**Borrelia burgdorferi** Binds Fibronectin through a Tandem β-Zipper, a Common Mechanism of Fibronectin Binding in Staphylococci, Streptococci, and Spirochetes*

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BBK32 is a fibronectin-binding protein from the Lyme disease-causing spirochete *Borrelia burgdorferi*. In this study, we show that BBK32 shares sequence similarity with fibronectin module-binding motifs previously identified in proteins from *Streptococcus pyogenes* and *Staphylococcus aureus*. Nuclear magnetic resonance spectroscopy and isothermal titration calorimetry are used to confirm the binding sites of BBK32 peptides within the N-terminal domain of fibronectin and to measure the affinities of the interactions. Comparison of chemical shift perturbations in fibronectin F1 modules on binding of peptides from BBK32, FnBPA from *S. aureus*, and SfbI from *S. pyogenes* provides further evidence for a shared mechanism of binding. Despite the different locations of the bacterial attachment sites in BBK32 compared with SfbI from *S. pyogenes* and FnBPA from *S. aureus*, an antiparallel orientation is observed for binding of the N-terminal domain of fibronectin to each of the pathogens. Thus, these phylogenetically and morphologically distinct bacterial pathogens have similar mechanisms for binding to human fibronectin.

Lyx borreliosis is the most prevalent and widespread vector-borne human infection in the northern hemisphere (1). The mechanisms through which *Borrelia burgdorferi* colonizes the host are poorly understood, but the pathogen seems to have evolved a number of strategies that allow it to bind to host tissue. The *B. burgdorferi* genome has been completely sequenced (2) and is remarkable for the large number of sequences encoding predicted or known lipoproteins, including outer surface proteins, many of which are likely to be involved in interactions with host tissue.

Immunolocalization studies suggest that fibronectin (Fn)\(^1\) can bind uniformly over the surface of *B. burgdorferi* (3). Fn is a human extracellular glycoprotein involved in important physiological processes such as cell migration and wound healing. Fn is targeted by the pathogenic bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* through bacterial cell wall-attached Fn-binding proteins (FnBPs) (4). These FnBPs belong to a class of adhesins called “microbial surface components recognizing adhesive matrix molecules” (4–6). The abilities of FnBPs to mediate adhesion to host tissue and invasion of non-phagocytic host cells (7–9) have generated considerable interest in the structural biology of FnBP/Fn binding. The first high resolution structural data for these interactions (10) revealed a novel mechanism of protein-protein interaction and led us to propose a new model for binding of *S. pyogenes* and *S. aureus* to the N-terminal domain (NTD) of Fn (10). The NTD of Fn contains five F1 modules (1–5F1), each with a consensus fold containing a double-stranded antiparallel β-sheet and a triple-stranded antiparallel β-sheet. In this model, structurally disordered Fn-binding repeats (FnBRs) within the bacterial FnBPs bind 1–5F1 or 2–5F1 in the NTD of Fn, primarily by forming an additional β-strand on the triple stranded β-sheet of each F1 module. We have shown previously that these FnBRs contain strings of F1 binding motifs in the correct order to bind the consecutive F1 modules in the NTD (11). FnBPs from both *S. pyogenes* and *S. aureus* contain multiple FnBRs (10).

BBK32, a 47-kDa Fn-binding lipoprotein identified in *B. burgdorferi* (12), is up-regulated under conditions that mimic those encountered by the bacterium during transmission from tick to mammalian host (12) and has also been shown to be present during systemic infection in the murine host (13). In humans, BBK32 antibodies are observed in infected persons (14), suggesting that the protein is present during human infection. Antibodies to BBK32 can partially protect mice from tick-borne infection (13).

BBK32 consists of a C-terminal globular domain and an N-terminal region lacking well-defined secondary structure (15). In prior studies, the Fn-binding activity of BBK32 was localized to a 32-residue peptide within the unstructured domain that shares sequence homology (in the N terminus of the peptide) to the upstream Fn-binding region of an FnBP (SfbI) from *S. pyogenes* (16). This region of SfbI had been shown to bind to the gelatin-binding domain (GBD) of Fn (17) (Fig. 1). More recently, it has been shown that, like the FnBPs from *S. aureus* and *S. pyogenes*, the disordered region of BBK32 undergoes a significant conformational change on binding to the NTD of Fn with an increase in β-sheet content in the complex (15). This suggests that BBK32 could interact with the NTD of Fn in a manner similar to that of the FnBPs of *S. aureus* and *S. pyogenes*. In this study, we show that BBK32 has sequence similarity to F1 binding motifs within *S. aureus* and *S. pyogenes* FnBPs. Although the similarity is limited, we successfully locate a string of F1 binding motifs in the
correct order to bind sequential F1 modules in the NTD of Fn. Comparison of NMR chemical shift perturbations of residues in F1 modules on binding of peptides from BBK32, FnBP, and Sbi provide further evidence for a common mechanism of F1 binding between these pathogens.

