Complement Inhibitor Factor H Binding to Lyme Disease Spirochetes Is Mediated by Inducible Expression of Multiple Plasmid-Encoded Outer Surface Protein E Paralogs[1]

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***Borrelia burgdorferi* spirochetes can circumvent the vertebrate host's immune system for long periods of time. *B. burgdorferi* sensu stricto and *B. afzelii*, but not *B. garinii*, bind the complement inhibitor factor H to protect themselves against complement-mediated opsonophagocytosis and killing. We found that factor H binding and complement resistance are due to inducible expression of a wide repertoire of outer surface protein E (OspE) lipoproteins variably called OspE, p21, ErpA, and ErpP. Individual *Borrelia* strains carry multiple plasmid-encoded OspE paralogs. Together the OspE homologs were found to constitute an array of proteins that bind factor H via multiple C-terminal domains that are exposed outwards from the *Borrelial* surface. Charged residue substitutions in the key binding regions account for variations between OspE family members in the optimal binding pH, temperature, and ionic strength. This may help the spirochetes to adapt into various host environments. Our finding that multiple plasmid-encoded OspE proteins act as virulence factors of *Borrelia* can provide new tools for the prevention and treatment of borreliosis. *The Journal of Immunology*, 2002, 169: 3847–3853.**

Lyme disease is a tick-transmitted zoonosis caused by the *Borrelia burgdorferi* spirochetes (1, 2). In man, *B. burgdorferi* initially causes an erythema migrans-type lesion at the site of the tick bite. Following its prolonged survival in the skin, *B. burgdorferi* can disseminate and cause late stage manifestations, whose nature to some extent depends on the infecting genospecies. *B. burgdorferi* sensu stricto often causes arthritis, whereas *B. afzelii* is most often associated with cutaneous sequelae, and *B. garinii* with neurological manifestations or complications (3).

Like all invasive pathogens *B. burgdorferi* must have means to evade the complement system (4), an important component of both the innate and adaptive immune systems. Strikingly, *Borrelia* survives in its mammalian hosts despite vigorous Ab responses. Strains of *B. burgdorferi* belonging to either the *B. burgdorferi* sensu stricto or the *B. afzelii* genospecies are resistant to human serum, whereas strains of the *B. garinii* subtype are serum sensitive (5). The serum-resistant strains are able to promote factor I-mediated cleavage of the key complement component C3b by binding the regulators factor H and factor H-like protein 1 from serum onto the spirochetal surface (6). Among the pathogenic streptococci, binding of factor H to the bacteria has been shown to be mediated via the surface-expressed M proteins in *Streptococcus pyogenes* (7) and by the PspC family member Hic protein in type 3 pneumococci (8, 9). Recent studies suggest that *B. burgdorferi* could also use specific surface molecules to evade complement attack (6, 10).

The genome of *Borrelia* exhibits several interesting features. Compared with other pathogenic microorganisms, e.g., to pneumococcus with 2236 predicted coding regions (11), *B. burgdorferi* has only 851 genes encoded on its linear chromosome, while many other genes reside in numerous linear and circular extrachromosomal plasmid elements (12–14). The various plasmids appear to be essential for *B. burgdorferi* virulence expression and Lyme disease pathogenesis. Spontaneous plasmid loss during in vitro cultivation of *B. burgdorferi* can result in attenuated and/or avirulent isolates (15). The plasmids encode many proteins, which are differentially expressed by *B. burgdorferi* as it is transmitted between its arthropod vector and mammalian hosts. The differentially expressed proteins include several homologs of outer surface protein E (OspE),[3] OspF, and Elp lipoprotein families (16, 17). As the functions of these plasmid-encoded, surface-expressed lipoproteins have been unknown, we studied whether they bind complement factor H and thus act as immune evasion molecules of *B. burgdorferi*. Binding of factor H would be critical for the survival of *Borrelia* spirochetes when they infect mammalian hosts and spread into their tissues. We show that factor H binds to a spectrum of the OspE family of proteins whose expression is induced as the spirochetes are transmitted from ticks to mammals.

Materials and Methods

Expression of recombinant Borrelia *proteins*

Outer surface lipoproteins from *B. burgdorferi* sensu stricto 297 and B31 strains were expressed in *Escherichia coli* as GST or polyhistidine-tagged

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[3] Abbreviations used in this paper: OspE, outer surface protein E; VBS, veronal-buffered saline.

