Spirochetal pathogens, such as the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, encode an abundance of lipoproteins; however, due in part to their evolutionary distance from more well-studied bacteria, such as Proteobacteria and Firmicutes, few spirochetal lipoproteins have assigned functions. Indeed, *B. burgdorferi* devotes almost 8% of its genome to lipoprotein genes and interacts with its environment primarily through the production of at least 80 surface-exposed lipoproteins throughout its tick vector–vertebrate host lifecycle. Several *B. burgdorferi* lipoproteins have been shown to serve roles in cellular adherence or immune evasion, but the functions for most *B. burgdorferi* surface lipoproteins remain unknown. In this study, we developed a *B. burgdorferi* lipoproteome screening platform utilizing intact spirochetes that enables the identification of previously unrecognized host interactions. As spirochetal survival in the bloodstream is essential for dissemination, we targeted our screen to C1, the first component of the classical (antibody-initiated) complement pathway. We identified two high-affinity C1 interactions by the paralogous lipoproteins, ElpB and ElpQ (also termed ErpB and ErpQ, respectively). Using biochemical, microbiological, and biophysical approaches, we demonstrate that ElpB and ElpQ bind the activated forms of the C1 proteases, C1r and C1s, and represent a distinct mechanistic class of C1 inhibitors that protect the spirochete from antibody-mediated complement killing. In addition to identifying a mode of complement inhibition, our study establishes a lipoproteome screening methodology as a discovery platform for identifying direct host–pathogen interactions that are central to the pathogenesis of spirochetes, such as the Lyme disease agent.

**Significance**

Spirochetal pathogens encode an abundance of lipoproteins that can provide a critical interface with the host environment. *Borrelia burgdorferi*, the model species for spirochetal biology, must survive an enzootic life cycle defined by fluctuations between vector (tick) and vertebrate host. While *B. burgdorferi* expresses over 80 surface lipoproteins—many of which likely contribute to host survival—the *B. burgdorferi* lipoproteome is poorly characterized. Here, we generated a platform to rapidly identify targets of *B. burgdorferi* surface lipoproteins and identified two paralogs that confer resistance to antibody-initiated complement killing that may promote survival in immunocompetent hosts. This work expands our understanding of complement evasion mechanisms and points toward a discovery approach for identifying host–pathogen interactions central to spirochete pathogenesis.
For both survival during exposure to the bloodmeal in the tick midgut and dissemination of the spirochete throughout the vertebrate host, protection against host defenses is essential. The complement system is the most immediate threat to survival that pathogens must contend with in the blood. This system is composed of a set of soluble and membrane-associated proteins that interact and activate a multistep proteolytic cascade upon detection of microbial surfaces, ultimately forming complexes that can damage microbial membrane integrity, recruit immune cells, and enhance phagocytosis (15–18). The three canonical pathways of complement system activation are each triggered by the recognition of molecular patterns on pathogenic surfaces. The lectin pathway proceeds by the recruitment of serine proteases (MASPs) to mannose-binding lectin bound to the microbial surface by recognition of mannose or related sugars. The alternative pathway is triggered when complement factor C3 undergoes spontaneous cleavage in proximity of a microbial surface; it also serves as the central regulator of the spirochete life cycle, binds to C4b to inhibit C1r, blocking C1r proteolytic activity. When C3 and C5 activation. BBK32 binds the C1 complex by recognition of mannose sulfate, BBK32 binds C1 (34, 35) and, as expected, shows that ElpB and ElpQ promote resistance to antibody-dependent complement killing. The discovery of a unique role for ElpB and ElpQ in evading complement provides a validation of our lipoproteome screening methodology, which may be leveraged again in future studies to better understand the host–pathogen interface of the most prominent vector-borne pathogen in North America.

Results

Screening the B. burgdorferi Surface Lipoproteome Identifies High-Affinity Interactions between ElpB and ElpQ with Human Complement Component C1. Utilizing a previously described lipoproteome library, we developed a whole-cell binding assay to screen 80 strains of B. burgdorferi B31-e2 that each ectopically overproduce a single distinct C-terminally His-tagged, surface-localized lipoprotein from the B. burgdorferi lipoproteome (10) for the ability to adhere to candidate ligands. As nonadherent controls, we included the parental strain B31-e2, as well as a strain that overproduces the lipoprotein BB0460, which was reported to be largely periplasmic (10). To validate our approach, we first screened the library for strains that bind to human fibronectin. As expected, the two strains that bound fibronectin most strongly overexpressed the B. burgdorferi outer surface lipoproteins BBK32 and RevA, each of which have been shown to bind human fibronectin (45–49) (SI Appendix, Fig. S1 and Table S1).

