

Fibronectin Binding Protein BBK32 of the Lyme Disease Spirochete Promotes Bacterial Attachment to Glycosaminoglycans

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Borrelia burgdorferi, the agent of Lyme disease, causes a multisystemic illness that can affect the skin, heart, joints, and nervous system and is capable of attachment to diverse cell types. Among the host components recognized by this spirochete are fibronectin and glycosaminoglycans (GAGs). Three surface-localized GAG-binding bacterial ligands, Bgp, DbpA, and DbpB, have been previously identified, but recent studies suggested that at least one additional GAG-binding ligand is expressed on the spirochetal surface when the spirochete is adapted to the mammalian host environment. BBK32 is a surface lipoprotein that is produced during infection and that has been shown to bind to fibronectin. In this study, we show that, when BBK32 was produced from a shuttle vector in an otherwise nonadherent high-passage *B. burgdorferi* strain, the protein localized on the bacterial surface and conferred attachment to fibronectin and to mammalian cell monolayers. In addition, the high-passage strain producing BBK32 bound to purified preparations of the GAGs dermatan sulfate and heparin, as well as to these GAGs on the surfaces of cultured mammalian cells. Recombinant BBK32 recognized purified heparin, indicating that the bacterial attachment to GAGs was due to direct binding by BBK32. This GAG-binding activity of BBK32 is apparently independent of fibronectin recognition, because exogenous heparin had no effect on BBK32-mediated bacterial binding to fibronectin.

Lyme disease, the most prevalent vector-borne illness in the United States, results from infection by the spirochete *Borrelia burgdorferi*. Following the bite from an infected *Ixodes* tick, these spirochetes establish a local infection in the skin, often giving rise to a characteristic rash termed erythema migrans. Some *B. burgdorferi* strains are capable of dissemination to multiple secondary sites, such as distant skin sites, brain, heart, or joints, and the presence of spirochetes in these tissues results in diverse clinical manifestations, including arthritis, carditis, neuroborreliosis, and acrodermatitis (44, 52, 53; for a review, see reference 46).

For many bacterial pathogens, attachment to host tissues is believed to be a critical step during colonization and is typically mediated by adhesins, i.e., surface proteins that promote bacterial attachment to host cells (16). Consistent with its ability to cause a multisystemic infection in mammals, *B. burgdorferi* attaches to a variety of cell types in vitro, including lymphocytes, platelets, epithelial cells, endothelial cells, and neuroglia (9, 11, 19, 20, 49, 50). Among the host cell components recognized are integrins (7, 8), fibronectin (21, 39, 49), and proteoglycans (23, 24, 29). Proteoglycans consist of a core protein covalently linked to glycosaminoglycans (GAGs), which are long, linear, and highly sulfated disaccharide repeats (for a review, see reference 48). Classes of GAGs, such as heparin, heparan sulfate, dermatan sulfate, and chondroitin-6-sulfate, differ in structure and are often functionally distinguished by their differential sensitivities to cleavage by specific lyases.

In addition to binding to intact proteoglycans, *B. burgdorferi* is capable of attachment to GAG chains (24, 29, 30), and culture of *B. burgdorferi* under “host-adapted” conditions in dialysis membrane chambers implanted in the rat peritoneal cavity (1) results in significantly enhanced binding to both endothelial cells and heparin, suggesting that GAG binding may play an important role within the mammalian host (37). In fact, production of the GAG-binding adhesin Vsp2 by the related relapsing fever spirochete *Borrelia turicatae* is associated with efficient colonization of the joint and blood of infected SCID mice, while production of a related protein, Vsp1, which does not efficiently bind GAGs, is associated with infection of the brain (5, 33, 38, 54). For *B. burgdorferi*, different classes of GAGs mediate in vitro bacterial attachment to different types of mammalian cells, e.g., heparan sulfate largely mediates attachment of *B. burgdorferi* to cultured endothelial cells, whereas dermatan sulfate and heparan sulfate promote spirochetal attachment to cultured glial cells (31). In addition, the particular GAG-binding specificity varies with the particular *B. burgdorferi* strain, resulting in corresponding differences in the spectrum of cell types to which each strain binds. Thus, a *B. burgdorferi* strain that binds dermatan sulfate and heparan sulfate attaches to both glial and endothelial cells, whereas another strain that binds only dermatan sulfate selectively binds glial cells (36).