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fusion proteins and purified. GST fusion constructs for OspE, p21, OspF, BbK2.10, BbK2.11, ElpA1, ElpA2, ElpB1, ElpB2, OspA, and OspC were generated and purified to homogeneity as previously described (16–18). The GST moiety was removed from all fusion proteins with thrombin before use in the binding assays. Polyhistidine-tagged ErpA, ErpP, ErpL, and ErpX proteins were generated by cloning PCR-amplified products representing the mature portion of each protein into the pBAD-TOPO vector (Invitrogen, Carlsbad, CA) using the respective primers: erpA, 5′-TG CAAAGCAATGGAGAGGTAAAGGTC; erpA, 3′-TTTTAAATTTCTTT TAAGCTCTTC; erpP, 5′-TGCAAAATTCATACTTCATATGATGAG; erpP, 3′-TTTTAAATTTTTTTTAAGCACTTCTAGTGG; erpL, 5′-TG CAAGAATTATGCAAGTGGTG; erpL, 3′-TTCTTTTTTATCTTCTTC TATTCCC; erpX, 5′-TGCAAGATTGATGCAACTGGTAAAGATGC; and erpX, 3′-CTGACTGTAACTGATGTATCCTTTAATTTC. All resulting constructs were sequenced to confirm that the inserted DNA was in-frame and that no errors were incorporated during PCR amplification. Fusion constructs were grown in tryptone-yeast broth supplemented with 100 μg ampicillin to an OD_{600} of 0.7 before arabinose was added to a final concentration of 0.2% to induce expression of the fusion proteins. Polyhistidine-tagged proteins were affinity-purified using nickel chromatography as instructed by the manufacturers (Amersham Pharmacia Biotech, Piscataway, NJ; and Invitrogen, Carlsbad, CA).

Surface plasmon resonance

Surface plasmon resonance binding experiments were conducted using the Biacore 2000 biosensor instrument (Biacore, Uppsala, Sweden). The proteins were amine-coupled to carbonyl groups of CM5 sensor chips according to the manufacturer's instructions. The buffer was chosen according to the pI of the analyte: maleate and sodium acetate buffers were used for proteins whose calculated pI values were over or below 6, respectively. The coupling levels of proteins varied between 500-3000 resonance units. The binding of complement factor H (Calbiochem, San Diego, CA; purity >95% by SDS-PAGE) to immobilized proteins was assayed at 22 and 37°C or only at 25°C. Recombinant OspE, OspF, and Elp family proteins (Fig. 1*a*) were coupled to a CM5 chip and the binding of fluid phase factor H was analyzed. Factor H at different concentrations in 50 mM veronal-buffered saline (VBS) was used as the ligand. The pH dependence of binding was examined with a set of adjusted pH values and a series of different factor H concentrations in 150 mM PBS. The binding kinetics were fitted to the simultaneous K_a/K_d determination feature of the Biaevaluation 3.1 software package and a natural logarithmic Langmuir binding (1:1) and mass transfer model. The sequences of the proteins used in the Biacore assays and peptide mapping were retrieved from the National Center for Biotechnology Information sequence database and aligned using the ClustalX 1.8 software package (19) (http://www-igmbc.u-strasbg.fr/BioInfo/clustalx, ftp://ftp-igmbc.u-strasbg.fr). A phylogenetic analysis was performed based on sequence comparisons of the proteins; ClustalX was used to generate an N-J-tree (Fig. 4*a*), which was plotted using the Phylip software package (http://evolution.genetics.washington.edu/phylip.html).

Indirect immunofluorescence analysis

For immunofluorescence experiments with *B. burgdorferi*, midguts were dissected out of *B. burgdorferi* strain 297-infected ticks (nymphal stage) before feeding (n = 12) or after feeding to repletion (n = 10). Extracted midguts were diluted in 20 μl PBS (pH 7.4) before being disrupted by repeated pipetting. Ten microliters of each sample (midguts from unfed or fed ticks) were spotted onto fluoro slides (Erie Scientific, Portsmouth, NH), and indirect immunofluorescence assays were performed as described previously (17). For these experiments, rat polyclonal antiserum directed against OspA, OspC, OspE, ElpB1, or OspF was used as the primary Ab at 1/25 dilutions. Alexa 488-labeled goat anti-rat IgG (H and L chain specific) was diluted in PBS/0.2% BSA at 1/1000 and used as the secondary Abs. Slides were washed and fixed in acetone before fluorescent imaging was performed. For each protein analyzed, multiple fields were viewed before images of representative fields were captured using a Spot digital camera and associated software (Diagnostic Instruments, Sterling Heights, MI).

