Patarakul et al., 1999; van Dam et al., 1997). This is accomplished, at least in part, by the bacteria camouflaging themselves with the host-derived complement regulators factor H (CFH), factor H-like protein 1 (FHL1), and the factor H-related proteins CFHR1, CFHR2, and CFHR5 (Alitalo et al., 2001; Hammerschmidt et al., 2012; Hellwage et al., 2001; Kraiczy et al., 2001a, 2001b; McDowell et al., 2003; Siegel et al., 2010). Binding of those host complement regulators is mediated by surface-exposed lipoproteins that were initially termed CRASPs (complement regulator-acquiring surface proteins) (Kraiczy et al., 2001a, 2001b, 2001c, 2003, 2004b; Wallich et al., 2005). While isolating and characterizing the 5 CRASPs of *B. burgdorferi* type strain B31, we identified BbCRASP-1 as being encoded by *cspA* (ORF BBA68), BbCRASP-2 as being encoded by *cspZ* (ORF BBH06), and BbCRASP-3, BbCRASP-4, and BbCRASP-5 as being identical to the previously named ErpP, ErpC, and ErpA proteins, respectively (Casjens et al., 2000; Hartmann et al., 2006; Kraiczy et al., 2003, 2004a, 2004b; Kraiczy et al., 2003; Stevenson et al., 1996).

Within the last decade, distinct CRASPs interacting with human CFH have been identified among additional borrelial species including *B. afzelii*, *B. spielmanii*, *B. garinii*, *B. bavariensis*, *B. lusitaniae*, *B. valaisiana*, *B. bissettii*, *B. andersonii*, *B. turdi*, *B. tanukii*, and *B. japonica* (Alitalo et al., 2001, 2005; Bhide et al., 2009; Dieterich et al., 2010; Herzberger et al., 2007; Kraiczy et al., 2001a; McDowell et al., 2003; Metts et al., 2003; Stevenson et al., 2002; van Burgel et al., 2010; Wallich et al., 2005). Some of these molecules can bind different animal CFH molecules, suggesting that these proteins share identical or similar mechanisms to interact with the key inhibitor of the alternative pathway of diverse hosts, *e.g.* mouse, rat, dog, sheep, cattle, horse, cat, pig, goat, and chicken (Alitalo et al., 2004; Bhide et al., 2009; Haupt et al., 2007; Hovis et al., 2006; Rogers and Marconi, 2007; Stevenson et al., 2002; van Burgel et al., 2010).

CFH and FHL1 are the key fluid-phase regulatory proteins of the alternative pathway of complement. Both glycoproteins control complement activation at the level of C3b by competing with factor B for binding to C3b, accelerating the decay of the C3 convertase (decay-accelerating activity), and acting as cofactors for factor I-mediated degradation of C3b (Zipfel and Skerka, 2009). The CFH protein family also consists of additional 6 factor H-related proteins (CFHR): CFHR1, CFHR2, CFHR3, CFHR4A, CFHR4B, and CFHR5. All share high degrees of similarity at their carboxy-termini with the carboxy-terminal short consensus repeats (SCRs) 18–20 of CFH (Józsi and Zipfel, 2008; Zipfel et al., 1999; Zipfel and Skerka, 2009). CFHR1 regulates complement at the level of C5 by inhibiting C5 convertase activity and assembly of the terminal membrane attack complex (Heinen et al., 2009). The biological function(s) of CFHR2 is (are) as yet unclear. Like CFH, CFHR5 displays cofactor activity for factor I-mediated inactivation of C3b, thereby inhibiting activity of the fluid-phase C3 convertase (McRae et al., 2001, 2005).

A variety of gene and protein names have been used for CRASP proteins in the literature, leading to some confusion about their identities and functions. One objective of this review is to clarify the nomenclature of these particular molecules.

Characteristics of CspA

The CspA protein (in the literature also referred to as BbCRASP-1, CRASP-1, BBA68, or class 2 CFH binding protein, FHBP) is a surface-exposed, 25.9-kDa lipoprotein. The *cspA* gene is located on the linear lp54 replicon of *B. burgdorferi* B31 (Table 1) (Kraiczy et al., 2004b). Sequence analysis of the B31 genome revealed that *cspA* is part of a large

paralogous gene family, PFam54. *B. burgdorferi* type strain B31 contains 11 apparently intact PFam54 genes, located on 4 different linear plasmids (Casjens et al., 2000, 2012; Wywial et al., 2009). The lp54 replicon of strain B31 contains a tandem array of 7 PFam54 genes (ORF numbers *bba64* to *bba70*), including *cspA*, plus 2 truncated PFam54 ORFs (*bba71* and *bba72*) (Casjens et al., 2000, 2012; Wywial et al., 2009). Other borrelial isolates can carry a distinct PFam54 gene array, as was demonstrated by comparative sequence analyses of 10 *B. burgdorferi* genomes originated from the US and Europe (Wywial et al., 2009).