To identify surface lipoproteins that target the CP, we screened the library for binding to purified, immobilized, human C1 complex. In addition to binding fibronectin and dermatan sulfate, BBK32 binds C1 (34, 35) and, as expected, spirochetes overexpressing BBK32 bound specifically to C1 in our screen (Fig. 1A, blue). Interestingly, strains overexpressing of novel virulence factors (36). Nevertheless, a transposon library of B. burgdorferi has previously proved useful for genome-wide screens to identify many virulence factors (37). Unfortunately, functional redundancy of lipoproteins may limit its utility in exploring the genome for host interactions. Alternatively, gain-of-function studies have allowed researchers to detect the acquisition of new virulence-associated functions, such as complement resistance or cell attachment (22, 34, 38, 39). This is accomplished through ectopic lipoprotein production in a high-passage strain that, due to stochastic plasmid loss, lacks many virulence-associated functions and is noninfectious. To comprehensively identify B. burgdorferi lipoproteins located on the outer surface of the spirochete, Dowdell et al. (10), using a strong constitutive promoter, ectopically produced epitope-tagged versions of all 127 putative lipoproteins encoded by B. burgdorferi strain B31 in the high-passage strain B31-e2, finding that more than 80 are detected on the outer surface.

In this study, we used this library of B31-e2 clones to establish a surface lipoproteome screening methodology. Based on the serum-resistance phenotype of a bbk32-deficient mutant described above and the observation that the complement evasion system of Lyme disease spirochetes has evolved to be functionally overlapping, we targeted our lipoproteome screen toward the human C1 complex. Erp proteins, whose genes share a high degree of homology in their promoter regions (23, 40–42; for review, see ref. 43), are comprised of three families, including the Erp family, based on their mature protein sequences (41, 44). We found that two Erp family members, ElpB and ElpQ (formerly termed ErpB and ErpQ, respectively), bind C1 with high affinity and block its activity through inhibition of the C1s protease subcomponent. Furthermore, we show that ElpB and ElpQ promote resistance to antibody-dependent complement killing. The discovery of a unique role for ElpB and ElpQ in evading complement provides a validation of our lipoproteome screening methodology, which may be leveraged again in future studies to better understand the host–pathogen interface of the most prominent vector-borne pathogen in North America.

For both survival during exposure to the bloodmeal in the tick midgut and dissemination of the spirochete throughout the vertebrate host, protection against host defenses is essential. The complement system is the most immediate threat to survival, although in proximity of a microbial surface; it also serves as the central regulator of the spirochete life cycle, binds to C4b to inhibit C1r, blocking C1r proteolytic activity. When C3 and C5 activation. BBK32 binds the C1 complex by recognition of mannose sulfate, BBK32 binds C1 (34, 35) and, as expected, shows that ElpB and ElpQ promote resistance to antibody-dependent complement killing. The discovery of a unique role for ElpB and ElpQ in evading complement provides a validation of our lipoproteome screening methodology, which may be leveraged again in future studies to better understand the host–pathogen interface of the most prominent vector-borne pathogen in North America.
lipoproteins ErpB or ErpQ (referred to in Fig. 1 as “ElpB” and “ElpQ,” respectively, for reasons described below) also bound strongly to C1, exhibiting a relative signal higher than that of the BBK32-expressing strain (Fig. 1A).

ErpB and ErpQ are members of the B. burgdorferi OspEF-related protein family (Erps), characterized by Marconi, Stevenson, and others (42, 43, 50). The genes encoding those Erp proteins were identified based on the similarity of their promoter sequences, and most of these genes are located on circular plasmid 32 (cp32) DNA elements (23). The analysis of the mature protein sequences indicates that Erp proteins fall into three families: OspE, OspF, and OspE/F-like leader peptides (Elp) (41, 44). Most OspE family members, including the above mentioned ErpP/ErpC/ErpA (CRASP-3/CRASP-4/CRASP-5), bind to the complement regulators factor H or factor H-related proteins (14). OspF and six other Erp proteins have been shown to bind heparan sulfate (38). Finally, Elp family proteins, defined by homology of their OspE/F-like leader peptides (41), which heretofore have had no known shared function, include ErpB and ErpQ. To distinguish ErpB and ErpQ from other Erp proteins based on both homology and apparent function, herein we refer to them as ElpB, ElpQ, and other proteins that share amino acid homology, as Elp rather than Erp proteins.