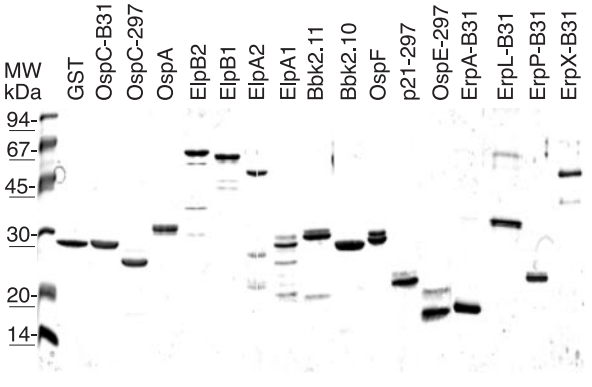

FIGURE 1. SDS-PAGE gel analysis of recombinant outer surface lipoproteins of *B. burgdorferi* used for surface plasmon resonance analyses. The proteins were expressed as GST- or polyhistidine-tagged fusion proteins in *E. coli*. After purification and removal of the tags, the proteins were run in SDS-PAGE and stained with Coomassie blue.

Peptide scanning

The amino acid sequences chosen for peptide scanning analysis from the National Center for Biotechnology Information protein sequence databank (http://www.ncbi.nlm.nih.gov) were OspE-N40 (AAA22959), OspE-297 (AAC34953), p21–297 (AAC34957), ErpA-B31-cp1 (AAF07400), ErpP-B31-cp9 (AAF07678), OspE-IP90 (AAC62927), and p21-DK1 (CAA69689). p21-DK1 is an OspF family member from *B. afzelii* DK1. The combined phylogenetic and peptide mapping data would support that the name of this protein be changed so as not to reflect an association with OspE orthologs. OspE-IP90 is a C-terminally truncated sequence from the *B. garinii* IP90 strain that does not bind factor H. All sequences were fragmented into 15-aa peptides with a 3-aa transition and a 12-aa overlap. The peptides were synthesized as spots onto polyethylene glycol-derivatized cellulose membranes (AIMS Scientific Products, Braunschweig, Germany; http://www.aims-scientific-products.de) using the peptide scanning instrument AutoSpot Robot ASP222 (Abimed Analysen-Technik, Langenfeld, Germany). Subsequently, a protein overlay assay with radiolabeled factor H (6, 20) was conducted. The membranes were washed and exposed on a phosphorimager plate. Amino acid sequences of the tested proteins were aligned using the Megalign and ClustalX programs.

Results

The first aim of this study was to search for *Borrelia* proteins that mediate complement resistance. As the lead molecule we used OspE from the *B. burgdorferi* N40 strain, which we previously found to bind factor H (21). Candidate OspE-related, OspF-related, and Elp lipoproteins encoded by respective genes were selected from various *Borrelia* strains, expressed in recombinant form, and examined for their affinities for factor H using the surface plasmon resonance technique. The 14 selected proteins from the three different families and from the *B. burgdorferi* sensu stricto 297, N40, and B31 strains were OspE-297, OspE-N40, p21–297, ErpA-B31, ErpP-B31, OspF-297, Bbk2.10–297, Bbk2.11–297, ErpL-B31, ElpA1–297, ElpA2–297, ElpB1–297, ElpB2–297, and ErpX-B31 (Fig. 1) (18). Factor H bound to OspE-N40, OspE-297, p21–297, ErpA-B31, and ErpP-B31, which all belong to the same protein family (Figs. 2-4). The binding interactions were dependent on pH and ionic strength, indicating their ionic nature.

The binding affinities of the proteins for factor H were determined in biosensor analyses by simultaneous K_a/K_d simulated kinetics. The K_d values for OspE-297 and p21–297 were 8.2 and 8.4 nM, respectively, at 25°C in 50 mM VBS, pH 7.4 (Fig. 2, *c* and *d*). At physiological ionic strength (150 mM VBS, pH 7.4, 22°C) the K_d values for ErpA-B31 and ErpP-B31 were 102 and 129 nM, respectively (Fig. 3, *c* and *d*). At 37°C similar K_d values resulted in higher on rates ($\sim 10^5$ $M^{-1}s^{-1}$) and off rates ($\sim 10^{-2}s^{-1}$) for both ErpA and ErpP. The concentration of factor H in human serum is 3.3 μM or higher. This suggests that in blood the OspE proteins on *Borrelia* readily become saturated by factor H molecules. The various OspE paralogs have 80% sequence homology with each other and form a distinct protein family (Fig. 4). While OspE-297 and p21–297 bound factor H optimally at acidic pH (not shown) and ErpP-B31 bound at neutral pH, ErpA-B31 binding covered the widest pH range (Fig. 3) that overlaps the stability range of factor H at pH 5–9.