CspA binds the human complement regulators CFH and FHL1 *via* SCR domains 5–7 (CFH and FHL1) and SCR domains 19 and 20 (CFH) (Kraiczy et al., 2001a, 2004b) (Fig. 1; Table 1). Intriguingly, no other tested PFam54 paralog of strain B31 can interact with complement regulators. This suggests that the paralogs possess other, unknown function(s) (Kraiczy et al., 2004b, 2006; McDowell et al., 2005). Furthermore, the high genetic variability and the lack of a clear synteny and orthology of the PFam54 genes among the species as well as between isolates of the same species suggest that the ability to interact with complement regulators may be an incidental rather than the primary function and therefore PFam54 paralogs possess other, as yet unknown function(s) (Wywial et al., 2009).

Crystals of CspA revealed that it consists of 5 crossing α-helices (A–E), connected by short 3_{10} turns and short loops to form a ‘helical-lollipop’ (Cordes et al., 2005). Based on computer models, it had been hypothesized that CspA (and other CFH-binding proteins) contain coiled-coil elements that form the CFH binding site (McDowell et al., 2005). However, CspA of *B. burgdorferi* does not contain any coiled-coil structures (Cordes et al., 2005), disproving the coiled-coil hypothesis and demonstrating that such structures are not necessary for CspA to bind CFH. The threedimensional structure of CspA also revealed that the protein is a homodimer. The 2-paired monomers form a large cleft at the dimer interface (Cordes et al., 2005). The architecture of the cleft is suitable for accommodation of a single short consensus repeat (SCR) domain of CFH suggesting that the dimer interface forms the CFH binding site (Cordes et al., 2006). In addition, the dimensions of the cleft would exclude antibodies, suggesting that internal residues are hidden in an immunologically privileged site. This could relieve pressure for variation in the ligand-binding domain. More recently, distinct amino acid residues have been identified that are involved in binding of CFH and FHL1 as well as for sustaining the architecture of the homodimer (Kraiczy et al., 2009). Based on studies with mutagenized proteins, the dimer interface appears to mediate binding of both complement regulators, while the C-terminal region contains a structurally sensitive interaction site. Although some amino acid substitutions introduced in helix E lead to loss of CFH and FHL1 binding, they also cause disruption of the CspA dimer, suggesting that this region is responsible for structure maintenance rather than being involved directly in binding with complement regulators. Differences in the amino acid sequences in the C terminus of PFam54 orthologs encoded on either lp54 (BBA64, BBA65), lp28-4 (BBI36, BBI38, BBI39), or lp38 (BBJ39.1 and BBJ41) may explain the inability of those proteins to bind CFH and FHL1.

Several studies have examined expression levels of the *cspA* gene during the spirochete’s natural mammal-tick infectious cycle. Transcripts from this gene are largely undetectable in spirochetes residing in the midguts of unfed ticks. However, almost all spirochetes transmitted by feeding ticks produce CspA, indicating rapid gene/protein induction during tick feeding (Bykowski et al., 2007; von Lackum et al., 2005). Two weeks after transmission to mammals, *cspA* transcripts become undetectable (Bykowski et al., 2007; Lederer et al., 2005; McDowell et al., 2006; Wallich et al., 2003). As a consequence of the short duration of *cspA* expression during mammalian infection, humans generally do not produce robust levels of antibodies against CspA (McDowell et al., 2006; Rossmann et al., 2006).

Noteworthy, detection of anti-CspA antibodies in serum samples obtained from Lyme disease patients has only been successful when non-denaturated CspA protein was utilized. In contrast, reactive antibodies of the same serum samples could not be detected when using conventional Western blotting, which includes denaturation of the protein by SDS-PAGE. Those data indicate that antibodies which develop against CspA during natural infection are largely restricted to natively folded structural determinants (Rossmann et al., 2007). *B. burgdorferi* again produce CspA during transmission from infected mammals to feeding, naïve ticks (Bykowski et al., 2007; Lederer et al., 2005; McDowell et al., 2006; Wallich et al., 2003). A recent report indicated that expression of *cspA* orthologous genes of *B. burgdorferi*, *B. afzelii*, *B. bavariensis*, and *B. garinii* may be induced during incubation of spirochetes with human or certain animal sera (*e.g.* dog, mouse, and guinea pig or hamster) (Kisova-Vargova et al., 2011) while in contrast, Tokarz et al. (2004) showed that incubation of spirochetes with blood did not change the expression of *cspA*.

Immune serum generated against CspA is able to prevent infection by needle inoculation of cultured bacteria, but does not efficiently protect infected mice from disseminated spirochetal infection (Wallich et al., 2003). These results are consistent with the known expression profile of CspA, being produced during the initial stage of mammalian infection, but not during later, disseminated stages (von Lackum et al., 2005). Tick challenge experiments with CspA-immunized mice need to be performed to answer the question of whether this particular protein can serve as an effective vaccine to protect humans and domestic animals against *B. burgdorferi* infection.