MATERIALS AND METHODS

**Recombinant Proteins and Synthetic Peptides—**Uniformly (U) \(^{15}\)N-labeled module pairs 2F13F1 and 4F15F1 (residues 62–151 and 152–244 of mature human Fn, respectively) were expressed in Pichia pastoris and purified using procedures similar to those described previously (10, 18). BBKFF, BBKFo, BBKTT, BBKTTb, BBKT, PyFo5, AuTw1, and AuFo3 were purchased from Alta Bioscience (Birmingham, UK). The N and C termini of these synthetic peptides were capped by AuTw1, and AuFo3 were performed at pH 5. For experiments with BBKTT, BBKTTb, and BBKT, three-dimensional nuclear Overhauser effect spectroscopy-HSQC and total correlation spectroscopy-HSQC spectra of U-15N-1H were recorded at 25 °C in 10 mM sodium/potassium phosphate buffer, pH 7.4, with a VP-ITC microcalorimeter (MicroCal LLC, Northampton, MA). BBKTTb and2F13F1 concentrations were determined by measuring the absorbance at 280 nm. Both the cell (containing the 2F13F1 module pair, 0.22 mM) and syringe solutions (containing BBKTb, 3.07 mM) were degassed at 15 °C for 20 min. One preliminary injection was 2 μl, and 37 injections were of 6 μl with a stirring speed of 310 rpm and a 3-min delay between injections. To take into account heat of dilution, a blank titration was performed by injecting peptide solution into buffer, and the linear prediction of this heat of dilution was subtracted from the main experiment. Data were analyzed using Origin software (OriginLab Corp.), fitting them to a single binding site model.

**RESULTS**

**Prediction of F1-Binding Peptides from BBK32 using S. pyogenes and S. aureus FnBP sequences—**Fig. 1 shows a sequence alignment of residues 126–190 of BBK32 with FnBRs from S. pyogenes and S. aureus (10). Sequence homology between the upstream Fn-binding region (GBD-binding) region of SfbI, an FnBP from S. pyogenes, and BBK32 was reported previously (10). We showed recently that directly C-terminal of the upstream Fn-binding region (GBD-binding) region of SfbI, an FnBP from S. pyogenes, and BBK32 was reported previously (10).
stream Fn-binding region in SfbI is an NTD-binding region containing five FnBRs. Within these FnBRs, we identified motifs that bind to specific F1 module pairs. In this study, we used the limited similarity between these F1 binding motifs and the BBK32 sequence to successfully identify F1 binding motifs of BBK32 in the correct order to bind to sequential F1 modules in the NTD of Fn. Thus, we used Fig. 1 to predict 2F1-3F1-(BBKTT, BBKTTb), 2F1-(BBKTW), 4F1-5F1-(BBKFF), and 4F1-(BBKFo) binding peptides from BBK32.

Binding of BBK32 Peptides to 2F1-3F1—Chemical shift perturbations observed for the backbone amide 1H and 15N nuclei for residues in 4F1 on addition of BBKFF and BBKFo are shown in Fig. 2. On addition of BBKFF to 4F1, significant chemical shift changes are observed in residues in 4F1, with changes in 5F1 restricted to residues near the previously identified interface between the two F1 module pairs (22). Residues 231 and 232 in the D–E loop and residues 236 and 238 in the E strand of 5F1 undergo 15N chemical shift perturbations of 1 ppm on addition of BBKFo but have only small perturbations on addition of BBKFF to 4F1. In 4F1, residues 188 and 190 in the D–E loop, residues 191–195 in the E strand, and a single residue (Val-180) in strand D undergo the largest chemical shift perturbations on addition of BBKFo. The Kd values for binding of BBKFF and BBKFo to 4F1 were determined to be 20 ± 6 and 590 ± 50 μM, respectively. The higher affinity binding of BBKFF is consistent with the binding of this peptide to both 4F1 and 5F1, whereas BBKFo binds only 4F1. Thus, Fig. 2 demonstrates that BBKFF and BBKFo bind to their predicted targets in the N-terminal domain of Fn. In addition, the chemical shift perturbation data support an antiparallel orientation of binding of BBK32 to 4F1 and the importance of the D–E loop and E strand residues in peptide binding.