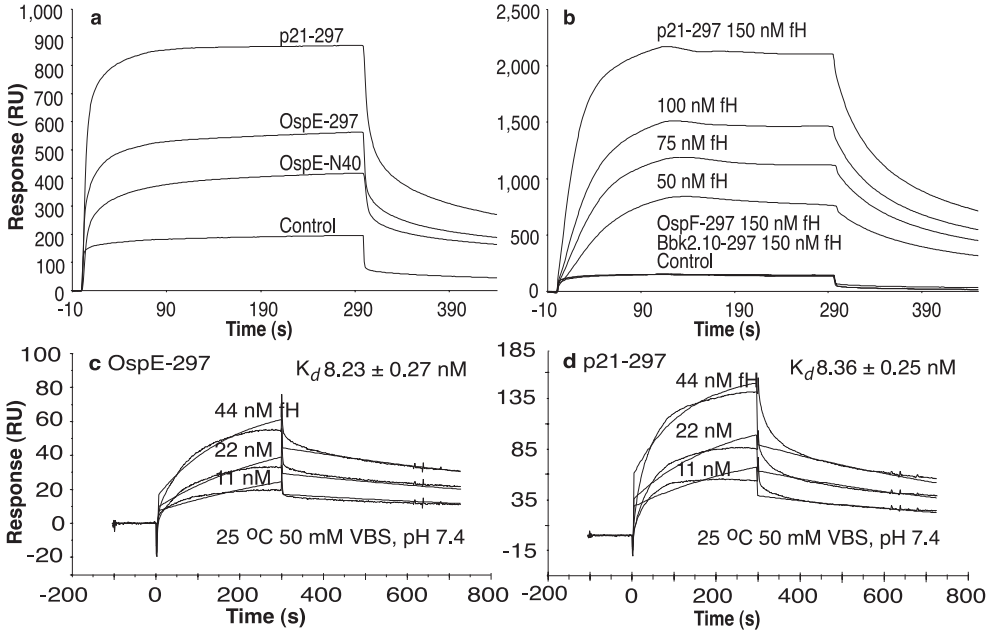

FIGURE 2. Surface plasmon resonance analysis of factor H binding to *Borrelial* OspE and p21 proteins. OspE-297 and p21–297 bound factor H with high affinity in 50 mM VBS 333 nM factor H at 25°C (*a*) and at 37°C (*b*). An empty flowcell was used as the negative control. The binding affinities were tested using a dilution series of factor H in 50 mM VBS, pH 7.4, at 25°C. They were 8.23 nM (*c*) and 8.36 nM (*d*) for OspE-297 and p21–297, respectively, which suggests that at the serum concentration of factor H (3300 nM) practically all OspE proteins on the surface of *Borrelia* bacteria bind a factor H molecule. The fitted kinetics of the interaction are indicated by a smoothed line. RU, resonance units.

In contrast to the various OspE homologs, no significant binding was seen to any of the five OspF or four Elp orthologs. A weak binding of ErpX-B31 to factor H was seen at pH 5.5, but not at neutral pH (not shown). Except for this weak binding, no proteins shown in Fig. 1 other than the OspE paralogs bound factor H (not shown). In addition, our prior studies have excluded factor H binding to numerous other surface molecules of *B. burgdorferi*, including OspA-297, OspC-Bbia, OspD-N40, P35/Bbk32-Bbia, and

