**Borrelia burgdorferi** Protein BBK32 Binds to Soluble Fibronectin via the N-terminal 70-kDa Region, Causing Fibronectin to Undergo Conformational Extension*  

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**Background:** The BBK32 adhesin of *Borrelia burgdorferi* interacts with fibronectin and contributes to infectivity.  
**Results:** BBK32 binds to fibronectin modules 2–5FN and 8FN through a tandem β-zipper and induces conformational change.  
**Conclusion:** The same extended site on fibronectin is targeted by different bacterial adhesins.  
**Significance:** Studies of the mechanism of BBK32-FN interaction enhance understanding of *B. burgdorferi* infection.

BBK32 is a fibronectin (FN)-binding protein expressed on the cell surface of *Borrelia burgdorferi*, the causative agent of Lyme disease. There is conflicting information about where and how BBK32 interacts with FN. We have characterized interactions of a recombinant 86-mer polypeptide, “Bbk32,” comprising the unstructured FN-binding region of BBK32. Competitive enzyme-linked assays utilizing various FN fragments and epitope-mapped anti-FN monoclonal antibodies showed that BBk32 binding involves both the fibrin-binding and the gelatin-binding domains of the 70-kDa N-terminal region (FN70K). Crystallographic and NMR analyses of smaller BBk32 peptides complexed, respectively, with 2–3FN1 and 8–9FN1, demonstrated that binding occurs by β-strand addition. Isothermal titration calorimetry indicated that BBk32 binds to isolated FN70K more tightly than to intact FN. In a competitive enzyme-linked binding assay, complex formation with BBk32 enhanced binding of FN with mAbIII-10 to the 10FNIII module. Thus, BBk32 binds to multiple FN type 1 modules of the FN70K region by a tandem β-zipper mechanism, and in doing so increases accessibility of FNIII modules that interact with other ligands. The similarity in the FN-binding mechanism of BBK32 and previously studied streptococcal proteins suggests that the binding and associated conformational change of FN play a role in infection.

Fibronectin (FN)5 is a versatile glycoprotein that plays important roles in diverse physiological processes such as embryogenesis and development (1, 2). It exists in a dimeric soluble form in body fluids and as an insoluble component of many extracellular matrices. Each monomer of FN consists of 12 type 1 (FN1), 2 type 2 (FNII), and 15–17 type 3 (FNIII) modules; the two monomers are linked by a disulfide bond near the C terminus (3) (see Fig. 1A). In the soluble form, intramolecular interactions result in a more compact conformation (4). FN becomes extended when deposited into extracellular matrices in the process known as FN assembly (5).

Bacterial virulence factors contribute to the invasion and, ultimately, colonization of a host (6). Although patterns of bacterial virulence are organism- and disease-specific, many pathogens utilize FN to adhere to mammalian cells and tissues (7, 8); in some cases FN binding results in invasion (9–11). The most studied FN-binding proteins are FNBPA and SfbI of *Staphylococcus aureus* and *Streptococcus pyogenes*, respectively. FNBPA and SfbI contain multiple FN-binding repeats (FNBRs) that bind 1–5FN1 from the N terminus of FN (see Fig. 1) (12). The intrinsically disordered FNBRs form β-strands with the E-strands (most C-terminal of the five strands in an FN1 module) of sequential FN modules via a tandem β-zipper mechanism (13, 14). When ligated in this manner, the compact form of FN undergoes a conformational change to an extended form so that the integrin-binding sites on 10FNIII modules are exposed (15). Integrins function as cell surface receptors for FN, and the conformational change allows pathogens coated with FN to undergo integrin-dependent host cell invasion (9–11). Although FNBRs bind 1–5FN1 or 2–5FN1, the “functional upstream domain” (FUD) comprising residues 423–471 of SfbI (16) and UR-FnZ-2 comprising residues 370–428 of FnZ from *Streptococcus equi* subsp. *zooepidemicus* (17) bind to an extended site comprising modules 2–5FN1 and 8FN1 in a proposed extended tandem β-zipper (see Fig. 1). This extended interaction appears to be important for efficient invasion (11). Antibody inhibition studies indicate that the β-zipper formed by FUD may extend to encompass 9FN1 and the linker region