Binding of BBK32 Peptides to 2F1-3F1—The sequence alignment in Fig. 1 was used to identify 2F1 and 3F1 binding motifs in BBK32. A comparison of chemical shift perturbations of 4F1 resonances on addition of BBKTT (Fig. 3A) and BBKTW (Fig. 3B) clearly shows that both modules in 2F1 were involved in binding to BBKT, whereas BBKTW binds 3F1. In addition, the chemical shift perturbations observed primarily for residues in 3F1. In particular, residues 144 in the D–E loop and residues 145 and 146 in the E strand of 3F1, which show the largest 1H chemical shift changes (of 4F1 residues) on addition of BBKT, undergo only very small changes on binding of BBKTW. Significant changes in the chemical shift of some 3F1 residues are observed on addition of the shorter peptide. These residues (for example, Ser-117 in strand B) are in strands A and B and in the D–E loop, which would be predicted, on the basis of the structure of the homologous 4F1-F1 module pair (22), to form part of an interface between the 2F1 and 3F1 modules. The chemical shift perturbation data support an antiparallel orientation of binding of BBK32 to 2F1, with the N-terminal part of the peptide binding to the C-terminal module in the pair. In BBKT, the C terminus was extended, but this clearly has very little effect on the chemical shift changes in 2F1. This suggested that the C terminus of BBKT correctly marked the boundary of the 2F1 binding motif. The Kd values for the interactions were then compared (Fig. 3D). BBKT bound with a Kd of 230 ± 60 μM, whereas BBKT bound with a Kd of 38 ± 6 μM. This is not consistent with our previous comparisons of F1 binding motifs, where the module-pair binding peptides bind with higher affinity than the single module binding peptides. Thus, the binding of an additional peptide, BBKTWb, which had a shorter N terminus but longer C terminus, was tested. In a comparison of Fig. 3, A and C, it is clear that the main changes in chemical shift perturbation occur in the E strand of 3F1, suggesting that part of the 3F1 binding motif has been removed by truncating the N terminus of the peptide. The Kd for binding of BBKTb...
Fig. 3. The absolute value of chemical shift perturbations of the \( ^1H \) and \( ^{15}N \) backbone amide resonances in \( ^3F1{^2}F1 \) on binding of the BBK32 peptides (Fig. 1). A, BBKTT; B, BBKT; and C, BBKTL. \( ^3F1{^2}F1 \) concentration was 0.2, 0.19, and 0.06 mM in A, B, and C, respectively. \( \beta \)-Strand secondary structure for the F1 modules is indicated at the top (A–E for \( ^3F1 \) and A–E–' for \( ^2F1 \)). Residues that could not be traced because of spectral overlap or because they disappear on peptide binding are marked with X. Proline residues are indicated with \( P \), D and E, titrations of chemical shift perturbation (\( \Delta \delta \)) used to calculate the \( K_D \) for the interaction of \( ^3F1{^2}F1 \) with BBKTT (D) and BBKTL (E). Residue numbers and whether the \( ^H \) or \( ^{15}N \) shift was used are indicated. The curves are drawn using the \( K_D \) calculated from at least eight chemical shift perturbations of the \( ^{15}N \) or \( ^H \) backbone amide resonances of \( ^3F1{^2}F1 \). Chemical shift perturbations are normalized to 1 at saturation. F, ITC profile for the interaction of BBKTL with \( ^3F1{^2}F1 \). Top, heat differences obtained for 37 injections. Bottom, integrated curve with experimental points (○) and the best fit (—). Data were fitted using a one-site model, resulting in the following: stoichiometry, \( n = 0.985 \pm 0.002; K_D = 30 \pm 0.4 \mu \mbox{M}; (H = 9262 \pm 23.8 \text{cal mol}^{-1} \cdot \text{K}^{-1}; S = -6.99 \text{cal mol}^{-1} \cdot \text{K}^{-1}) \). FnBR/NTD interactions are dependent on ionic strength; they are weaker on addition of salt (U. Schwarz-Linek, unpublished data). However, to facilitate the ITC experiment, binding is measured in the presence of 10 mM phosphate buffer.