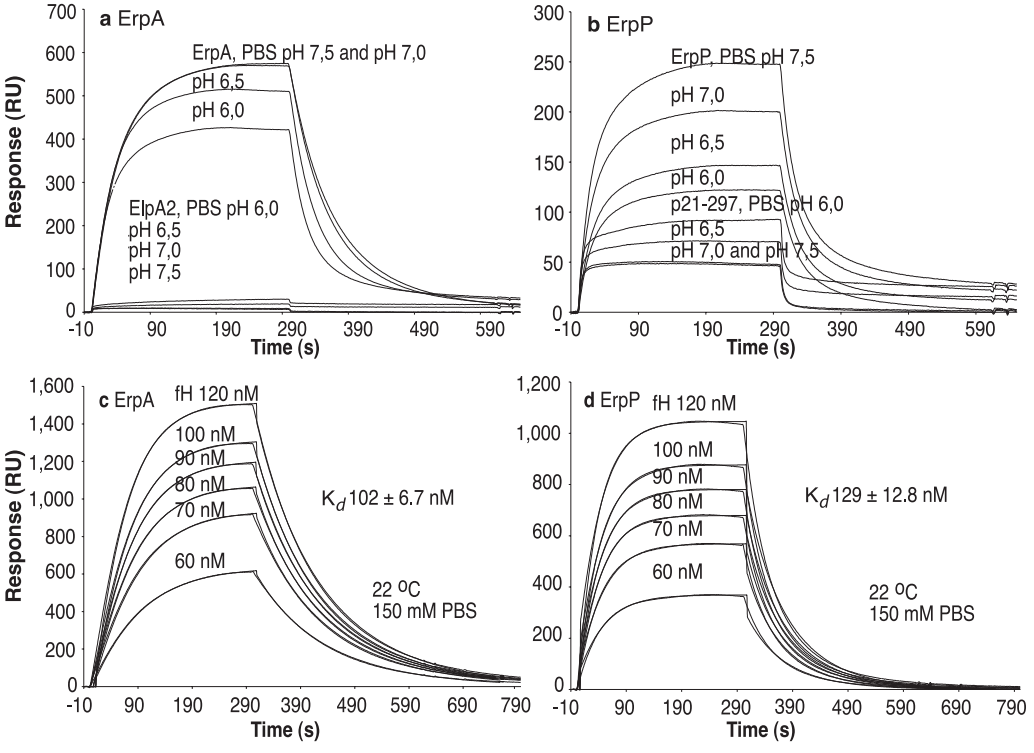

FIGURE 3. Surface plasmon resonance analysis of factor H binding to *Borrelial* ErpA and ErpP proteins. The OspE orthologs ErpA-B31 and ErpP-B31 from the B31 strain showed the strongest binding (with K_d values of 102 and 129 nM, respectively) of factor H in 150 mM PBS at various pH conditions. An empty inactivated flowcell was used as the negative control. Binding was seen under varying ionic and temperature conditions, suggesting that these proteins enable immune evasion in various host environments. The fitted kinetics are indicated by a smoothed line. RU, resonance units.

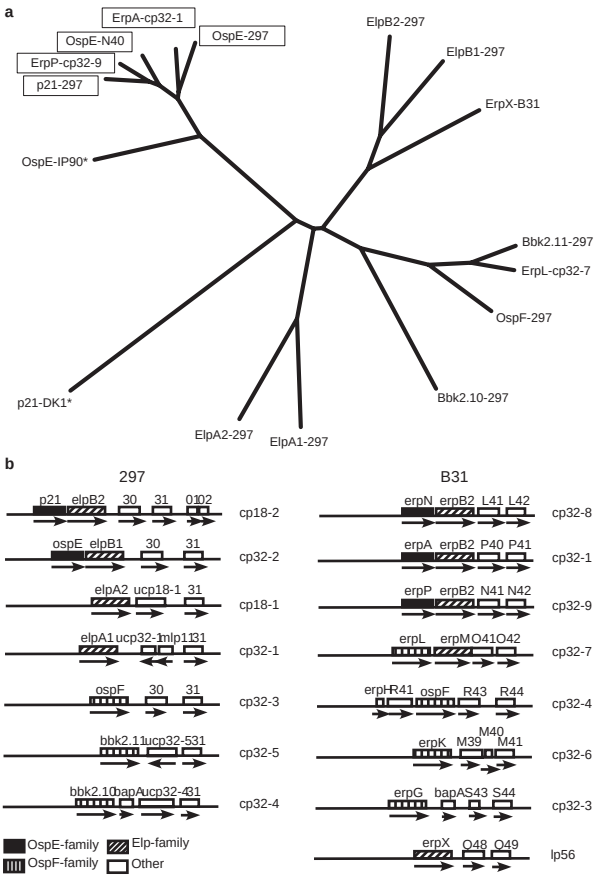

FIGURE 4. The phylogenetic relationship of plasmid-encoded surface proteins that bind or do not bind factor H. *a*, Based on their sequence differences, the proteins fall into three evolutionarily unrelated families (18) with significant homologies to OspE, OspF, or Elps. Proteins that bind factor H (boxed) all belong to the OspE family of surface lipoproteins. OspF or Elp family members did not exhibit appreciable binding to human factor H. Proteins analyzed by peptide mapping only (see Fig. 6) are marked with an asterisk. *b*, Regions in the *B. burgdorferi* 297 and B31 plasmids containing gene loci for members of the OspE, OspF, and Elp protein families (32).