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§5 The abbreviations used are: FN, fibronectin; FN70K, fibronectin N-terminal 70-kDa region; FNBPA, fibronectin-binding protein A; FNBR, fibronectin-binding repeat; FnII/III, fibronectin type 1/2/3 module; FUD, functional upstream domain; HSQC, heteronuclear single-quantum coherence; ITC, isothermal titration calorimetry; TOCSY, total correlation spectroscopy; r.m.s.d., root mean square deviation; b, biotinylated.
between 9FN and 1FNIII (16). Other studies have demonstrated that a peptide from FnB6 of Streptococcus dysgalactiae uses the tandem B zipper mechanism to bind to 1-2FN (13), as do FnZ (residues 370–391) (17) and type I collagen (residues 778–799) in binding to 6FN (18) (see Fig. 1).

BBK32, an FN-binding protein (19) from the Lyme disease-causing spirochete Borrelia burgdorferi, contributes to optimal infectivity as the infectious process progresses from colonization to dissemination (20). BBK32 has an FN-binding sequence that is non-repetitive (21) and also differs from FNBPA, SfbI, and FnZ in being linked to the bacterial cell surface by its N terminus rather than the C terminus (22). NMR studies (23) suggested that peptides (BBKFF and BBKTT; see Fig. 1) from an intrinsically disordered segment (residues 21–205 (22)) of BBK32 bind to tandem 4–5FN or 2–3FN modules, respectively, by the tandem B zipper mechanism (22, 23). However, the specific residues of BBK32 involved in the interaction were not identified. A lack of sequence similarity between BBK32 and other FNBPs precluded the identification of the interacting residues based on sequence alone. The sequence 120–205 of BBK32 (hereafter called Bbk32) also contains a motif similar to the LAGESGET motif identified previously in UR-FnZ-2 and SfbI (21, 23), which was subsequently shown to bind to 8FN (17). When the Bbk32 motif (LSGESGET) is aligned with the SfbI and FnZ 8FN-binding regions, the downstream BBKFF and BBKTT sequences are spaced approximately correctly to interact with 2–3FN and 4–5FN (see Fig. 1B) (23). Thus, this sequence alignment suggests that Bbk32 also binds to an extended region of FN comprising 2–3FN and 4–5FN modules. However, there is evidence that a sequence within the same region of BBK32, underlined in Fig. 1B, binds to isolated FNIII modules (24) and, as observed for the anastellin fragment derived from 1FNIII of FN (25), induces FN aggregation.

Here, we have employed complementary techniques to characterize the interaction of Bbk32 with FN. We report experimental evidence that supports the hypothesis that BBK32 uses the tandem B-zipper mechanism to engage an extended binding site on FN, and we explore the effects of such ligation on the conformation of FN.

**EXPERIMENTAL PROCEDURES**

**FN, FN70K, and FN Fragments**—A schematic and nomenclature for FN and FN fragments are shown in Fig. 1A. Throughout the study, FN residues are numbered based on the mature protein; i.e. the N-terminal glutamine after removal of the 31-residue propro-sequence is numbered 1. FN was purified from a fibrinogen-rich plasma fraction by heat precipitation (60 °C; 5 min) of the fibrinogen followed by chromatography (26). Expression and purification of polyhistidine-tagged monoclonic 1–2FN, N–2FNIII, 6–9FN, 7–10FN, 7–14FNIII, and 2–14FNIII and dimeric 6FN–C, 1FNIII–C, and 2FNIII–C were accomplished using recombinant baculovirus and affinity chromatography as described previously (27–29). Expression and purification of unlabeled 2–3FN and 8–9FN, and of uniformly 15N-labeled 8–9FN, were performed as described previously (13, 17). Proteolytic FN70K was prepared as described previously (30). Concentrations were determined using extinction coefficients at 280 nm, which were calculated using the ProtParam tool from ExPAsy. The molarity of FN or FN fragments, whether monomeric or dimeric, was calculated based on the mass of the monomer. A full-length FN monomer was assumed to have an average molecular mass of 250 kDa.