A simple hypothesis to explain these results is that *B. burgdorferi* produces multiple GAG-binding adhesins and that the repertoire of adhesins produced by a given strain is responsible for its particular GAG binding specificity. In fact, three GAG-binding surface proteins have been identified. Bgp (*Borrelia* GAG-binding protein) is a heparin-binding hemagglutinin that is both secreted into the media and localized on the bacterial

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surface (6, 35). Decorin binding protein A (DbpA) and DbpB are surface lipoproteins that, in addition to binding the proteoglycan decorin (22), bind to GAGs (17, 37). Bgp, DbpA, and DbpB do not appear to entirely account for the enhanced GAG-binding activity displayed by host-adapted bacteria (37), suggesting that this pathogen encodes at least one additional GAG-binding pathway.

Upon prolonged culture, *B. burgdorferi* commonly loses plasmids that are required for full infectivity in mice but not required for in vitro growth (2, 25, 27, 34, 41, 45). *B. burgdorferi* strain B314 is a high-passage derivative of strain B31 that has lost many plasmids, including the plasmid carrying *dbpA* and *dbpB* (43), and is incapable of attaching to mammalian cells in vitro (17). The absence of potentially redundant binding pathways encoded by strain B314 greatly simplifies characterization of the cell binding activities of ectopically expressed adhesins, and we recently showed that expression of *dbpA* or *dbpB* from a shuttle vector (47) conferred on *B. burgdorferi* strain B314 distinct GAG- and mammalian cell-binding phenotypes (17). Strain B314 also lacks lp36, which encodes BBK32, a 47-kDa surface lipoprotein that recognizes the extracellular matrix protein fibronectin (18, 39). In the present study, we produced BBK32 in B314 and demonstrated that the protein promotes spirochetal attachment to host cells not only by recognizing fibronectin but also by recognizing GAGs.

MATERIALS AND METHODS

Bacterial strains and cell lines. All strains of *B. burgdorferi* were cultured in BSK-H complete medium (Sigma Chemical Co., St. Louis, MO) at 33°C. *Borrelia* transformants were cultured in BSK-H complete medium supplemented with kanamycin (200 µg/ml). The high-passage B314 *B. burgdorferi* strain was a generous gift from Tom Schwan (RML, Hamilton, MT). 293 human kidney epithelial, HEP-2 human epithelial, C6 rat glioma, and EAhy-926 human endothelial cells were cultured as described previously (36).

Plasmids and cloning. To generate a recombinant maltose-binding protein (MBP)–BBK32 fusion protein that lacked the BBK32 signal sequence and lipid moiety, codons 21 to 354 of the strain B31 BBK32 were amplified using forward primer 5'-CGGAATTCGATTTATTCATAAGATAT-3' and reverse primer 5'-GCAAGCTTTAAGTACCAACGCCATTCTT-3' (restriction sites underlined) and cloned into the pMal-c2 vector (Novagen, Madison, WI). The resulting plasmid, pMBP-BBK32, was transformed into *Escherichia coli* BL21 and its sequence confirmed by DNA sequencing. To construct a shuttle vector that promoted expression of cloned genes during in vitro culture of *B. burgdorferi*, the strain B31 *ospC* promoter (nucleotides -184 to -9), which is active during growth in BSK-H (43), was amplified using PCR with forward primer 5'-CCC AAGCTTTTAATTTAGCATATTTGGCTTTG-3' and reverse primer 5'-ACG CGTCGACCCCTCTTTTATTTATGAATTATT-3' (restriction sites underlined). This 175-bp amplicon was ligated into the HindIII and SalI sites of shuttle vector pBSV2 (47) (generously provided by Patricia Rosa, RML, Hamilton, MT) to generate pJF21. To modify pJF21 to produce BBK32 in vitro, the entire BBK32 gene, encoding full-length BBK32 (354 amino acids), was amplified by PCR using forward primer 5'-ACGCGTCGACATGAAAAAGTTAAAG TAAATATTTGG-3' and reverse primer 5'-CGCGGATCCGTACCAACGCC ATTCTTGTCATGATCC-3' and was inserted into the SalI and BamHI sites of pJF21 to generate pBBK32_{B31}, herein referred to as "pBBK32."

Electroporation of *B. burgdorferi* B314. Electrocompetent B314 spirochetes were transformed with 30 µg of plasmid DNA and cultured in BSK-H complete medium at 37°C for 24 h as previously described (17). Aliquots of the culture were mixed with 1.8% analytical grade agarose (Bio-Rad, Hercules, CA) and plated onto a solidified BSK-H-agarose layer in sterilized 100- by 20-mm tissue culture dishes (Falcon) in the presence of kanamycin (200 µg/ml). Plates were incubated at 37°C in a 5% CO₂ atmosphere for 2 weeks. Colonies were selected and cultured at 37°C in liquid BSK-H medium containing kanamycin (200 µg/ml) until mid-logarithmic phase and then stored at -80°C in BSK-H containing 20% glycerol.