The genome of B. burgdorferi strain B31, the parental strain of B31-e2, encodes not only ElpB and ElpQ, but also ElpM, ElpO, and ElpX (42, 43, 50) (SI Appendix, Table S4). Despite being encoded on separate cp32 plasmids, elpB and elpO are identical at the amino acid sequence level, and for simplicity, ElpO will be referred to as ElpB hereafter. In strain B31, the Elp proteins (i.e., ElpB, ElpM, ElpQ, and ElpX) are 44 to 59% identical and 59 to 76% similar and exhibit their highest identity in the N-terminal and C-terminal protein regions (SI Appendix, Fig. S2 and Table S4). Strain B31-e2, a high passage derivative of strain B31, retains only three cp32 plasmids and does not carry elpQ, elpM, elpO, or elpX (51). However, this strain does encode elpB; thus, our finding here that strain B31-e2 does not bind to immobilized C1 absent ectopic production of ElpB or ElpQ indicates that the endogenous level of ElpB production and surface localization in this strain is insufficient to promote spirochetal binding in this assay.

To confirm the results of our screen, and because little is known about the function of Elp proteins, we individually tested strains producing each Elp in the ELISA-based spirochete binding assay against the C1 complex, including bovine serum albumin (BSA) as a negative control (Fig. 1A, Inset). Spirochetes expressing BBK32 (a C1-binding protein) and BB0460 (a lipoprotein previously suggested to be largely periplasmic) (10), were used as positive and negative controls, respectively. Strains producing ElpB, ElpQ, or BBK32 all exhibited statistically significant binding to C1 relative to BSA, whereas ElpM, ElpX, and BB0460 did not (Fig. 1A, Inset).

To further investigate the ability of ElpB and ElpQ to directly bind to human C1, we purified recombinant GST-tagged fusion proteins (GST-ElpB and GST-ElpQ). Consistent with data obtained from the spirochete binding assay (Fig. 1A), GST-ElpB and GST-ElpQ bound with high affinity to immobilized C1 in an enzyme-linked immunosorbent assay (ELISA-type) binding assay, exhibiting apparent equilibrium dissociation constants (K_d) of 3.4 nM and 3.8 nM, respectively (Fig. 1B and Table 1). To gain insight into the interaction of ElpB and ElpQ with soluble C1, we used surface plasmon
resonance (SPR) whereby GST-ElpB and GST-ElpQ were immobilized on SPR sensor chips. When C1 was used as an analyte, strong C1-binding was observed, with GST-ElpB and GST-ElpQ exhibiting steady-state calculated $K_D$ values of 5.6 and 11 nM, respectively (Fig. 1C and Table 1). Together, these data confirm that ElpB and ElpQ individually promote spirochete binding to human C1 via direct interaction with this molecule.

**ElpB and ElpQ Selectively Bind the Activated Forms of C1r and C1s.** The C1 complex is composed of C1q and a heterotramer of C1r and C1s (i.e., C1r2C1s2) (SI Appendix, Fig. S3A). C1q is a nonenzymatic component and functions in pattern recognition, while C1r and C1s are serine proteases that catalyze the initial proteolytic reactions of the CP. To clarify whether ElpB and ElpQ bind to C1 by interacting with individual subcomponents, we carried out an ELISA-type binding assay using purified immobilized C1q and activated forms of C1r and C1s (i.e., C1r enzyme and C1s enzyme). Relative to the negative control GST-BB0460, no significant interaction was detected for either GST-ElpB or GST-ElpQ with human C1q, (SI Appendix, Fig. S3B). In contrast, each protein bound with high affinity to C1r enzyme ($K_D$ of GST-ElpB/C1r = 41 nM; GST-ElpQ/C1r = 11 nM) as well as to C1s enzyme ($K_D$ of GST-ElpB/C1s = 6.7 nM; GST-ElpQ/C1s = 4.7 nM) (Table 1 and SI Appendix, Fig. S3 C and D).

To examine the function of ElpB and ElpQ in binding C1 subunits when produced by *B. burgdorferi*, we first assessed the relative amounts of ElpB and ElpQ in bacterial lysates by conventional immunoblotting, detecting the His tag on the ectopically produced lipoprotein. ElpB and ElpQ migrated with their predicted apparent molecular masses (61 kDa and 55 kDa, respectively). Full-length ElpQ was produced at vastly higher levels than ElpB, and the presence of a prominent lower molecular weight ElpB band—presumably a stable degradation product—suggested that ElpB, but not ElpQ was subjected to proteolytic cleavage (SI Appendix, Fig. S4). Pronase treatment was then used to assess the surface localization of each protein, as previously described (10). As expected, ElpB and ElpQ were predominantly expressed on the spirochetal surface (SI Appendix, Fig. S4). *B. burgdorferi* B31-e2 producing BBK32 or BB0460, analyzed in parallel, were exclusively or predominately, respectively, localized on the bacterial surface.