The ability of CspA to facilitate complement resistance was demonstrated by the generation of a CspA-deficient *B. burgdorferi* B31 strain (Brooks et al., 2005; Kenedy et al., 2009). While CspA-deficient spirochetes were highly susceptible to human complement, *B. garinii* producing surface-exposed CspA displayed a serum-resistant phenotype revealing that CspA alone is sufficient for mediating resistance to human serum.

Studies with *B. burgdorferi* mutant strains indicated that the *cspA* gene is transcribed using the housekeeping sigma factor, RpoD (σ^{70}), with neither of the alternative RNA polymerase sigma factors RpoS (σ^{S}) and RpoN (NtrA, σ^{54}) playing a significant role (Bykowski et al., 2007; McDowell et al., 2006). Transcription of *cspA* increased during the mammal-to-tick and tick-to-mammal transmission process, but not during established infection (Bykowski et al., 2007). Expression of *cspA* was downregulated shortly after transmission of the spirochetes to the mammalian host. Furthermore, wholegenome array analysis using ‘host-adapted’ or ‘blood-treated’ B31 spirochetes also revealed that *cspA* expression is downregulated in response to different environmental stimuli (Brooks et al., 2003; Tokarz et al., 2004). In contrast to *B. burgdorferi*, *in vitro* cultivated *B. afzelii* spirochetes subjected to environmental conditions simulating the natural mammalian-tick infection cycle produced lower levels of CspA at ambient temperature than at elevated temperatures (Kraiczy et al., 2001a).

Another intriguing feature of the PFam54/*cspA* paralogous genes comes from *in silico* RNA secondary structure modeling studies. The sequences immediately downstream of the stop codon carry highly conserved RNA stem-loop structures (Delihas, 2009a). Other borrelial RNA stem-loop motifs are associated with a potential transposon (Delihas, 2009b), suggesting that the PFam54-linked motifs may be involved with expansion of this gene family throughout the borrelial genome.

Characteristics of CspZ

CspZ (in the literature also referred to as BbCRASP-2, CRASP-2, or BBH06) is a surface-exposed 23.2-kDa lipoprotein. It can bind both CFH and FHL1 *via* SCR domain 7 (Table 1

and Fig. 1) (Hartmann et al., 2006; Siegel et al., 2008b). The *cspZ* gene is located on the linear lp28-3 replicon of *B. burgdorferi* B31 and on related lp28-3 plasmids of other borrelial isolates (Hartmann et al., 2006; Siegel et al., 2008b). In contrast to *cspA* and other members of the PFam54 gene family, there are no other genes within the *B. burgdorferi* B31 genome that are related to *cspZ* (Casjens et al., 2000, 2012; Fraser et al., 1997; Hartmann et al., 2006).

Analyses of a number of geographically dispersed borrelial isolates revealed that *cspZ* sequences are, overall, well conserved among species associated with Lyme disease, *e.g. B. burgdorferi*, *B. afzelii*, *B. garinii*, *B. spielmanii*, and *B. bavariensis* as well as *B. bissettii* (identity ≥97.2%) (Kraiczy et al., 2008; Rogers et al., 2009a; Rogers and Marconi, 2007; Schutzer et al., 2011, 2012). Despite these high degrees of conservation, there are some significant differences among CspZ proteins. There is a species-linked polymorphism in *B. afzelii*, *B. garinii*, and *B. spielmanii cspZ* genes, which encode an N-terminal domain of 44 amino acids that is absent from *B. burgdorferi* CspZ proteins. CspZ derived from *B. burgdorferi* bind both CFH and FHL1, while the CspZ orthologs of *B. garinii*, *B. afzelii*, and *B. spielmanii* do not bind these complement regulators (Rogers et al., 2009a; Rogers and Marconi, 2007). The N-terminal extension found in *B. garinii* CspZ proteins does not prevent binding of CFH or FHL1, indicating that other elements are involved in ligand binding. By investigating a number of additional *B. burgdorferi* isolates, Rogers et al. (2009a) showed that some natural CspZ orthologs probably lack CFH binding activity due to a 4-amino acid insertion at the N terminus of CspZ and/or point mutations at 3 distinct positions (amino acids 51, 150, and 193). Computer modeling of CspZ suggests that the 4-amino acid insertion is located in a loop between 2 α-helices and thus may destabilize the architecture of the protein and indirectly affect the interactions between CspZ and complement regulators (Kraiczy et al., 2009). In addition, through site-directed mutagenesis and by exogenous expression of mutant CspZ proteins in a borrelial strain that lacks all known CRASPs, a structurally sensitive domain has been identified within the C terminus of CspZ (amino acids 204–211), suggesting that this particular region forms a contiguous binding domain (Siegel et al., 2008b). Changes of amino acid residues within or outside the 4 proposed binding regions may have significant impacts on CFH and FHL1 binding. Those data support a hypothesis of multiple contact sites for the CspZ and its complement regulator ligands. Elucidation of the three-dimensional structure of CspZ will likely yield important clues in our understanding of the molecular interactions between this protein and the host complement regulators of the alternative pathway.