Generation of BBK32 antiserum. MBP and MBP-BBK32 were purified from pMAL-c2 and pMBP-BBK32, respectively, using an amylose column (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. Five- to 6-week-old BALB/c mice were immunized with 200 µg of either MBP or MBP-BBK32 in complete Freund's adjuvant. The animals were boosted twice with 200 µg of the same proteins in incomplete Freund's adjuvant at 2-week intervals, and antisera were prepared after terminal cardiac puncture.

Proteinase K treatment, SDS-PAGE, and Western blotting. To detect BBK32 protein, lysates from 1×10^7 bacteria were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). BBK32 and FlaB were identified by immunoblotting using a polyclonal antibody against MBP-BBK32 (diluted 1:10,000) or monoclonal antibody CB1 (a gift from J. Benach, Stony Brook University, Stony Brook, NY) against FlaB (diluted 1:200), respectively. Surface localization of BBK32 was determined as previously described (39). Briefly, 1×10^7 bacteria were centrifuged and pellets were washed twice with phosphate-buffered saline (PBS). After the final wash, pellets were gently lifted with 5 mM MgCl₂ in PBS supplemented with 4 mg/ml proteinase K (Roche, Indianapolis, IN) and incubated at room temperature for 30 min. To inactivate proteinase K, 150 µg phenylmethylsulfonyl fluoride was added to each pellet. Pellets were washed twice with 5 mM MgCl₂ in PBS, lysed, and separated by 10% SDS-PAGE. Proteins were identified by immunoblotting as described above.

Radiolabeling *B. burgdorferi*. Radiolabeled spirochetes were prepared by culturing at 33°C in BSK-H medium supplemented with 60 µCi of [³⁵S]methionine and [³⁵S]cysteine per ml. When cultures achieved mid-log-phase density (about 5×10^7 bacteria per ml), spirochetes were centrifuged at $10,000 \times g$, washed three times with 0.2% bovine serum albumin (BSA) in PBS, and stored as aliquots at -80°C in BSK-H containing 20% glycerol.

Attachment of radiolabeled bacteria to purified GAGs and fibronectin. Prior to each assay, wells from Nunc 96-well break-apart microtiter plates were coated with either 1 mg/ml purified human fibronectin or 5 mg/ml dermatan sulfate, heparin, or chondroitin-6-sulfate in PBS at 4°C overnight. Wells were washed three times with 0.5% Tween in PBS. Frozen aliquots of radiolabeled spirochetes were thawed and resuspended at 1×10^8 cells/ml in BSK-H and were incubated at room temperature for 2 h. Radiolabeled spirochetes were then diluted 1:3 with 10 mM glucose, 50 mM NaCl, and 10 mM HEPES, at pH 7.0, and added in quadruplicate wells at 1×10^6 spirochetes/well. To enhance substrate-spirochete contact, plates were centrifuged at $190 \times g$ for 5 min and then rocked at room temperature for 1 h. Unbound spirochetes were removed by washing wells four times with 0.2% BSA in PBS. Plates were then air dried, and the percentage of bound bacteria in each well was determined by liquid scintillation.

Attachment of radiolabeled bacteria to mammalian cells. One day before each assay, mammalian cells were lifted with 0.05% trypsin and 0.53 mM EDTA (Gibco BRL) and plated in Nunc 96-well break-apart microtiter plates previously UV sterilized and coated with 2 µg/ml MBP-Inv497, an MBP fused to the invasive protein from *Yersinia pseudotuberculosis* (28). Just before the addition of radiolabeled spirochetes, cell monolayers were washed twice with PBS.

Inhibition of binding with exogenous GAGs. Radiolabeled spirochetes were prepared as described above and were incubated for 30 min at room temperature in BSK-H supplemented with 2 mg/ml GAGs. Following incubation, spirochetes were diluted 1:3 with 10 mM glucose, 50 mM NaCl, and 10 mM HEPES at pH 7.0 before addition to wells containing cell monolayers or coated with fibronectin or GAG.

Enzymatic removal of specific classes of GAGs. Monolayers were incubated for 2 h with 0.5 U/ml heparinase I, heparitinase, or chondroitinase ABC (Sigma) at 37°C in RPMI 1640 supplemented with 1% BSA, 10^{-2} trypsin inhibitory units/ml aprotinin, and 165 µg/ml phenylmethylsulfonyl fluoride. After the monolayers were washed twice with PBS, radiolabeled spirochetes were added to treated monolayers as described above.