Finally, we probed these bacterial lysates using purified human C1 or the C1 subcomponent proteases to test for potential protein–protein interactions. Lysates from spirochetes expressing BBK32 (a C1r-binding positive control) contained a species that bound strongly to C1 complex, C1r proenzyme, and C1r enzyme but, as expected, to neither form of C1s (Fig. 2A vs. B). In all cases the C1/C1r-binding species comigrated with epitope-tagged BBK32 (SI Appendix, Fig. S4A). The negative-control BB0460 lysates contained no species that bound detectably to any complement protein probe (Fig. 2A and B). Consistent with the data shown in Fig. 1 and SI Appendix, Fig. S3, single bands coincident with ElpB and ElpQ, as judged by an α-6xHis blot (SI Appendix, Fig. S4A), bound to C1 complex, C1r enzyme (i.e., activated C1r), and C1s enzyme (i.e., activated C1s) (Fig. 2A and B, Top and Middle). Furthermore, this binding was reduced in the lysates of cells treated with pronase.

Interestingly, we found that C1r proenzyme failed to bind either ElpB or ElpQ spirochete lysates (Fig. 2A, Bottom). C1s proenzyme also showed lower relative binding to ElpB compared to the activated form of C1s but due to the high levels of ElpQ production, comparison of relative binding of this protein to the proenzyme and activated forms of C1s was more difficult to assess (Fig. 2B, Middle and Bottom). To quantitatively investigate these interactions, we measured the relative affinities of pro- and active forms of both C1r and C1s for recombinant GST-ElpB and GST-ElpQ by SPR. Indeed, while GST-ElpB and GST-ElpQ bound to C1r enzyme with $K_D$ values of 100 nM and 97 nM, respectively, neither protein exhibited detectable binding for C1r proenzyme (Fig. 2C and SI Appendix, Fig. S5). Similarly, GST-ElpB and GST-ElpQ bound C1s enzyme with ∼70-fold and ∼38-fold higher affinity, respectively, than C1s proenzyme ($K_D$ of 3.9 nM vs. 270 nM; $K_D$ of 4.5 nM vs. 170 nM) (Fig. 2D, Table 1, and SI Appendix, Fig. S5).

**ElpQ Inhibits C1s Cleavage of C2 and C4.** Having established that ElpB and ElpQ were capable of direct interaction with human C1 via specific recognition of the protease subcomponents, using ElpQ we explored a potential mechanism of action for C1 inhibition. To facilitate clarity in our gel-based cleavage assays and to eliminate the GST-tag from the mechanistic analysis, we generated an ElpQ construct lacking this epitope. The “tagless” ElpQ behaved nearly identically in SPR C1s-binding assays and ELISA-based complement assays when compared to GST-ElpQ (SI Appendix, Fig. S6).

BBK32, which binds to C1r but not C1s, is capable of directly inhibiting purified C1r enzyme cleavage of C1s proenzyme (34). In contrast, recombinant ElpQ failed to block this reaction at protein concentrations several orders of magnitude greater than the C1r/ElpQ $K_D$ (SI Appendix, Fig. S7A). ElpQ also failed to prevent the cleavage of the small peptide C1r

### Table 1. ELISA-type and SPR binding assays

| GST-fusion protein | Complement protein | ELISA $K_D$ (nM)† | SPR $K_D$ (nM)‡ |
|--------------------|--------------------|-------------------|----------------|
| GST-ElpB           | C1                 | 3.4 ± 0.4         | 5.6 ± 1.5      |
|                    | C1r enzyme         | 41 ± 4.3          | 100 ± 27       |
|                    | C1s enzyme         | 6.7 ± 0.7         | 3.9 ± 0.48     |
|                    | C1r proenzyme      | —                 | NB             |
|                    | C1s proenzyme      | —                 | 270 ± 55       |
| GST-ElpQ           | C1                 | 3.8 ± 1.2         | 11 ± 2.0       |
|                    | C1r enzyme         | 11 ± 1.9          | 97 ± 35        |
|                    | C1s enzyme         | 4.7 ± 1.0         | 4.5 ± 1.0      |
|                    | C1r proenzyme      | —                 | NB             |
|                    | C1s proenzyme      | —                 | 170 ± 73       |

†NB, no detectable binding.
‡$K_D$, determined by quantitative ELISA.
§$K_D$, determined by SPR.
substrate Z-Gly-Arg-sBzl (52), whereas BBK32 did so readily (SI Appendix, Fig. S7). Similarly, unlike futhan, a small molecule active site C1s inhibitor (52), 25 μM ElpQ (i.e., >5,500-fold over the measured $K_D$) (Table 1) failed to inhibit the cleavage of the C1s peptidic substrate Z-L-Lys thiobenzyl by C1s (Fig. 3A). Thus, in the C1s/ElpQ complex, the active site of C1s remains accessible to small peptide substrates.