There appears to be a connection between ability/inability of a given strain's CspZ to bind CFH and that bacterium's ribosomal spacer sequence type (RST). The RST grouping is also linked to a strain's tendency toward hematogenous dissemination and invasiveness (Iyer et al., 2001; Rogers et al., 2009a; Wormser et al., 2008). This relationship is linked to 3 CspZ phylogenetic clusters (groups 1–3). Isolates belonging to the highly invasive RST1 and moderate invasive RST3 genogroups fall into groups 1 and 3, which are able to bind CFH. In contrast, group 2 CspZ proteins do not bind CFH and are found in bacteria of the minimally invasive RST2 genogroup. These correlations suggest that CspZ may help *B. burgdorferi* to disseminate in the mammalian host, as would be expected of a protein involved in the resistance to the host innate immune system.

The *cspZ* gene is not transcribed by borreliae within the midguts of unfed nymphal ticks (Bykowski et al., 2007; Coleman et al., 2008). During bacterial transmission from feeding ticks to mammalian hosts, only a low percentage of spirochetes produce CspZ. However, within 2 weeks post infection, *cspZ* transcript levels increase significantly, reaching levels that are considerably higher than those observed in cultured bacteria. Note that the expression profile of CspZ is opposite that of CspA, which is produced at high levels during

transmission, then quickly repressed as mammalian infection is established (Bykowski et al., 2007; von Lackum et al., 2005). Human patients with early or late manifestation of Lyme disease generally exhibit a strong antibody response to CspZ, also indicating substantial production of this protein during natural infection (Bykowski et al., 2007; Kraiczy et al., 2009; Rogers et al., 2009a). Moreover, the antibody titers tend to be higher in sera from patients with late-stage disease than with sera obtained from early Lyme disease patients. These observations indicate that spirochetes produce CspZ throughout disseminated and persistent infection of humans and experimental animals. However, for establishment of murine infection, CspZ appears to be not absolutely required (Coleman et al., 2008). Thus, it is not yet known how much CspZ contributes to the ability of Lyme spirochetes to infect their mammalian hosts.

CspZ can provide spirochetes with resistance to host complement. When a strain of *B. garinii* that normally lacks CRASPs was transformed such that it produced CspZ on its surface, the bacterium acquired ability to bind CFH and became resistant to complement-mediated killing (Siegel et al., 2008a). Contradictory data have been reported from a CspZ-deficient *B. burgdorferi* strain that resists complement-mediated killing by human serum (Coleman et al., 2008). However, that observation might be due to CspA compensating for the loss of CspZ in the studied *B. burgdorferi* strain. As noted in the preceding paragraph, wild-type *B. burgdorferi* produce substantially greater levels of *cspZ* transcripts during mammalian infection than they do when grown in culture medium (Bykowski et al., 2007). Moreover, CspZ protein is often undetectable in cultured spirochetes. This is a likely explanation for an observation that a *B. burgdorferi* strain specifically deleted of *cspA* (*cspZ*$^{+}$, *cspA*$^{-}$) was unable to bind CFH (Coleman et al., 2008). The low level of CspZ expression by cultured wild-type *B. burgdorferi* may also explain the inability of CspZ-directed antibodies to protect mice from challenge with cultured *B. burgdorferi* (Rogers et al., 2009a).

As are all the other borrelial CRASP-encoding genes, *cspZ* transcription is directed by the housekeeping sigma factor, RpoD (Bykowski et al., 2007, 2008). Mutant spirochetes, deficient of either the alternative sigma factors, RpoN and RpoS, express *cspZ* at levels comparable to wild-type *B. burgdorferi* (Bykowski et al., 2007, 2008). Expression of *cspZ* is influenced by Rrp1 (BB0419) and Rrp2 (BB0763), parts of 2 distinct two-component sensory transduction systems (Rogers et al., 2009b; Yang et al., 2003). Rrp1 produces cyclic di-GMP in response to unknown signals, which appears to influence expression levels of a wide variety of borrelial genes, many of which are involved in mammalian infection (Rogers et al., 2009b).

Immunofluorescence microscopy of intact spirochetes indicated surface exposure of CspZ (Hartmann et al., 2006; Siegel et al., 2008a). The majority of surface-exposed proteins of *B. burgdorferi* are susceptible to protease treatment *in situ*, in particular to proteolytic enzymes exhibiting broad substrate specificity. An uncommon feature of native CspZ is its insensitivity to proteolytic degradation by proteinase K and trypsin (Coleman et al., 2008; Hartmann et al., 2006). However, several other *Borrelia* spp. outer membrane proteins that have been confirmed to be surface-exposed, such as the *B. burgdorferi* OspA, Oms66, ErpB, ErpM, ErpK, ErpX, and *B. turicatae* Vsp proteins, are also innately resistant to protease degradation *in situ* (El-Hage et al., 2001; Exner et al., 2000; Zückert et al., 2001). CspZ and these other surface proteins may naturally fold such that protease target sequences are hidden, might be glycosylated or otherwise modified, or may interact with other surface components that shield them from proteases (Benz and Schmidt, 2002; El-Hage et al., 2001; Exner et al., 2000; Zückert et al., 2001). Alternatively, resistance to proteolytic degradation might also be due to CspZ not being exposed at the outer surface (Coleman et al., 2008).