RESULTS

Expression of BBK32 in strain B314 results in localization of the lipoprotein on the spirochetal surface and confers spirochetal binding to fibronectin and cultured mammalian cells. Some strains of *B. burgdorferi* do not express BBK32 during in vitro culture (14, 39). To ensure high-level expression of BBK32 in our standard nonadherent *B. burgdorferi* strain B314, we first modified the shuttle vector pBSV2 (47) to carry the promoter for *ospC*, which is expressed by this strain during in vitro culture (43) (see Materials and Methods). The BBK32

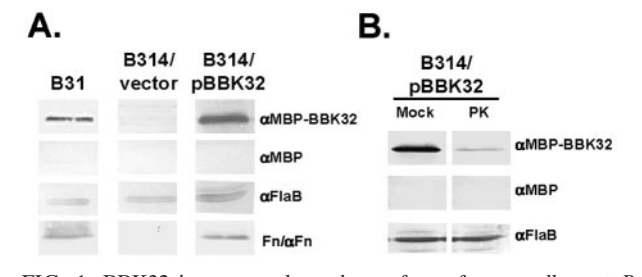


FIG. 1. BBK32 is expressed on the surface of a nonadherent *B. burgdorferi* strain. (A) Lysates of low-passage *B. burgdorferi* B31, *B. burgdorferi* B314 harboring pBBK32 (“B314/pBBK32”), or the control vector pJF21 (“B314/vector”) were separated by 10% SDS-PAGE and immunoblotted with the indicated antibodies (α, anti) or far-Western blotted with fibronectin (“Fn/αFn”). (B) Intact spirochetes were briefly digested with proteinase K, and lysates prepared from these spirochetes were separated by 10% SDS-PAGE and immunoblotted with the indicated antibodies.

gene from low-passage *B. burgdorferi* strain B31 was inserted into the modified shuttle vector pJF21 to generate pBBK32. Strain B314, which as mentioned above lacks the plasmid encoding BBK32 (18), was transformed with pBBK32, and immunoblotting of bacterial lysates with antiserum raised against an MBP-BBK32 fusion, or, as a control, against MBP, revealed that B314/pBBK32 produced abundant BBK32, whereas B314 harboring the control vector pJF21 produced none (Fig. 1A). In fact, B314/pBBK32 produced BBK32 at higher levels than did the low-passage strain B31. Far-Western blotting of lysates of B314/pBBK32 with purified fibronectin revealed that the BBK32 produced by this strain was competent for binding fibronectin (Fig. 1A, bottom). BBK32 produced by B314/pBBK32, but not control periplasmic FlaB protein, was degraded by exposure of spirochetes to proteinase K, demon-

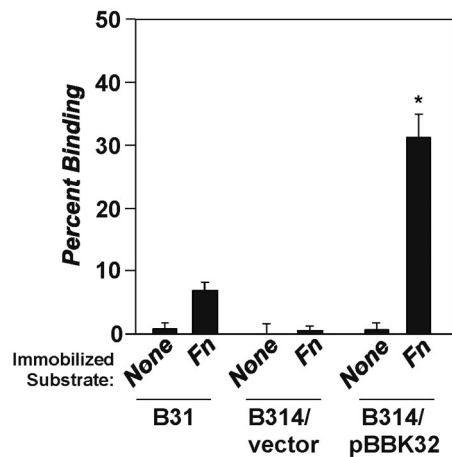


FIG. 2. Expression of BBK32 in a nonadherent *B. burgdorferi* strain promotes efficient attachment to fibronectin. Radiolabeled strains B31, B314/pJF21 (“B314/vector”), and B314/pBBK32 were added to mock-coated wells or wells coated with fibronectin. The percentage of cells stably bound was determined by liquid scintillation counting. Each bar represents the mean of four independent determinations \pm the standard deviation. Asterisk indicates that binding of B314/pBBK32 to fibronectin was significantly greater ($P < 0.05$) than binding of B314/vector.

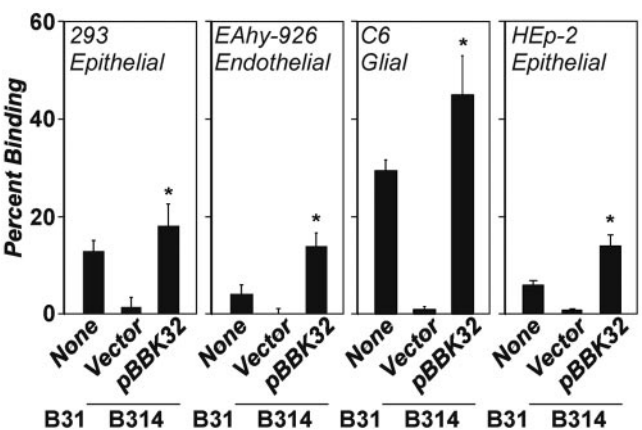


FIG. 3. BBK32 promotes spirochetal attachment to cultured epithelial cells independently of fibronectin. Radiolabeled strains B31, B314/pJF21 (“vector”), and B314/pBBK32 were added to wells containing monolayers of 293 epithelial, EAhy-926 endothelial, C6 glial, or HEP-2 epithelial cells (Fn deficient), and the percentage of cells stably bound was determined. Each bar represents the mean of four independent determinations \pm the standard deviation. Asterisks indicate that binding of B314/pBBK32 to the denoted cell lines was significantly greater ($P < 0.05$) than binding by B314/vector.