We next tested whether ElpQ was capable of inhibiting C1s-mediated cleavage of native substrates. The cleavage of C2 or C4 by purified C1s was monitored by SDS/PAGE in the presence of increasing concentrations of ElpQ (Fig. 3B–E). Whereas BBK32 failed to block C2 cleavage by C1s (Fig. 3D, lane 3), as judged by the generation of the C2 cleavage product C2b (‘→ C2b”) (Fig. 3B), ElpQ blocked C1s-mediated C2 proteolysis and the concomitant formation of C2b in a dose-dependent fashion (Fig. 3D, lanes 6 to 13). Likewise, while BBK32 failed to prevent C4 cleavage by C1s (Fig. 3E, lane 3), as judged by generation of the C4 cleavage product C4α (‘→ C4α”) (Fig. 3C), ElpQ did so in a dose-dependent manner (Fig. 3E, lanes 6 to 13). Densitometry analysis resulted in calculated ElpQ IC_{50} values of 1.4 μM and 11 μM for C2 and C4, respectively. Similarly, a tagless version of ElpB blocked C2 cleavage by C1s, and with an IC_{50} (0.87 μM) like that of ElpQ (SI Appendix, Fig. S8). The observation that ElpQ inhibited the cleavage of large endogenous C1s substrates but not a small peptide C1s substrate suggests that ElpQ inhibits C1s in a manner that leaves the active site of C1s accessible to small peptides.

ElpQ Inhibits the CP of Complement. Collectively, the data above identify an interaction between surface-expressed B. burgdorferi lipoproteins ElpB and ElpQ with human C1 and

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**Fig. 2.** ElpB and ElpQ preferentially bind activated forms of C1r and C1s. (A) Extracts from untreated (“−”) or pronase-treated (“+”) 1 × 10^{7} strain B31-e2 spirochetes were separated by SDS/PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The filters were probed with purified C1 complex (Top), activated C1r enzyme (Middle) or C1r proenzyme (Bottom), and bound probe revealed by anti-C1r antibody, followed by HRP-conjugated anti-mouse antibody. Shown is a representative of three experiments. (B) Filters prepared identically to A were probed with purified C1 complex (Top), activated C1s enzyme (Middle) or C1s proenzyme (Bottom), and bound probe revealed by anti-C1s antibody, followed by HRP-conjugated anti-mouse antibody. Shown is a representative of three experiments. (C and D) Biosensors immobilized with GST-ElpB (Upper) or GST-ElpQ (Lower) were tested by SPR for binding to the indicated concentrations of the enzyme or proenzyme forms of C1r (C) or C1s (D). Injection series were each performed in triplicate. For both C and D, steady-state affinity fits were determined by T200 Biacore Evaluation software and $K_D$ values are reported in Table 1.
Complement-Mediated Killing.

Ectopic Production of ElpQ Protects Spirochetes from Complement-Mediated Killing.

Fig. 3. ElpQ inhibits the proteolytic activity of complement C1s. (A) Enzymatic cleavage by C1s of the small peptide substrate Z-Lys-sBzl was assayed with 5,5′-dithiobis-(2-nitrobenzoyl acid) (Ellman's reagent) in the presence of 25 μM BBK32-C (noninhibitory control) or ElpQ at 25 °C for 1 h. Experiments were performed in triplicate. Absorbance was read at 412 nm and signals were normalized to negative control no-substrate wells. (B and D) Proteolytic cleavage of C2 by C1s enzyme produces ~70 kDa C2b and ~35 kDa C2a after 1 h at 37 °C. Lanes 1 to 5: Control reactions in the presence (“+”) or absence (“−”) or 25 μM ElpQ, 25 μM BBK32-C (noninhibitory control), 6.25 nM C1s, and 685 nM C2. (Note that the amount of C1s loaded is below the level of detection by SDS/PAGE). (D, lanes 6 to 13) C2b accumulation in the presence of 6.25 nM C1s, 685 nM C2 and a twofold dilution series (from 16 to 0.13 μM) of ElpQ. In B, the fraction of C2b relative to total input C2 in the same lane determined by densitometry analysis data are normalized to C2 (lane 4) and C1s digested C2 (lane 5). A representative gel is shown. The experiment was performed three times. (C and E) C4, which consists of three polypeptide chains—C4α (97 kDa), C4β (33 kDa), C4γ (33 kDa)—is cleaved by C1s enzyme for 1 h at 37 °C to produce C4α′ (88 kDa). (E, lanes 1 to 5) SDS/PAGE profile in the presence (“+”) or absence (“−”) of 25 μM ElpQ; 25 μM BBK32-C (noninhibitory control); 6.25 nM C1s, and 616 nM C4. (Lanes 6 to 13) SDS/PAGE profile in the presence of 6.25 nM C1s, 616 nM C4, and a twofold dilution series (from 25 to 0.20 μM) of ElpQ. In C, the fraction of C4α′ relative to input C4) in the same lane and normalized to C1s + C4 positive-control (lane 5) and negative-control C4 (lane 4) was determined by densitometry analysis.