Characteristics of ErpA, ErpC, and ErpP

B. burgdorferi type strain B31 encodes three 17- to 20-kDa surface-exposed lipoproteins that have high affinities for CFH and some CFHRs (Fig. 1) (Hammerschmidt et al., 2012; Haupt et al., 2007; Hellwage et al., 2001; Siegel et al., 2010). These are ErpA, ErpC, and ErpP (also collectively described as OspE, although each protein has a distinct sequence) (Table 1) (Casjens et al., 1997b, 2000; Stevenson et al., 1996). Several additional alternative names are found in publications: ErpA has also been described as BbCRASP-5, ErpI, ErpN, BBP38, and BBL39; ErpC has also been called BbCRASP-4; and ErpP has been called BbCRASP-3 or BBN38 (Hovis et al., 2006; Kraiczy et al., 2004a; McDowell et al., 2004; Metts et al., 2003). Type strain B31 carries 3 separate but identical *erpA* genes, one each on cp32-1, cp32-5, and cp23-8, and an *erp* gene redundancy that has also been found in some other *B. burgdorferi* isolates (Stevenson and Miller, 2003; Stevenson et al., 2000b). Of note, the *erpC* gene was not identified in the sequenced subculture of type strain B31 (B31-MI), due to its lack of cp32-2, although that plasmid is maintained by other subcultures of strain B31 (Casjens et al., 2000; Stevenson et al., 1996, 2006). Orthologs that have been characterized in other *B. burgdorferi* strains were given various names. Adding to confusion, some nomenclatures give the same name to distinct proteins/genes, such as the very different proteins of *B. burgdorferi* strains N40 and 297 that have both been named ‘P21’. As noted above, the approximately 20-kDa CRASPs have been collectively named OspE. However, as we describe below, the proteins in the OspE group do not all possess the same functions. In the Erp nomenclature, each distinct protein has a unique name, *e.g.* ErpA, ErpC, Erp63, Erp64, *etc.*

Almost all of the examined approximately 20-kDa Erp/CRASPs bind human CFH through that complement regulator’s SCR20 domain (Kraiczy et al., 2001a). They also bind CFHR-1, CFHR-2, and CFHR-5 through those proteins’ C-terminal SCR domains (which are >90% identical to the C-terminal SCR20 of CFH). None of these molecules, however, bind complement regulator FHL1. Despite their high degrees of sequence similarity, the strain B31 ErpA, ErpC, and ErpP proteins differ in their affinities for CFH, CFHR-1, CFHR-2, and CFHR-5 (Hammerschmidt et al., 2012; Haupt et al., 2007; Siegel et al., 2010). There are several lines of evidence, indicating that native ErpA, ErpC, and ErpP do not confer resistance of *B. burgdorferi* to complement-mediated lysis *in vitro* under conditions where high concentrations of human serum (up to 50%) were applied. A CspA-deficient B31 strain as well as B313, a derivative of B31, lacked CFH binding and were highly sensitive to human complement despite the production of ErpA and ErpN or ErpA, respectively (Brooks et al., 2005; Hartmann et al., 2006; Rossmann et al., 2007; Schott et al., 2010). Moreover, heterologous production of ErpA, ErpC, or ErpP in a strain of *B. garinii* that lacks naturally occurring CRASPs did not provide resistance to complement-mediating bacteriolysis (Hammerschmidt et al., 2012; Siegel et al., 2010), suggesting that these particular Erp proteins may act in concert with CspA and/or CspZ to help the bacteria evade complement-mediated killing, confirming earlier findings with CRASP-deficient spirochetes. However, simultaneous overproduction of ErpP and ErpA in the serum-sensitive CspA-deficient B31 strain imparts serum resistance, thus under the expression of an artificial promoter, Erp proteins are sufficient to restore serum resistance (Kenedy and Akins, 2011).

Type strain B31 encodes 13 unique Erp proteins, most of which have highly divergent sequences (Casjens et al., 2000; Stevenson et al., 1998a). Other members of the Erp protein family, such as ErpX or ErpY of type strain B31 and Erp proteins of *B. lusitaniae* can also bind CFH under certain conditions, although it is not clear whether those interactions are of biological significance (Dieterich et al., 2010; Stevenson et al., 2002).