**Monoclonal Antibodies**—Mouse anti-human monoclonal antibodies (mAbs) mAbIII-10, 4D1, 7D5, 5C3, and L8 were described previously (16, 31, 32). Locations of epitopes on FN are indicated in Fig. 1A.

**Polypeptides and Peptides**—Bbk32 was expressed from a pGEX-5X-2 plasmid as a GST fusion protein in Escherichia coli BL21 (DE3). Cultures were grown to an A600 of ~0.6 before induction of expression using 0.4 mm isopropyl-thio-β-D-galactopyranoside. The fusion protein was expressed at 30 °C for ~16 h. The GST tag affinity purification was performed using a GSTrap HP (GE Healthcare) column as per the manufacturer’s instructions. Fractions containing GST-Bbk32 were pooled and dialyzed against 50 mm Tris-HCl, 150 mm NaCl, 1 mm CaCl2, pH 7.5. To proteolytically cleave the GST tag from Bbk32, factor Xa (NEB UK) was added at a final fusion protein:protease ratio of 500:1 (w/w), and the solution was incubated at 4 °C overnight. The solution was diluted 10-fold with H2O, the pH was reduced to 4.6, and then the solution was passed through a 1-ml Hitrap Q XL (GE Healthcare) anion exchange column equilibrated in 20 mm N-methylpiperazine, pH 4.6. Bound protein was eluted from the column using a continuous linear gradient into 20 mm N-methylpiperazine, 1 x NaCl, pH 4.6, over 40 ml at a flow rate of 1 ml/min. Fractions containing cleaved Bbk32 were pooled, dialyzed against H2O, and then lyophilized and stored at −20 °C. FUD was expressed and purified as described previously (16). BBK32EN and BBK32TWL were synthesized by Severn Biotech and Alta Biosciences, respectively. BBK32TWL was capped at the N and C termini by acetylation and amidation, respectively. The concentration of FUD was determined using a molar adsorption coefficient at 280 nm of 2980 M cm−1 × cm2. The concentration of Bbk32 was determined using the bicinchoninic acid (BCA) assay (Pierce) with FUD as the standard dissolved into saline buffer. The concentration of BBK32EN and BBK32TWL was determined by the mass of lyophilized material assuming a water content of 15% (w/w). Biotinylation of FUD or Bbk32 with N-hydroxysulfo-ssuccinimide-biotin (Pierce) was performed as described previously (16). Throughout the study, biotinylated probes are designated by the prefix “b-“.

**FN-Ligand Binding Assays**—Enzyme-linked assays were carried out for direct binding of b-Bbk32 with adsorbed FN and FN fragments and for blocking of b-Bbk32 binding to adsorbed proteins by soluble FN fragments and mAbs. Isothermal titration calorimetry (ITC) was used to compare interactions of Bbk32 and FUD with FN and FN70K. A competitive enzyme-linked assay was also performed to compare effects of Bbk32 or FUD in complex with FN on binding of mAbIII-10 to adsorbed FN and the effect of mAbIII-10 or heparin in complex with FN in solution on binding of b-Bbk32 to adsorbed FN70K. These experiments were carried out as described previously (15, 16) except that in enzyme-linked assays 20 mm Tris, 100 mm NaCl, pH 7.4, buffer was used, and in ITC experiments sodium phosphate buffer with 100 mm NaCl was used. ITC to study the interaction between BBK32EN and 8–9FN and a con-
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RESULTS