To assess the ability of ElpB and ElpQ to resist CP killing, with BBK32 and BB0460 as positive and negative controls, respectively. Based on a previously described assay to initiate the CP (53), we incubated these strains with either anti-__B. burgdorferi__ polyclonal antibodies or nonspecific antibodies (as a negative control), then added normal human serum to provide complement components and lysozyme to facilitate disruption of spirochetal integrity (Materials and Methods). After dilution into BSK-II media and 72-h incubation to allow for growth of surviving bacteria, we enumerated living spirochetes. Although the ElpQ-producing strain did not grow quite as well after exposure to nonspecific antibodies and lysozyme, all the four strains grew to high titers (i.e., 1.6 × 10^9/mL and 1.1 × 10^9/mL), indicating that the lysozyme treatment alone was insufficient to kill bacteria efficiently (SI Appendix, Table S3). We speculate that the high-level ectopic overproduction of protein by the ElpQ producer may slightly diminish the overall fitness of this strain.

To assess the ability of ElpB and ElpQ to counter classical complement killing, we calculated relative survival: that is, the degree of killing required not only anti-__B. burgdorferi__ polyclonal antibodies or nonspecific antibodies (as a negative control), but was abrogated by prior heat treatment of serum to inactivate complement (SI Appendix, Table S3). Conversely, production of BBK32 conferred high-level protection, with relative
survival of 0.45, or ~230-fold higher than the negative control BB0460 ($P < 0.001$) (Fig. 5, blue). *B. burgdorferi* B31-e2 producing ElpQ displayed a relative survival of 0.62, 321-fold higher than the control ($P < 0.0001$) (Fig. 5, red). *B. burgdorferi* B31-e2 expressing ElpB, which appeared to produce a small fraction of ectopic protein compared to the ElpQ-producing strain when assessed by immunoblotting (Fig. 1 A and B), exhibited a relative survival of 0.03, 15-fold higher than control, but this difference did not reach statistical significance (Fig. 5, green). Nevertheless, the dramatically enhanced resistance to CP-mediated killing conferred by ElpQ, along with the trend in protection by the ElpB-producing strain, indicates that the inhibition of C1 and blockade of the CP observed in biochemical assays reflects an activity that protects bacterial viability.

**Discussion**

Lyme disease spirochetes are typical of other spirochetal pathogens in that they encode many lipoproteins (11). Although the proportion of lipoproteins located in the periplasm varies among spirochetes (8, 10, 54, 55), surface lipoproteins are critical to pathogenesis and provide an important means by which pathogenic spirochetes interact with the host environment (56, 57). Of the ~125 lipoproteins encoded by the *B. burgdorferi* genome, the majority localize to the outer membrane (10), although functions for relatively few of these proteins have been elucidated. Adding to the complexity of understanding lipoprotein function, several of the best-characterized *B. burgdorferi* outer surface lipoproteins, such as OspC and BBK32, have been shown to provide multiple independent functions during murine borreliosis (58–62).

Building on the generation of a comprehensive lipoprotein library (10), we developed an ectopic overexpression screening methodology to identify novel interactions between host macromolecules and the *B. burgdorferi* surface lipoproteome expressed in its native environment in the outer membrane of intact spirochetes. This methodology has the potential to uncover diverse host interactions that take place at the spirochete surface and may be valuable in the study of other pathogenic bacteria as well.