Based on the first high resolution structural data for Fn/bacterial FnBP interactions (10), we recently proposed a model for \( S. aureus \) and \( S. pyogenes \) binding to Fn in which structurally disordered Fn-binding repeats in the C-terminal region of FnBPs bind to the NTD of Fn primarily by formation of short antiparallel \( \beta \)-strands on the triple-stranded \( \beta \)-sheet of sequential F1 modules of the NTD (4, 10, 11). In this tandem \( \beta \)-zipper mechanism of FnBR/Fn binding, we showed that each F1 module is recognized by short motifs within the bacterial FnBRs, resulting in the formation of a high affinity binding site for Fn (11).

The unstructured N-terminal region of BBK32 from the Lyme disease pathogen \( B. burgdorferi \) has been shown to undergo a significant conformational change with an apparent increase in \( \beta \)-sheet content (15) on binding to the NTD of Fn. This was reminiscent of the results of similar experiments performed using a streptococcal FnBP (23) and consistent with BBK32 binding to Fn using the tandem \( \beta \)-zipper mechanism. In addition, BBK32 had been shown to share sequence homology with the GBD-binding region of SfbI from \( S. pyogenes \) (16). In SfbI from \( S. pyogenes \), binding sites for the NTD of Fn lie C-terminal to the GBD binding site. With the hypothesis that this may also be true for BBK32, we used sequence alignments of BBK32 with NTD-binding regions from FnBPs of \( S. pyogenes \) and \( S. aureus \) (Fig. 1) and identified potential binding sites within BBK32 (BBKTT and BBKF, Fig. 1) for module pairs from the NTD of Fn.

Comparison of chemical shift perturbations on binding of BBK32 peptides to F1 module pairs shows the involvement of \( ^3F1, ^3F1, ^4F1, \) and \( ^5F1 \) in the binding to BBK32. As observed previously for SfbI from \( S. pyogenes \), the orientation of binding of BBK32 to Fn is antiparallel; that is, the C terminus of

**DISCUSSION**

Based on sequence alignments of BBK32 with FnBP from \( S. aureus \) and \( S. pyogenes \), the results of structural studies show that the BBK32 peptides bind to Fn primarily by formation of short antiparallel \( \beta \)-strands on the triple-stranded \( \beta \)-sheet of sequential F1 modules of the NTD (4, 10, 11). In this tandem \( \beta \)-zipper mechanism of FnBR/Fn binding, we showed that each F1 module is recognized by short motifs within the bacterial FnBRs, resulting in the formation of a high affinity binding site for Fn (11).

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Comparison of chemical shift perturbations on binding of BBK32 peptides to F1 module pairs shows the involvement of \( ^3F1, ^3F1, ^4F1, \) and \( ^5F1 \) in the binding to BBK32. As observed previously for SfbI from \( S. pyogenes \), the orientation of binding of BBK32 to Fn is antiparallel; that is, the C terminus of
BBKTT (BBKTw) binds 2F1, and the C terminus of BBKFF (BBKFo) binds 4F1. Thus, we have identified a sequence of F1 binding motifs in BBK32 in the correct order to bind sequential F1 modules (2-5F1) in the NTD of Fn. The antiparallel orientation of binding is consistent with the putative GBD-binding sequence lying N-terminal to the NTD-binding sequence (Fig. 5). As in the FnBRs FnBPA-1–11 and SfbI-1–4 from *S. aureus* and *S. pyogenes*, respectively (10), a 1F1 binding motif has not yet been identified in BBK32.