The Major Binding Region in FN for Bbk32 Is Located in the FN70K Domain—The sequence alignment in Fig. 1B suggests that Bbk32 mimics UR-FnZ-2 and FUD in binding to an extended site comprising 8–FNI and 2–5FNI modules. However, the region of Bbk32 that is predicted to bind to 3–4FNI contains residues that have been shown to bind to single or tandem FNIII modules in the 1–3FNIII region (24) (Fig. 1). To test the relative importance of the FNI and the adjacent FNIII modules, we examined the ability of b-Bbk32 to bind adsorbed FN or various FN fragments (Fig. 2A) in a direct binding assay. There was significant binding of b-Bbk32 to FN, FN70K, and N-3FNIII, whereas there was no detectable binding of b-Bbk32 at concentrations up to 10 nM to adsorbed 6–9FNI, 1–14FNIII, 2–14FNIII, FN70K-C, or 3FNIII-C (Fig. 2A). In an assay for inhibition of binding of b-Bbk32 to adsorbed FN by a soluble potential competitor, 40 nM soluble FN, FN70K, or N-3FNIII inhibited binding, whereas 40 nM 1–5FNI, 6–9FNI, or 8FNI-C did not (Fig. 2B, left panel). In a similar assay with potential competitors at 1000 nM, 1–5FNI competed for b-Bbk32 binding partially, whereas 6–9FNI, 7–9FNI, or 2–5FNI/FNIII competed for b-Bbk32 binding poorly. When 1–5FNI and 6–9FNI were combined, competition for b-Bbk32 binding was more efficient (Fig. 2B, middle panel). This synergism indicates that both 1–5FNI and 6–9FNI participate in the Bbk32-FN interaction, but are more effective when on the same molecule (as in the FN70K fragment). To investigate further the FNI modules involved in the interaction, we utilized mAbs to map the Bbk32-binding site. In a competitive enzyme-linked assay, 4D1 to 2FNI only slightly blocked b-Bbk32 binding to adsorbed FN, whereas 7D5 to 4FNI, 5C3 to 9FNI, and L8 to 9FNI/FNIII largely prevented b-Bbk32 from binding to adsorbed FN (Fig. 2B, right panel). This is the same inhibitory profile that was found with the four mAbs and FUD binding (15, 16). These results are consistent with the hypothesis, based on the sequence alignment, that Bbk32 binds to FN in the same region as FUD, that is, the FN70K domain (Fig. 1).

Binding of BBK32 Peptides to 2FNI and 8FNI Occurs by β-Strand Addition—Previous NMR studies indicated that BBKTT and BBKFF (within Bbk32) bind to 2–3FNI and 4–5FNI, respectively, in a tandem β-zipper interaction (23). To explore further how Bbk32 binds to FNI modules, we carried out structural studies, concentrating on peptides bound to tandem FNI modules at the two ends of the potential extended binding site, i.e. 2–3FNI and 8–9FNI. Two small peptides were utilized: BBK32EN, which contains the LSGESGEL motif and is predicted to bind 8FNI, and BBK32TwL, which is predicted to bind to 2FNI (Fig. 1).

A chemical shift perturbation assay, in which 1H15N HSQC spectra of 15N-labeled 8–9FNI were recorded with increasing concentrations of BBK32EN (6.0 mM), was grown in 1.4 M sodium/potassium phosphate, pH 6.9, using sitting drop vapor diffusion. The crystal was flash-cooled in liquid nitrogen using 20% (v/v) glycerol as the cryoprotectant. X-ray diffraction data were collected at the European Synchrotron Radiation Facility on the ID33 beamline. Data were processed using Mosflm (34) and scaled using Aimless (35). Molecular replacement using 2–3FNI only (Protein Data Bank (PDB) code 2CG7 (36)) as the model was accomplished using Phaser (37). The structure was refined as described previously (38), with final refinement performed using Phenix.refine (39). Two alternate side-chain conformations were modeled for 2–3FNI Thr-147 and for BBK32TwL Ile-182. Data collection, refinement, and validation statistics are in Table 1.