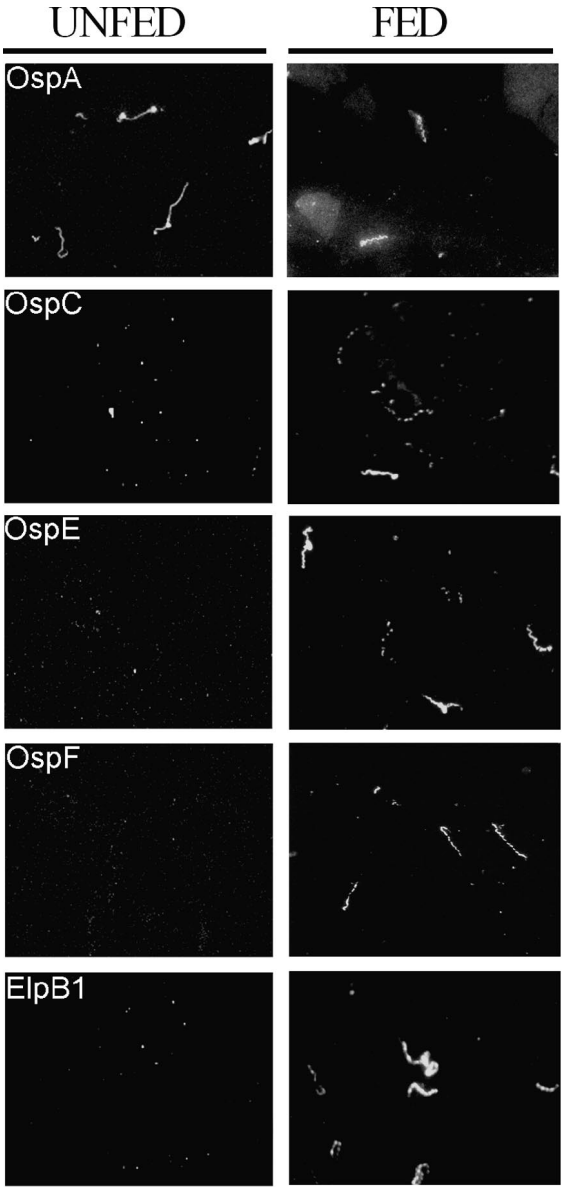

FIGURE 5. Expression of the OspE-related, OspF-related, and Elp lipoproteins in midguts from infected ticks before and after feeding. Midguts were carefully dissected out of infected ticks before or after a blood meal and probed with anti-OspA, -OspC, -OspE, -OspF, or -ElpB1 Abs. The *left* and *right panels* show representative midgut smears from ticks before feeding (unfed) and after feeding to repletion (fed). Magnification, ×1000 for all panels.

DbpA-N40 proteins from the respective *B. burgdorferi* sensu stricto strains (21).

Because the plasmid-encoded outer surface proteins of *Borrelia* have been suggested to be differentially expressed in the arthropod vectors and mammalian hosts, we next analyzed the lipoprotein expression patterns in *B. burgdorferi*-infected *Ixodes scapularis* ticks. We performed indirect immunofluorescence analysis on spirochetes within tick midguts before and after feeding to repletion. As shown in Fig. 5, no spirochetes were identified that expressed OspE, OspF, and ElpB1 in the midguts of unfed ticks. However, within fed ticks there was a pronounced induction of expression for OspE, OspF, and ElpB1. As a control for these experiments, the expression of OspA and OspC in unfed and fed tick midguts also was analyzed. As expected, there was a marked reduction in OspA and a concomitant increase in OspC expression during tick feeding (22).

While the expression of multiple OspE paralogs could guarantee the survival of *Borrelia* in various host animals (23), differences in complement sensitivity between *B. burgdorferi* strains may determine the spectrum of host specificities and even affect the ecology of *Borrelia* infections in nature, as has been elegantly shown by Kurtenbach et al. (24). Variations in the sequences of OspE paralogs could explain the finding that factor H can bind to the surface of *Borrelia* via at least two different sites, one on short consensus repeat (3) domains 5–7, also present in the short alternatively spliced variant factor H-like protein 1 of factor H, and another on the C-terminal short consensus repeat domains 19–20 (21, 25). These sites could be differentially used by OspE paralogs in the different genospecies and strains of *Borrelia*. To examine this possibility, we analyzed the putative factor H binding sites on the OspE proteins by peptide mapping (Fig. 6*a*). Five candidate regions (I–V) were detected. When highlighted in a sequence alignment (Fig. 6*b*), it can be seen that the binding sites are almost identical in the different OspE homologs but are absent from the OspF and Elp family members. No homologous sequences were found in the OspA and OspC proteins either. Sequence comparisons showed that the *B. burgdorferi* sequences for p21, OspE,