The chemical shift perturbation data provide important clues to the mechanism of binding. When chemical shift perturbations in 2F1 and 4F1 on binding of BBK32 are compared with those observed on binding of *S. aureus* and *S. pyogenes* Fn-binding peptides (Fig. 4), it is clear that all three pathogens bind to the same surface of the F1 modules. The similarity between the magnitude and direction of the largest shifts is striking given the differences between the bacterial peptide sequences. Furthermore, the evidence for the involvement in the binding of the E strand residues in each of the F1 modules, together with the previous evidence for an increase in β-strand content in BBK32 on binding to Fn (15), provide very strong evidence that BBK32 uses the tandem β-zipper mechanism previously identified for streptococcal binding to Fn (10). Although we have shown that BBK32 contains 2F1, 3F1, 4F1, and 5F1 binding motifs and have identified the approximate N and C termini of the 2-5F1 binding sequence, the sequence similarity of BBK32 with FnBPs from Gram-positive bacteria is insufficient for identification of the specific bacterial residues involved in F1 module binding. The length of BBKFF (18 residues) is consistent with the length of previously identified 4F15F1 binding peptides (PyFF5; 19 residues) (11). The optimal 2F13F1 binding peptide, however, seems to be at least 24 residues long. This is significantly longer than previously identified 2F13F1 binding peptides from *S. pyogenes* (PyTT5, 18 residues; PyTT4, 19 residues) (11) and may justify a different alignment of the sequences in Fig. 1, where, for example, gaps are included between the 2F1- and 3F1 binding motifs. Other alignments of the FnBPs (other than that shown in Fig. 1A) with higher similarity in regions of the 2F1- binding and 4F1-binding motifs of the Gram-positive FnBPs are possible if significant gaps are introduced into these Gram-positive FnBP sequences (Fig. 1B). In addition, sequence similarity cannot be used to identify the ‘register’ of the antiparallel β-zipper interaction with the NTD of Fn (i.e. to identify which BBK32 and Fn residues are opposed in the β-sheet). Having established this unexpected similarity between the NTD-binding mechanisms of *B. burgdorferi* and Gram-positive bacteria, we aim to obtain high resolution structural data for BBK32/NTD complexes to answer these questions.

As in *S. pyogenes* binding to Fn, binding of BBK32 to the NTD occurred in an antiparallel orientation. This is perhaps a little surprising, in that the BBK32 membrane attachment site
is near the N terminus, whereas FnBPs of \textit{S. pyogenes} and \textit{S. aureus} are attached to the bacterial cell wall near the C terminus of the protein. The conservation of the antiparallel orientation of Fn despite the difference in location of the bacterial attachment site suggests that if the role played by the \textit{S. pyogenes} and \textit{B. burgdorferi} FnBPs is similar, it is independent of the orientation of Fn with respect to the bacterial cell surface. That is, in SfbI, 1F1 binds closest (in sequence) to the cell-wall attachment site, whereas in BBK32, the GBD binding site and then 2F1 would lie closest to the bacterial surface (Fig. 5). This would be consistent with previous suggestions (4) that bacterial proteins play a role in activating Fn, so that the RGD sequence, in the central region of Fn, is accessible for integrin binding (4).

FnBP-mediated uptake of \textit{S. aureus} and \textit{S. pyogenes} may allow these bacteria to evade the host immune system or administered antibiotics, aiding the persistence of the bacteria within the host. We and others have suggested that multiple Fn-binding sites may play a role in integrin-clustering and subsequent uptake of \textit{S. pyogenes} and \textit{S. aureus} into epithelial cells (4). Unlike SfbI from \textit{S. pyogenes} and FnBPA from \textit{S. aureus}, which both contain multiple Fn binding sites (11, 24), so far only one Fn-binding site has been identified in BBK32. However, integrin clustering might also be achieved through binding of Fn to multiple copies of the FnBP. Whether BBK32 mediates uptake of \textit{B. burgdorferi} into epithelial or endothelial cells \textit{in vivo} has yet to be established, although \textit{in vitro} invasion of endothelial cells by \textit{B. burgdorferi} has been reported (25). It has also been shown \textit{in vitro} that \textit{B. burgdorferi} is able to attach to the apical surface of endothelial cells, migrate through the intercellular spaces and into the subendothelial matrix. Thus, cellular invasion, which has been suggested to play a role in hematogenous dissemination of \textit{S. aureus} and \textit{S. pyogenes}, may not be necessary for \textit{B. burgdorferi} migration.

The spirochete \textit{B. burgdorferi} is morphologically distinct from the Gram-positive pathogens \textit{S. aureus} and \textit{S. pyogenes}. For example, spirochetes are motile, tightly coiled, bacteria 8–30 µm in length, whereas \textit{S. aureus} and \textit{S. pyogenes} are non-motile spherical bacteria with a diameter of 0.5–1 µm (26). In addition, Gram-positive cocci and spirochetes are contained in different major lineages (kingdoms) of bacteria. Although the precise role(s) of Fn binding in infection have yet to be demonstrated for bacterial pathogens, the identification of a conserved mechanism of Fn binding between streptococci, staphylococci, and spirochetes hints at the importance of this mechanism for the bacteria and suggests that it may be more widespread in bacterial/Fn interactions than has been demonstrated so far.

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