strating that the BBK32 was produced predominantly on the surface of the transformant (Fig. 1B).

In order to determine whether production of BBK32 is sufficient to promote spirochete attachment to fibronectin, strain B314 harboring either the shuttle vector pJF21 or pBBK32 was tested for the ability to bind to mock-coated wells or wells coated with fibronectin. Transformants expressing BBK32, but not control transformants, specifically attached to immobilized fibronectin (Fig. 2). In fact, B314/pBBK32 bound to fibronectin significantly better than did strain B31, consistent with its higher level of BBK32 production.

Cultured mammalian cells commonly produce fibronectin (10), so BBK32 was tested for the ability to mediate attachment of strain B314 to different types of cultured mammalian cells. B314/pBBK32, but not B314 harboring the vector control, bound efficiently to 293 kidney epithelial cells, EA-Hy926 endothelial cells, and C6 glial cells (Fig. 3). For each cell line, the level of attachment of B314/pBBK32 exceeded that of strain B31, suggesting that, in these assays, the overproduction of BBK32 was more than sufficient to compensate for the absence of binding pathways mediated by DbpA or DbpB, which are not produced by B314/pBBK32 (17).

BBK32 recognizes glycosaminoglycans on the surface of mammalian cells and promotes spirochete attachment to purified GAGs. It has been reported that HEP-2 epithelial cells do not produce fibronectin (12), and we confirmed that these cells, when cultured under our laboratory conditions, did not produce detectable fibronectin (Table 1). Unexpectedly, B314/pBBK32 bound to HEP-2 cells virtually as efficiently as it bound to 293, EA-Hy926, and C6 cells, which produce easily detectable amounts of fibronectin (Fig. 3, Table 1), suggesting that fibronectin is not the sole receptor for BBK32 produced by mammalian cells. Bacteria that have been grown in dialysis membrane chambers in the rat peritoneum display considerably higher levels of GAG binding than do bacteria grown in

TABLE 1. Fibronectin is not detectable on the surface of HEP-2 epithelial cells^a

Cells	Relative fibronectin expression
None	0.00 ± 0.01
293 epithelial	0.15 ± 0.03*
EAhy-926 endothelial	0.14 ± 0.05*
C6 glial	0.16 ± 0.08*
HEP-2 epithelial	0.01 ± 0.01

^a Microtiter wells containing monolayers of the indicated cell lines were tested for the presence of fibronectin by ELISA using a polyclonal antibody against fibronectin. Stably bound antifibronectin antibody was quantitated with an anti-rabbit antibody conjugated with horseradish peroxidase, and the absorbance at 650 nm was determined. Each point represents the mean of four independent determinations ± the standard deviation. Wells containing cell monolayers with relative fibronectin expression significantly ($P < 0.05$) greater than wells without mammalian cells are indicated with an asterisk.

vitro, and this “host-induced” GAG binding activity could not be entirely accounted for by DbpA, DbpB, or Bgp, the previously identified GAG binding surface proteins of *B. burgdorferi* (37). Therefore, we tested whether BBK32 might recognize GAGs on the host cell surface, using 293 epithelial cells, a cell line which we have previously shown to produce GAGs recognized by *B. burgdorferi* (36).

Specific classes of GAGs on the surface of 293 cells were enzymatically removed by digestion with lyases prior to binding by B314/pBBK32 or, for comparison, strain B31. Removal of heparin-, heparan sulfate-, or chondroitin sulfate-related GAGs with heparinase, heparitinase, or chondroitinase ABC, respectively, significantly reduced host cell binding by both of these bacterial strains (Fig. 4A). Strain B31 appeared somewhat less dependent on heparin- and heparan sulfate-related GAGs for binding to 293 cells than did strain B314/pBBK32, consistent with the fact that B31 produces DbpA and DbpB, which rec-