Multi-label immunofluorescence analysis confirmed that a bacterium's entire repertoire of Erp proteins is simultaneously co-expressed (El-Hage et al., 2001; El-Hage and Stevenson, 2002). All *erp* genes are carried on circular replicons of the cp32 family and all examined *B. burgdorferi* isolates naturally carry multiple, distinct cp32 plasmids (Table 1) (Casjens et al., 2012; Schutzer et al., 2012; Stevenson and Miller, 2003; Stevenson et al., 2001). In addition, numerous related cp32 replicons have been identified in all Lyme disease-associated spirochetes, including *B. afzelii*, *B. garinii*, *B. bavariensis*, and *B. spielmanii* as well as *B. lusitaniae*, and other members of the genus *Borrelia* such as the relapsing fever spirochetes *B. hermsii* and *B. turicatae* (Brissette et al., 2008; Casjens et al., 2012; Schutzer et al., 2011, 2012; Stevenson et al., 2000a, 2006). Of note, cp32 plasmids of examined relapsing fever *Borrelia* species do not contain any *erp* loci (Stevenson et al., 2000b). Several lines of evidence indicate that cp32 replicons are genomes of lysogenic prophages, which could be responsible for the observed horizontal transmission of *erp* genes among *Borrelia* (Casjens et al., 1997a, 2000; Chenail et al., 2012; Eggers et al., 2001; Eggers and Samuels, 1999; Stevenson et al., 1998b; Stevenson and Miller, 2003; Zhang and Marconi, 2005).

All Erp family members contain a typical consensus signal peptidase II cleavage sequence at the N terminus (Stevenson et al., 2000b). A variety of techniques have confirmed that all Erp proteins are located in the borrelial outer membrane and are exposed to the external environment (El-Hage et al., 2001; Kraiczy et al., 2003; Lam et al., 1994; Siegel et al., 2010). An additional feature of Erp proteins is their high content of the charged amino acids lysine and glutamate. Lysine residues, in particular, are proposed to be involved in the interaction of Erp proteins with host CFH (Alitalo et al., 2004, 2005). Truncations of the N- and C terminus of ErpA and ErpP completely disrupt binding of CFH, supporting the hypothesis that discontinuous or conformational domains are required for binding of CFH (Kraiczy et al., 2003; Metts et al., 2003).

Three-dimensional structures have yet to be determined for any Erp protein, so it is not entirely clear whether hypothesized coiled-coil structures are present, and if those or other higher-ordered structures are required for proper ligand binding (Alitalo et al., 2002, 2004, 2005; Kraiczy et al., 2003; McDowell et al., 2004; Seling et al., 2010). The contribution of individual amino acid residues in binding of CFH has been clearly demonstrated for the Erp63 protein of *B. spielmanii* strain TIsar3 (Seling et al., 2010). Exchange of a single amino acid residue completely abolished ability to bind CFH, yet did not alter the ability of Erp63 to bind plasminogen, suggesting that the entire architecture of this particular molecule was not impaired and that this particular amino acid residue directly interacts with CFH.

Unexpectedly, spirochetes that lack *cspA* and *cspZ*, but contain *erpA*, *erpC*, or *erpP*, do not bind CFH to their surface, although the complement regulators CFHR-1, CFHR-2, and CFHR-5 were bound (Fig. 1) (Hammerschmidt et al., 2012; Siegel et al., 2010). Those results indicate that ErpA, ErpC, and ErpP *in situ* possess different binding properties than do the isolated recombinant proteins. One possible explanation is that other membrane proteins proximate to these molecules impair interactions between Erps and the large CFH protein, while permitting binding of the smaller CFHR-1, CFHR-2, and CFHR-5 molecules. These findings warrant continuative investigations of Erp protein architecture and protein-protein interactions to define the functions of these ubiquitous, infection-associated surface proteins.

Erp proteins are produced during mammalian infection, but are largely repressed during tick colonization (Akins et al., 1995; Hefty et al., 2001; McDowell et al., 2001a, 2001b; Miller and Stevenson, 2006; Miller et al., 2003; Stevenson et al., 1995, 1998a; Wallich et al., 1995, 2005). The spirochetes continually synthesize Erp proteins throughout mammalian infection, although levels of expression vary over time (Miller and Stevenson, 2006; Miller et al.,

2003). Analysis of the mechanisms governing *erp* gene expression revealed that all *erp* operons are controlled by the housekeeping sigma factor RpoD, although some are also transcribed to varying extents by the alternative sigma factor RpoS (Caimano et al., 2004; Eggers et al., 2004, 2006). Regulation of *erp* transcription depends on a DNA region adjacent to the promoter the *erp* operator, to which 3 cytoplasmic proteins bind. BpaB binds to the *erp* operator and represses *erp* transcription. EbfC competes with BpaB and functions as an anti-repressor. BpuR binding enhances the repressive effects of BpaB (Babb et al., 2004, 2006; Burns et al., 2010; Jutras et al., 2012a, 2012b; Riley et al., 2009). The alarmones autoinducer- 2 (AI-2) and cyclic di-GMP also influence *erp* transcription levels (Rogers et al., 2009b; Stevenson and Babb, 2002; von Lackum et al., 2006). Noteworthy, some inconsistencies in regulation of *erp* genes have been reported for some *B. burgdorferi* strains, possibly due to genetic differences that result in different levels of regulatory factors or affinities of DNA binding protein alleles (Das et al., 1997; Miller et al., 2005). Nucleotide differences found in *erp* promoter and operator regions may also contribute to strain-to-strain variations in *erp* expression levels.