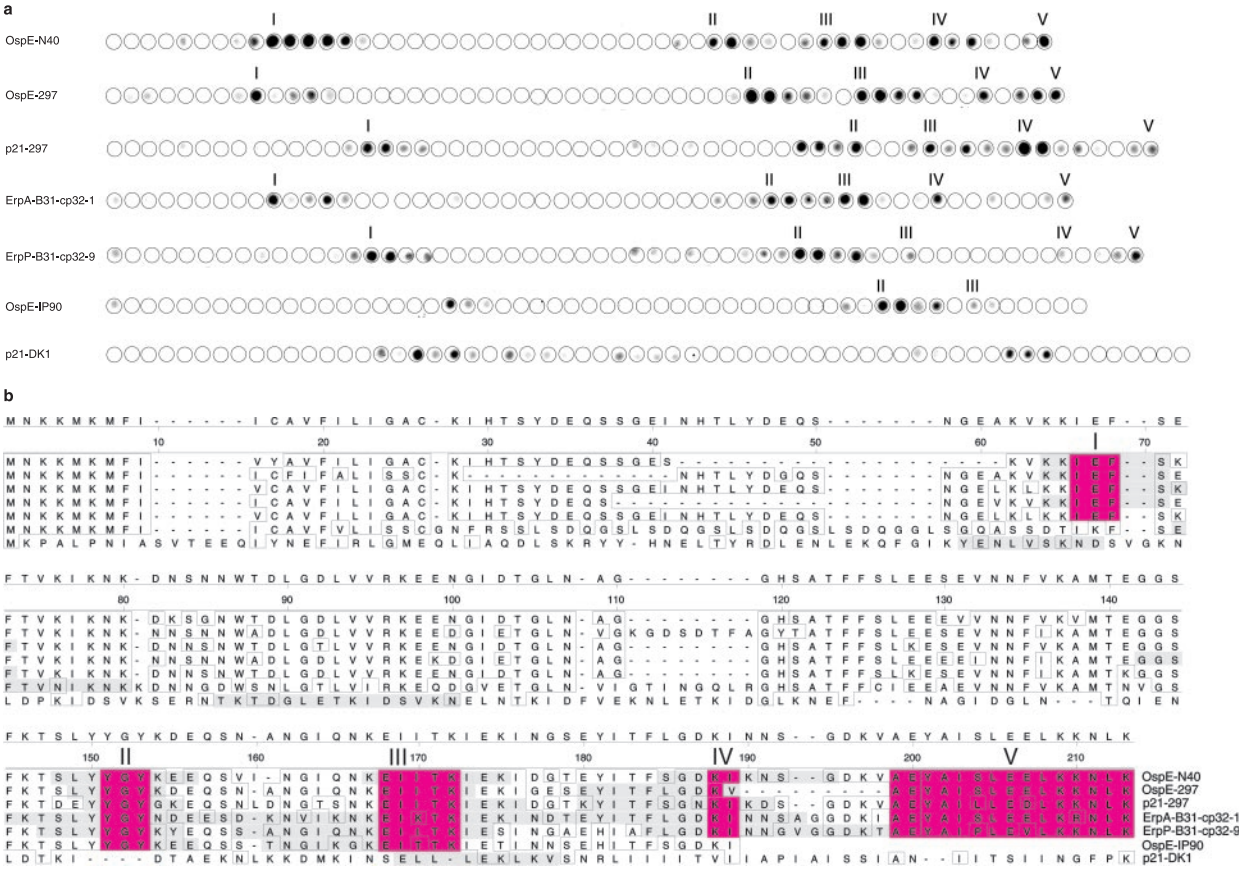

FIGURE 6. Peptide mapping of the factor H binding regions in OspE proteins. OspE-related proteins were fragmented into 15-aa peptides with 3-aa transitions, and the peptides were synthesized as spots onto a polyethylene glycol-derivatized cellulose membrane. Radiolabeled factor H was allowed to bind to the peptides. After washing, the membrane was subjected to autoradiography. The circles (*a*) represent the 15-aa peptides covering the entire sequences of the designated proteins with 3-aa transitions starting from the N termini. The intensity of radioactivity inside the circles represents the strength of factor H binding to each peptide. Altogether five putative factor H binding regions (marked I–V), which correspond to homologous regions in the distinct OspE proteins, can be identified. In *b* areas corresponding to the binding peptides are shaded gray, and the deduced common binding regions, shared by all factor H binding borrelial proteins, are colored red. These represent potential linear binding motifs in the tertiary structure of OspE-related proteins. The key binding region is located in the outward projecting C termini of the OspE proteins, which bind to the C terminus of factor H (21). OspE-IP90 represents a truncated OspE family member from the complement-sensitive *B. garinii* strain, and p21-DK1 is a more distantly related protein from *B. afzelii*.

ErpA, and ErpP from the 297 and B31 strains were more closely related to each other than to the *B. garinii* IP90 OspE.

Most binding regions found in the OspE homologs were clustered in the C terminus, which projects outward from the spirochetal surface. The C-terminal factor H binding region is relatively well conserved, while more variable regions are located in the N-terminal parts of the proteins. Lack of critical binding sites from the N terminus was verified by creating an OspE-297 mutant that lacked the 34 most N-terminal amino acid residues but still maintained factor H binding capacity (not shown). Features in common with the core regions of the putative binding sites (Fig. 6*b*) are that they contain and are flanked by charged residues, especially lysines, and are predicted to be exposed on the surface of the spirochete. The pI values of the OspE paralogs vary noticeably among the family members and across their sequences. In general, they have a zwitterionic charge distribution with numerous positively and negatively charged amino acid residues throughout their primary sequences. Variations in the regions flanking the binding domains as well as charged residue substitutions in the binding domains also seem to modulate the pH optimum of the whole protein for factor H binding. In general, conservation of the binding regions supports a critical role for the factor H-OspE interactions.