### Table 1

Data collection, refinement and validation statistics for the 2–3FNI-BBK32TwL structure

| Data collection | ESRF ID33EH1-adsc-q315 |
|-----------------|------------------------|
| Wavelength (Å)  | 1.0039                 |
| Number of images| 130                    |
| Exposure time (s)| 1.0                    |
| Oscillation angle (°) | 0.5               |
| Temperature (K)  | 130                    |
| Resolution (Å)   | 48.49–1.96 (2.03–1.96) |
| Space group      | P632                   |
| Unit cell parameters (Å, °) | 86.58, 86.58, 120.00 |
| No. of unique reflections | 10,524 (1033) |
| Mean I/σ(I)      | 12.9 (4.3)             |
| Average redundancy| 7.2 (7.0)             |
| Data completeness (%) | 99.8 (100.0) |
| Rmerge (%)       | 10.4 (59.0)            |

| Refinement |
|------------|
| Rwork (%)  | 16.1 (21.1) |
| Rfree (%)  | 19.9 (22.9) |
| r.m.s.d. bond distance (Å) | 0.007 |
| r.m.s.d. bond angle (°) | 0.97 |
| Average B factor | 25.9 |

| Ramachandran plot (%) |
|-----------------------|
| Core | 96 |
| Disallowed | 0 |
| No. of protein residues | 104 |
| No. of solvent atoms | 125 |

Values in parentheses refer to the highest resolution shell. ESRF, European Synchrotron Radiation Facility.
565, which is the loop between the 9FNI D- and E-strands (Fig. 3C) that was unassigned in the free form. The appearance of these peaks upon peptide binding suggests that these were previously in a region undergoing conformational exchange that is stabilized upon peptide binding. The peaks corresponding to the 8FNI D-E loop (Gly-516–Gly-520, Fig. 3C) are also significantly more intense in the spectra of the 8–9FNI-peptide complex indicating that these are also stabilized. It was evident, however, that the majority of residues, including those comprising the E-strand of 9FNI, underwent only subtle changes in chemical shift upon peptide binding, suggesting that they are not directly involved in the interaction (Fig. 3B). In the peptide-bound form, 13 residues remain unassigned. Of these, Glu-486, Glu-506, and His-550 are also unassigned in the unbound form. The remaining 10 unassigned residues are the sequence corresponding to the 8FNI E-strand, the intermodule linker, and the A-strand of 9FNI (Fig. 3C). All of these residues undergo exchange between the free and bound chemical shift in the intermediate or slow exchange regime during the chemical shift perturbation assay. Despite the inability to assign these residues in the spectrum of 8–9FNI bound to BBK32EN, the perturbation of these resonances upon the addition of BBK32EN clearly indicates that their environment is altered when 8–9FNI interacts with BBK32EN, as would be expected if BBK32EN binds to the 8FNI E-strand by β-strand addition. Similar results were obtained when studying the interaction of 8–9FNI with FnZ (17). To determine the energetics of the interaction between BBK32EN and 8–9FNI, an ITC experiment was performed in which BBK32EN was titrated into a solution of 8–9FNI. BBK32EN bound 8–9FNI with a $K_d$ of 25.7 $\mu M$ (Fig. 3D and Table 2). The interaction was driven by favorable enthalpy overcompensating unfavorable entropy (Table 2), providing additional evidence for β-strand addition.

Attempts to crystallize 2–3FNI, 4–5FNI, or 8–9FNI in complex with the Bbk32 peptides known to bind to the tandem FNI modules have been unsuccessful. However, the structure of 2–3FNI in complex with BBK32TwL was obtained (Fig. 4A and Table 1). The structures of 2–3FNI (PDB code 2CG7 (36)) and of 2–3FNI bound to BBK32TwL overlay with an r.m.s.d. of 1.13Å. Thus, the module pair itself is largely unaffected by peptide
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binding. The FNI modules retain their canonical fold of a two-stranded and a three-stranded β-sheet. The peptide interacts with the E-strand of the FNI module in an antiparallel orientation with interstrand hydrogen bonds and peptide backbone φ and ψ angles consistent with a β-strand conformation. Therefore, BBK32TwL binds FNI using a β-strand addition mechanism. Salt bridge interactions are formed between Glu-183 and Arg-83 (FNI C-strand), and between Glu-184 and Arg-101 (FNI E-strand) (Fig. 4B; underlined residues are from BBK32).