During mammalian infection, the entire repertoire of Erp proteins produced by the spirochetes are exposed to the external environment, and host adaptive immune systems produce robust antibody responses against these lipoproteins (Akins et al., 1995; Lam et al., 1994; Miller et al., 2003; Nguyen et al., 1994; Stevenson et al., 1998a; Wallich et al., 1995). Due to the large strain-tostrain variability of Erp protein sequences, the suitability of these molecules as vaccine components or for serodiagnostic tests is very restricted (Miller et al., 2000; Stevenson et al., 1998a). Antibodies directed against the identical Erp proteins produced by the challenge strain of bacteria are unable to effectively protect mice from infection (Hefty et al., 2002; Nguyen et al., 1994). It remains to be determined how an extracellular pathogen such as *B. burgdorferi* is able to produce such highly antigenic proteins on its outer surface during long-term infection of immunocompetent animals.

Conclusions

Independent of the geographical origin, all examined natural *B. burgdorferi* isolates contain *cspA* and numerous *erp* genes. In addition, a large number of investigated Lyme disease borreliae also harbor a *cspZ* gene. The ubiquity of these genes points toward an important role(s) for their encoded proteins in the Lyme disease spirochete's natural infectious cycle. The CRASPs' strong affinities and binding capacities for host complement regulatory proteins strongly supports their hypothesized roles in immune evasion in diverse mammalian hosts. Although the three-dimensional structure of CspA has been determined, and mutagenesis approaches with CspA, CspZ, and ErpA/ErpP have identified probable structural determinants involved in interactions with complement regulators, much remains to be learned about this particular group of molecules. Continued studies of borrelial CRASPs will further clarify the role(s) of these proteins in infection and in the pathogenesis of Lyme disease/Lyme borreliosis.

As with *B. burgdorferi*, sequence heterogeneity of CRASP-encoding genes is also found in serum-resistant *B. afzelii* and *B. spielmanii*. All examined isolates of those species contain a *cspA* gene located on an lp54 replicon and numerous *erp* genes (Herzberger et al., 2007, 2009; Kraiczy et al., 2001a; Wallich et al., 2005). Although *cspA* orthologous genes are presented in the genomes of serum-sensitive *B. valaisiana* and *B. lusitaniae* (Schutzer et al., 2012), tested orthologs do not bind CFH or FHL1 (Dieterich et al., 2010, and P. Kraiczy unpublished data). In contrast to CspA, several Erp homologs of *B. valaisiana* and *B. lusitaniae* do bind CFH and CFHR1 (Dieterich et al., 2010). Of note, no CRASP-encoding sequences have been identified in genomes of bacteria outside of the genus *Borrelia*. Moreover, many important human pathogens, *e.g. Staphylococcus aureus*, *Streptococcus*

pyogenes, *S. pneumoniae*, *Neisseria meningitidis*, *N. gonorrhoeae*, and *Haemophilus influenzae*, bind host complement regulators through highly diverse bacterial proteins (for review see Lambris et al., 2008). Investigations of Lyme disease spirochetes provide not only insight into the infectious mechanisms of these spirochetes, but also provide helpful comparisons and contrasts with many other infectious agents.

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Abbreviations

CFH	factor H
FHL1	factor H-like protein 1
CFHR	human factor H-related proteins
CRASP	complement regulator-acquiring surface protein
PF	paralogous family
CspA	complement regulator-acquiring surface protein 1
CspZ	complement regulator-acquiring surface protein 2
Erp	OspE/F-related surface protein