ognize chondroitin sulfates on this cell line (17). Combination lyase digestion to remove diverse classes of GAGs from 293 cells resulted in a further reduction of binding by B314/pBBK32 (Fig. 4A). The putative recognition of GAGs by BBK32 predicts that exogenous GAGs should inhibit attachment of B314/pBBK32 to 293 cells. Strain B314/pBBK32, or for comparison strain B31, was incubated with purified GAGs prior to binding to 293 cells. Heparin, a heparan sulfate analog, and dermatan sulfate both blocked binding, whereas chondroitin-6-sulfate had no effect (Fig. 4B). Finally, to test whether GAGs are sufficient to promote attachment of *B. burgdorferi* expressing BBK32, B314/pBBK32 was tested for the ability to attach to purified GAGs immobilized in microtiter wells. This strain, like strain B31, attached to wells coated with dermatan sulfate or heparin even more efficiently than to wells coated with fibronectin (Fig. 4C). As predicted, neither strain bound to wells coated with chondroitin-6-sulfate.

To determine whether attachment of strain B314/pBBK32 was due to a direct interaction between BBK32 and GAGs, recombinant MBP-BBK32 was tested for the ability to bind to purified heparin, or as a control, fibronectin, immobilized in microtiter wells. As expected, MBP-BBK32 bound to fibronectin, and with an efficiency 10-fold higher than MBP (Fig. 5). In addition, while MBP-BBK32 did not bind to chondroitin-6-sulfate, it recognized heparin as efficiently as it did fibronectin. These results indicate that BBK32 is capable of promoting *B. burgdorferi* attachment to mammalian cells by recognizing GAGs or fibronectin.

Bacterial attachment to fibronectin mediated by BBK32 is not inhibitable by heparin. The BBK32 fibronectin-binding domain has been localized by deletion analysis to residues 131 to 162 in the unordered N-terminal half of BBK32 (26, 40, 42). If the same domain also promotes GAG binding, exogenous

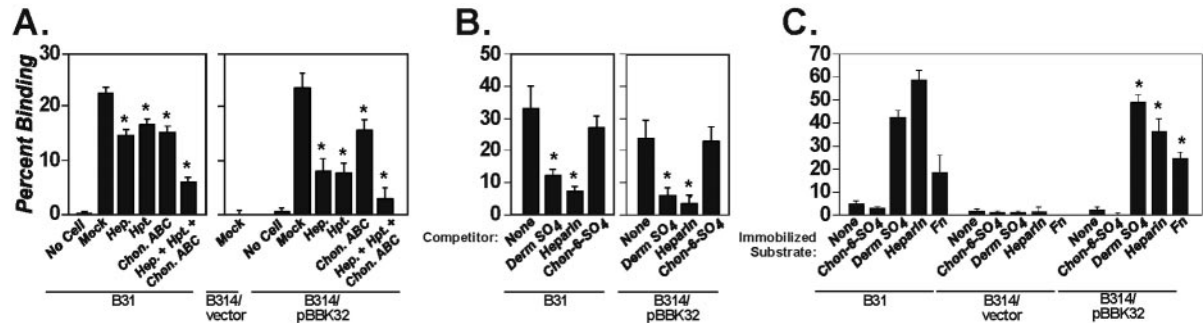


FIG. 4. *B. burgdorferi* producing BBK32 binds to GAGs on the surface of epithelial cells. (A) Radiolabeled strains B31, B314/pJF21 (“B314/vector”), and B314/pBBK32 were added to wells containing monolayers of 293 epithelial cells that had been mock treated or treated with heparinase, heparitinase, or chondroitinase ABC. The percentage of cells stably bound was determined by scintillation counting. Hep., heparinase digestion; Hpt., heparitinase digestion; Chon. ABC, chondroitinase ABC digestion. Asterisks indicate that binding of B314/pBBK32 to cells treated with the denoted lyases or combination of lyases was significantly ($P < 0.05$) less than binding to mock-treated cells. In addition, cell binding after treatment with a combination of lyases was significantly ($P < 0.05$) less than binding after any single lyase treatment. (B) Radiolabeled strains B31 and B314/pBBK32 were incubated with PBS alone or 2 mg/ml of the indicated GAG for 30 min prior to incubation with monolayers of 293 epithelial cells, and the percent of cells stably bound was determined. “Derm SO₄,” dermatan sulfate; “Chon-6-SO₄,” chondroitin-6-sulfate. Each bar represents the mean of four independent determinations ± the standard deviation (SD). Incubation of B314/pBBK32 with dermatan sulfate or heparin significantly reduced ($P < 0.05$; asterisks) spirochete attachment to epithelial cells compared to incubation with chondroitin-6-sulfate. Binding of B314/pBBK32 to cells treated with multiple lyases significantly reduced ($P < 0.05$) binding compared to spirochetes binding to cells treated with the indicated single lyases. (C) Radiolabeled strains B31, B314/pJF21 (“vector”), and B314/pBBK32 were added to mock-coated wells or wells coated with the indicated GAG or fibronectin, and the percentage of cells stably bound was determined. Each bar represents the mean of four independent determinations ± SD. Asterisks indicate that binding of B314/pBBK32 to wells coated with dermatan sulfate, heparin, or fibronectin was significantly greater ($P < 0.05$) than binding of B314/vector.