A clear limitation of the “gain-of-function” strategy described here is that overproduction of ectopically expressed lipoproteins may uncover artificial functions. Indeed, to investigate functional aspects of the lipoproteome most easily, the *B. burgdorferi* B31-e2 gain-of-function library was constructed using the flagellin promoter, *P*~flaB~, one of the strongest constitutive *B. burgdorferi* promoters (63). Although we did not quantify levels of ectopic lipoprotein production in this study, immunoblotting revealed that ElpQ was produced in vastly greater amounts than ElpB, with the likely consequence that only the ElpQ producer was shown to protect strain B31-e2 significantly from classical complement killing (Fig. 5). This limitation notwithstanding, ectopic overexpression for gain-of-function analyses have identified and analyzed the function of many *B. burgdorferi* adhesive lipoproteins that have been subsequently validated by genetic studies involving infectious *B. burgdorferi* strains (61, 64–68).

We applied the current lipoproteome screening strategy to the requirement that, as an extracellular pathogen that encounters host blood during both the tick bloodmeal and throughout dissemination and colonization of their vertebrate hosts, Lyme disease spirochetes must prevent complement-mediated opsonization and lysis at multiple stages in the enzootic cycle. Moreover, the complement system employs three distinct pathways for activation that together form a complex host defense. Reflecting this, nearly a dozen different *B. burgdorferi* outer surface lipoproteins have

![Fig. 4.](image-url) ElpB and ElpQ inhibit the CP of complement. (A–C) Normal human serum (NHS) was incubated with the indicated concentration of purified GST-fusion proteins, then added to wells precoated with human IgM. Deposition of (A) C4b, (B) C3b, or (C) C5b-9 was determined by the addition of the appropriate primary and secondary antibodies (Materials and Methods) enumerated by absorbance at OD405nm or OD450nm. Each well was normalized to wells with no inhibitor (100%) and no serum (0%). Curves were fit using nonlinear regression to determine IC50 values. (D) NHS was incubated with the indicated concentration of purified GST-fusion proteins and then added to preincubated sheep erythrocytes (Materials and Methods). Erythrocyte lysis was determined by OD405nm and normalized to lysis by deionized water (100%) and no serum (0%). Error bars indicate SEM. Each concentration was tested a minimum of three times.
been shown to directly interact with complement components, disrupting their activities (13, 14). At least three factors contribute to the multiplicity of lipoproteins devoted to thwart complement defense. First, distinct borreliial complement evasion proteins block different complement activation pathways. For example, BBK32 selectively targets C1r, the initiator protease of the CP, while OspC binds to C4b, the downstream activation product of both the classical and lectin pathways (20, 34, 35). Second, individual borreliial lipoproteins may target the same host protein but function at different stages of the enzootic cycle. *B. burgdorferi* CspA and CspZ both bind to factor H and prevent activation of the alternative pathway, while CspA is expressed exclusively in the tick midgut and prevents the bactericidal effects of the bloodmeal, whereas CspZ is produced early in vertebrate infection and fosters the establishment of infection in that host (32, 33, 69). Finally, although some Lyme disease spirochete strains are restricted to only a single vertebrate, other strains have the capacity to infect multiple vertebrate hosts (70) that encode polymorphic complement lipoproteins, ElpB and ElpQ, like BBK32, prevent antibody-mediated complement activation but target the C1 complex via distinct means. BBK32 does not bind C1s, but recognizes bothzymogen and activated forms of C1r, blocking its enzymatic activity. In contrast, ElpB and ElpQ bind to both C1r and C1s but selectively recognize activated forms of the proteases (Fig. 2 and SI Appendix, Fig. S5). Furthermore, we showed that ElpQ is incapable of directly blocking purified C1r activity (SI Appendix, Fig. S7) but prevents cleavage of both C2 and C4 by purified activated C1s enzyme (Fig. 3 B and C); ElpB possesses a similar activity (SI Appendix, Fig. S8). Finally, ElpQ did not prevent cleavage of small peptide substrates, and is unusual among microbial-derived serine protease inhibitors, many of which—such as ecotin or BBK32 (79)—target the protease active site (80, 81). A model detailing the mechanistic differences between BBK32 and ElpB/Q C1 inhibition is presented in Fig. 6.