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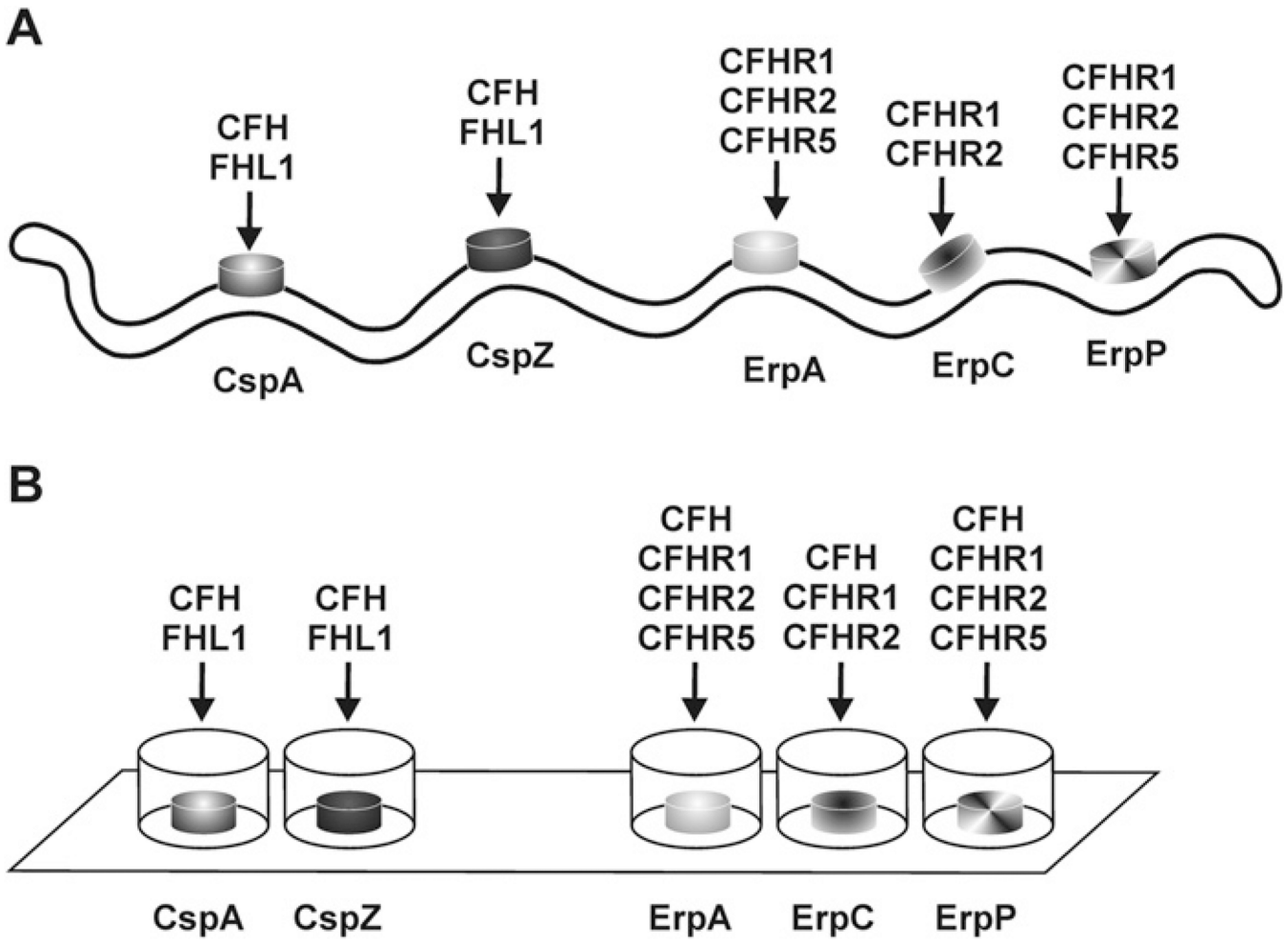

Fig. 1.

Binding capabilities of CRASP proteins of *B. burgdorferi* to distinct complement regulators. Schematic representation of the binding properties of native CRASP proteins (CspA, CspZ, ErpA, ErpC, and ErpP) exposed to the spirochetal outer surface (A) as well as of recombinant proteins immobilized on a solid, abiotic surface (nitrocellulose membrane or microtiter plate) (B).

Table 1

Characteristics of CRASP proteins

	CspA	**CspZ**	**ErpA**	**ErpC**	**ErpP**
Synonyms and other designations	CRASP-1 BbCRASP-1 BBA68 ZS7.A68 FHBP	CRASP-2 BbCRASP-2 BBH06	CRASP-5 BbCRASP-5 ErpI ErpN BBP38 BBL39 OspE	CRASP-4 BbCRASP-4	CRASP-3 BbCRASP-3 BBN38
Gene name	*cspA*	*cspZ*	*erpA*	*erpC*	*erpP*
Gene location in *B. burgdorferi* strain B31	lp54	lp28-3	cp32-1 cp32-5 cp32-8	cp32-2	cp32-9
Gene family	PFam54	None	PFam162	PFam162	PFam162
Gene expression in unfed tick	No	No	No	No	No
Gene expression in feeding tick	Yes	No	Yes	Yes	Yes
Gene expression in skin at tick feeding site	Yes (high expression)	Yes (low expression)	Yes	Yes	Yes
Gene expression in mice/disseminated infection	No	Yes (high expression)	Yes	Yes	Yes
Susceptibility to proteolytic degradation	Yes	No	Yes	Yes	Yes
Confers serum resistance	Yes	Yes	No	No	No
Interaction of complement regulators with native CRASP	CFH FHL1	CFH FHL1	CFHR1 CFHR2 CFHR5	CFHR1 CFHR2	CFHR1 CFHR2 CFHR5
Interaction of complement regulators with denaturated CRASP	CFH FHL1	CFH FHL1	CFH CFHR1 CFHR2 CFHR5	CFH CFHR1 CFHR2	CFH CFHR1 CFHR2 CFHR5
Interaction with plasminogen	Yes	Yes	Yes	Yes	Yes