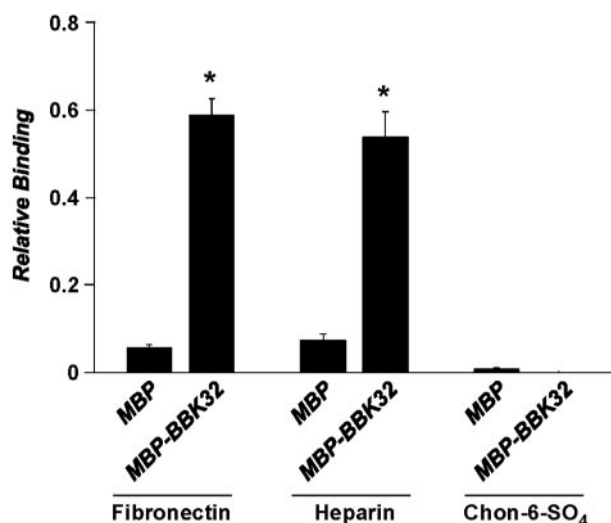


FIG. 5. BBK32 binds to heparin. Recombinant MBP-BBK32 or MBP proteins were added to fibronectin- or GAG-coated wells, and bound protein was quantitated by measuring absorbance at 650 nm after ELISA using MBP antiserum and an anti-rabbit horseradish peroxidase-conjugated secondary antibody. "Chon-6-SO₄," chondroitin-6-sulfate. Each bar represents the mean of four independent determinations \pm the standard deviation. Asterisks indicate that recombinant MBP-BBK32 bound wells coated with fibronectin or heparin significantly better ($P < 0.05$) than MBP.

GAGs might inhibit BBK32-mediated spirochetal attachment to fibronectin. We found that a high (2-mg/ml) concentration of heparin did not diminish attachment of B314/pBBK32 to immobilized fibronectin (Fig. 6). In contrast, binding of this strain to immobilized heparin was diminished fivefold by exogenous heparin, and this inhibition was specific to heparin because chondroitin-6-sulfate had no effect. These results suggest that recognition of fibronectin and GAG binding represent independent activities of BBK32.

DISCUSSION

The development of vectors capable of replication in *B. burgdorferi* has facilitated new approaches in the study of virulence-associated phenotypes of *B. burgdorferi* (13, 47). We previously utilized complementation of the high-passage non-adherent *B. burgdorferi* strain B314 as a straightforward assay for surface molecules that promote spirochetal attachment to mammalian cells and uncovered previously unrecognized GAG-binding activities of the decorin binding proteins DbpA and DbpB (17). Here we utilized the *ospC* promoter, which is active during in vitro culture, to ectopically express lipoprotein BBK32 in strain B314 and found that BBK32 promoted bacterial attachment to purified fibronectin and to mammalian cells in vitro, thus confirming that it indeed functions as a fibronectin-binding adhesin. In addition, production of BBK32 by strain B314 revealed a second adhesive activity, one that promotes binding to diverse mammalian cells through the recognition of dermatan sulfate and heparan sulfate.

It seems likely that the fibronectin- and GAG-binding activities of BBK32 are independent, because high concentrations of heparin could not diminish binding of B314/pBBK32 to

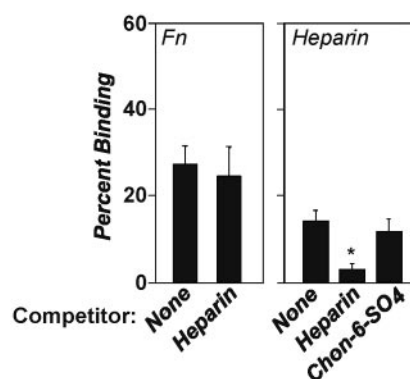


FIG. 6. Attachment of *B. burgdorferi* producing BBK32 to fibronectin is not inhibited by exogenous heparin. Radiolabeled B314/pBBK32 was incubated with the indicated 2 mg/ml heparin prior to addition to fibronectin- or heparin-coated wells, and the percentage of cells stably bound was determined. Each bar represents the mean of four independent determinations \pm the standard deviation. The asterisk indicates that incubation of B314/pBBK32 with heparin resulted in significantly less ($P < 0.05$) spirochetal binding to wells coated with heparin than incubation with chondroitin-6-sulfate.

immobilized fibronectin. These results confirm those of Johnson and coworkers, who showed, using far-Western assays, that heparin did not inhibit binding of fibronectin to BBK32 (39). In addition, the fibronectin-binding domain identified by these investigators, amino acids 131 to 162 (40), contains highly conserved acidic amino acids and seems unlikely to interact with GAGs, which are also highly negatively charged.