Previous work showed that expression of BBK32 by a high-passage, noninfectious *B. burgdorferi* strain enhanced serum resistance, and that simultaneous inactivation of the classical and lectin pathways eliminated this enhancement, indicating that BBK32 blocked one or both pathways. To confirm that the C1-binding activities of BBK32, ElpB, and ElpQ specifically blocked classical complement killing, we triggered this pathway by treating high-passage strains that ectopically produce these proteins with anti-*B. burgdorferi* antibody. Whereas BBK32, ElpB, and ElpQ provided

![Graph](https://doi.org/10.1073/pnas.2117770119)
no survival advantage when spirochetes were treated with serum supplemented with isotype control antibody, all three lipoproteins promoted survival when incubated with specific antibody, indicating that the C1r- or C1s-inhibitory activities of BBK32 or ElpB and ElpQ, respectively, protected spirochetes from classical complement killing. ElpQ provided the greatest degree of protection, enhancing the survival ratio 321-fold relative to BB0460, and no survival advantage when spirochetes were treated with serum supplemented with isotype control antibody, all three lipoproteins were produced in vivo and consistent with the hypothesis they function during mammalian infection (23, 82, 83). Blocking C1s, OspC facilitates invasion of B. burgdorferi in the context of other anticomplement factors — such as BBK32, OspC or OspE family members — that together inactivate multi-complement pathways to facilitate survival in the host. This complexity will require genetic techniques not routinely performed for B. burgdorferi at this time, but recent description of a CRISPR interference platform for silencing B. burgdorferi genes may facilitate these important investigations (86).

Materials and Methods

Bacterial Strains, Plasmids, and Lipoprotein Gain-of-Function Library. *Escherichia coli* strains DH5α and BL21(DE3) were used for plasmid cloning and propagation and protein purification, respectively, as cultured in LB-Miller broth, as described in *SI Appendix, Supplemental Materials and Methods*. An epitope-tagged *B. burgdorferi* lipoprotein expression (gain-of-function) library in the high-passage, noninfectious B31-e2 background strain (10) (*SI Appendix, Table S1*) was grown in supplemented BSK-II medium, as described in *SI Appendix, Supplemental Materials and Methods*. Plasmids are described in *SI Appendix, Table S1* and primers to generate several plasmids are described in *SI Appendix, Supplemental Materials and Methods*.

Quantitation of Binding of Gain-of-Function Library Clones to Immobilized Substrates. Binding of gain-of-function library clones to immobilized substrates was measured using a modification of a previously described ELISA-based assay (87), as described in *SI Appendix, Supplemental Materials and Methods*.

Quantitative ELISA or SPR to Assess *B. burgdorferi* Lipoprotein Binding to Purified Human C1 Components. To quantitate the ability of *B. burgdorferi* lipoproteins to bind purified components of the C1 complex, we adapted a previously described quantitative ELISA-based (65) or SPR-based assays, using a Biacore T200 (GE Healthcare), as previously described (34), with modifications as described in *SI Appendix, Supplemental Materials and Methods*.

Inhibition of C3d, C4d, C5b-9 Deposition, and Erythrocyte Hemolysis by Recombinant *B. burgdorferi* Lipoproteins. We adapted previously described ELISA-based assays to determine the effect of recombinant *B. burgdorferi* lipoproteins on CP-mediated deposition of C3d (34, 88) or C4d (34). Inhibition of CP-mediated erythrocyte hemolysis by recombinant *B. burgdorferi* lipoproteins was assayed using a modified version of the previously described CP hemolytic assay (12, 34, 89), as described in *SI Appendix, Supplemental Materials and Methods*.

Inhibition of C1r and C1s Enzyme Activity by Synthetic Peptide Cleavage. C1r enzyme assays were achieved by monitoring the autolytic activation of C1r proenzyme using the substrate Z-GlyArg-bzl, and C1s enzyme assays using Z-Lys-bzl, as described in *SI Appendix, Supplemental Materials and Methods*.

Gel-Based Assays to Detect Inhibition of C1r-Mediated Proenzyme C1s Cleavage or C1s-Mediated C2/C4 Cleavage. Enzymatic inhibition assays were performed using SDS/PAGE to monitor the cleavage of proenzyme C1s, as previously described, with modifications described in *SI Appendix, Supplemental Materials and Methods* (34). Similarly, to assess inhibition of C1s mediated cleavage of C2 or C4, C2- or C4-derived cleavage products were detected by SDS/PAGE after incubation of substrates with twofold dilutions of ElpQ or ElpB, as described in *SI Appendix, Supplemental Materials and Methods*.

CP-Mediated Serum-Killing Assay. The ability of *B. burgdorferi* B31-e2 strains to resist CP killing was based on a previously described assay that involved anti-B. burgdorferi polyclonal (or control) antibodies, human serum to provide complement, and lysozyme to facilitate disruption of spirochetal integrity of MAC-associated bacteria (53). After treatment, cultures were grown for 72 h and spirochetal titer were counted in duplicate by dark-field microscopy (*SI Appendix, Supplemental Materials and Methods*).

Data Availability. All study data are included in the article and *SI Appendix*.

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