Discussion

Our results clearly imply a specific function for the plasmid-encoded OspE homologs as complement evasion molecules of *B. burgdorferi*. By binding factor H they can inhibit the bactericidal effects of direct or Ab-mediated complement activation and phagocytosis (26). Surface-expressed OspE-related lipoproteins are therefore integral for *B. burgdorferi* escape of immune clearance and complement-mediated destruction. The fact that the expression of OspE is up-regulated on the spirochetes within tick midguts after a blood meal (Fig. 5) and upon transmission to mammals by ticks (17) is also consistent with this function during the infectious process. The physiological conditions in tick-ingested blood are different from those in the mammalian host. As the interaction between factor H and OspE proteins is preserved in conditions of a wide pH range, it is likely that *Borrelia burgdorferi* can protect itself from complement attack also in ingested blood in the tick midgut, where the pH is ~6.8 (27).

The serum resistance of *Borrelia* strains is associated with the ability of bound factor H to promote C3b cleavage by factor I (6). Soluble OspE can compete with the binding of factor H to the surface of *Borrelia*, and when preincubated in serum, OspE can enhance the cytolytic activity of serum against *B. burgdorferi* (21).

Collectively, our data indicate that the OspE family of factor H binding proteins is associated with virulence in the human host. The close homology of factor H molecules in different animals and the fact that *Borrelia* infects a wide variety of vertebrates suggest that OspE-related proteins are also associated with in virulence mammalian hosts other than man. Indeed, we (6) observed that *B. burgdorferi* and *B. afzelii* can bind rabbit factor H from growth medium, and recently Stevenson et al. (23) observed that factor H from a variety of animal species binds to *B. burgdorferi* OspE/F/Elp proteins (called Erps). Stevenson et al. (23) obtained results consistent with our data concerning the B31 strain. They further concluded and speculated that all the Erp proteins would bind factor H in different host environments and affect the host specificity of the pathogen. Based on the binding and sequence data available, our results suggest that at least in the case of human factor H, the binding is specific for the OspE-related proteins only. Of the other proteins using the sensitive Biacore assay, only the ErpX-B31 protein was found to weakly bind factor H at pH 5.5, but not under physiological conditions. No other OspF or Elp protein bound human factor H. Furthermore, the factor H binding C-termini of OspE family proteins have amino acid sequences that are absent from the other Erp (OspF and Elp) proteins.

Recent evidence (28) suggests that plasmids harboring the OspE paralogs in borrelial strains share homology with bacteriophages. The plasmids could thus mediate horizontal transfer of genetic information among *Borreliae*. If this mechanism were in current use for the transfer of genetic material in Lyme disease spirochetes, it could result in the transfer of complement resistance in the *Borreliae*, similar to the transfer of antibiotic resistance in pathogenic bacteria. Thus, OspE paralogs may represent mobile units with which complement resistance can be transmitted from one population or strain to another. Plasmids encoding the OspE/F/Elp orthologs are often lost from a *B. burgdorferi* strain upon several generations of in vitro culture, with a concomitant attenuation in virulence. This probably also explains the loss of serum resistance and reduced numbers of demonstrable factor H binding proteins in *Borrelia* strains cultured for prolonged periods in vitro (29).

A puzzling question is why *B. garinii* strains are sensitive to complement killing. The likely reasons are, first, that *B. garinii* carries low copy numbers of OspE genes and, second, that the OspE proteins in *B. garinii* differ from those in the other genospecies. Consistent with these conjectures, primers or probes based on known OspE sequences do not readily recognize DNA from *B. garinii*, and the only currently established *B. garinii* OspE sequence ends before the most C-terminal region, implicated here in factor H interactions (30). Unlike all other OspE-related proteins, the *B. garinii* OspE contains four repeats consisting of SLSDQG in its N-terminal part (30). A missing C terminus, critical amino acid differences, or a smaller number of OspE paralogs could explain the sensitivity of *B. garinii* to complement killing. More studies are needed to explain why the *garinii* strains are sensitive to complement attack. The complement sensitivity of *B. garinii* may explain its preference to cause infections in the CNS (31). *B. garinii* could survive within the CNS because the amount of cytotoxic complement is limited in this body compartment. How *B. garinii* can reach the CNS and which additional immune evasion strategies it uses remain to be investigated. Additional information about the pathogenesis of *B. garinii* infection, in particular, will be of help in the development of an efficient treatment against all three genospecies.

In conclusion, our study reveals that a distinct set of plasmid-encoded homologous proteins, the OspE protein family, is responsible for factor H binding-mediated complement resistance in *Borrelia burgdorferi*. Recombinatorial processes have led to a redundancy of OspE-like gene products whose expression becomes induced and which function under a variety of physiological conditions within the arthropod and mammalian hosts. Identification of the different virulence factors and the underlying mechanisms of immune evasion can provide new tools to prevent and treat borreliosis.

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