B. burgdorferi strain B314 producing BBK32 bound to HEp-2 cells, which did not produce fibronectin detectable by enzyme-linked immunosorbent assay (ELISA), suggesting that GAGs are sufficient to promote BBK32-mediated bacterial adhesion to mammalian cells. In addition, enzymatic removal of GAGs from 293 epithelial cells resulted in an eightfold decrease in binding by B314/pBBK32, suggesting that GAGs represent the major 293 cell receptor for BBK32. In contrast, enzymatic removal of GAGs expressed by Chinese hamster ovary (CHO) cells did not diminish binding by B314/pBBK32, and this recombinant strain bound to CHO cells deficient for GAG synthesis as efficiently as to wild-type CHO cells (J. R. Fischer, unpublished observations). Hence, the relative contribution of GAGs and fibronectin to cell attachment may vary with the target cell.

B. burgdorferi strain N40, when grown in dialysis membrane chambers in the rat peritoneum, a condition designed to mimic conditions of growth during mammalian infection (1), produces a marked increase in binding to purified heparin and to heparan sulfate produced by EAhy-926 endothelial cells (37). Recombinant derivatives of the previously identified GAG binding ligands, Bgp, DbpA and DbpB, were incapable of efficiently inhibiting GAG-mediated cell binding by host-adapted *B. burgdorferi* strain N40, suggesting that this strain produces other GAG-binding surface molecules. BBK32 is not produced by strain N40 during in vitro culture or in the unfed tick but is induced upon tick feeding and mammalian infection (15). Although the regulation of BBK32 in strain N40 appears to differ from that in B31 (39) and although it is unclear whether BBK32 of strain B31 is upregulated in the mammalian host (3),

BBK32 expression in strain B31 cultured in vitro is nevertheless induced upon exposure to mammalian blood (51), consistent with the hypothesis that BBK32 may promote cell adhesion of *B. burgdorferi* during mammalian infection. Thus, the present finding that BBK32 promotes spirochetal attachment to GAGs suggests that this adhesin may contribute to the observed induction of GAG-binding activity by strain N40. We cannot easily test this hypothesis with strain B314/pBBK32 because it lacks lp25, which is required for growth in implanted dialysis membrane chambers. However, our recent isolation of a defined BBK32 mutant of strain B31 will permit a rigorous test of the role of BBK32 in GAG binding by host-adapted bacteria (Fischer, unpublished).

Previously observed *B. burgdorferi* strain differences in GAG-binding specificity were hypothesized to result from the expression of different sets of GAG-binding adhesins, each with unique specificity (36). BBK32 now joins DbpA and DbpB as *B. burgdorferi* proteins that have been documented to mediate spirochetal attachment to GAGs when expressed on the bacterial surface (17). (Bgp is also a candidate GAG-binding adhesin, but ectopic expression of *bgp* in strain B314 did not result in a detectable increase in cell attachment (K. T. LeBlanc, unpublished observation). As predicted, each of these three adhesins demonstrates unique GAG-binding and cell-binding specificities. We previously showed that DbpA mediates bacterial attachment to purified dermatan sulfate and 293 cells (17). DbpB possesses these same activities but additionally promotes binding to purified chondroitin-6-sulfate and C6 glial cells. Neither DbpA nor DbpB is capable of promoting spirochetal attachment to EA-Hy926 endothelial cells. In contrast, we showed here that BBK32 promotes bacterial binding to purified heparin as well as to dermatan sulfate and promotes binding to GAGs expressed by 293 epithelial cells, C6 glial cells, and EA-Hy926 endothelial cells (Fig. 3) (Fischer, unpublished). Differences in GAG binding mediated by the Vsp proteins of *B. turicatae* are associated with differences in tissue tropism (5, 33, 38, 54), suggesting that the GAG-binding adhesins of *B. burgdorferi* similarly influence tissue colonization. Consistent with this notion, infection of wild-type and decorin-deficient mice suggested that decorin may promote the spread of the pathogen and its survival in tissues that express relatively high levels of decorin (4, 32). The present study indicates that BBK32 and DbpA/DbpB not only recognize different protein-associated receptors (fibronectin and decorin, respectively) but also possess unique GAG-binding activities that provide potentially nonredundant function during mammalian infection.

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