The side chain of Glu-185 participates in an interaction with the backbone nitrogen of Phe-67 (FNI A-strand) via an ordered water molecule (Fig. 4B). These side-chain interactions are also found in the structures of FNI bound by FNBPA or SfbI peptides (38, 40). There is an additional interaction between the side chains of Glu-181 and Lys-85 (FNI C-strand) that has not been observed in the other structures, but the FNBPA and SfbI peptides do not have a negatively charged residue at this position.

**ITC of the Interactions of Bbk32 with FN and FN70K**—Bbk32 peptides bind to the tandem FNI modules 2–3FNI and 4–5FNI with $K_d$ values of 20 and 30 μM, respectively (23) and to 8–9FNI with a $K_d$ of 25.7 μM (Table 2). Here, the affinity of the interaction between Bbk32 and intact FN or FN70K containing the full binding site was determined. ITC was carried out in sodium phosphate buffer with 100 mM NaCl, pH 7.4, at 25 °C, in parallel with experiments for FUD binding performed under the same conditions. The curves fit a model of two identical binding sites for both polypeptides in dimeric FN (Fig. 5, A and C, and Table 2) and one binding site in monomeric FN70K (Fig. 5, B and D, and Table 2). For both FUD and Bbk32 binding to FN or FN70K, Δ$H$ was highly favorable, and Δ$S$ was unfavorable (Table 2). As observed for FUD, Bbk32 bound to FN70K 20-fold more tightly than to FN, with a $K_d$ of 5.7 nM when compared with 120 nM for FN.

**Binding of Bbk32 to FN Causes Exposure of an Epitope in 10FNIII**—Previous studies revealed that FUD binding leads to exposure of a cryptic epitope in 10FNIII, indicating FN conformational change (15). To determine whether Bbk32 polypeptide also causes a conformational change in soluble FN, the exposure of the mAbIII-10 epitope in 10FNIII was monitored using a competitive enzyme-linked assay. Binding of mAbIII-10 to adsorbed FN was inhibited by the FN-polypeptide complex. At a fixed FN concentration of 10 nM (20 nM monomer), inhibition was dependent on Bbk32 concentration with a near maximal effect when the ratio of polypeptide/FN subunit was 1:1 (Fig. 6A). A similar curve was obtained for FUD as a positive control. We further assessed mAbIII-10 epitope exposure by a similar assay except that mAbIII-10 binding was blocked by increasing concentrations of soluble purified FN by itself or in complex with a 2.5-fold molar excess of the polypeptide (polypeptide/FN subunit). Bbk32, as well as FUD, caused FN to compete manyfold better than FN alone (Fig. 6B). It should be noted that the aforementioned assays were performed in 100 mM NaCl in which the mAbIII-10 epitope is cryptic (31). These data suggest that Bbk32 causes expansion of the FN conformation to a similar degree to FUD.

To corroborate the enhancement of Bbk32 binding by mAbIII-10 ligation of 10FNIII, we compared FN and the FN-mAbIII-10 1:1 (1 IgG per FN subunit) complex as competitors for binding of b-Bbk32 or, as a positive control, b-FUD to adsorbed FN70K. Using the type of competitive enzyme-linked assay shown in Fig. 6, complex formation with mAbIII-10 increased the ability of soluble FN to compete for binding of b-FUD (Fig. 7A) or b-Bbk32 (Fig. 7C) to FN70K. The enhancement was 2-fold for b-FUD and 5-fold for b-Bbk32. Heparin binds to 12–14FNIII (3, 41) and has been shown to cause an FN conformational change involving N-terminal FNI modules (15). Thus, we also examined the effect of heparin on the ability of soluble FN to compete with b-Bbk32 or b-FUD for binding to adsorbed FN70K (Fig. 7, B and D). Heparin caused...
increased binding of b-FUD by 1.5-fold and of b-Bbk32 by 2.5-fold. These results indicate that ligation of more C-terminal FNIII modules enhances binding of Bbk32 to its N-terminal binding site in FN.

**DISCUSSION**

Previously, Raibaud *et al.* (23) suggested that BBK32 bound to the FN modules 2–3FNI via a tandem β-zipper mechanism. Here, using NMR, crystallography, ITC, and various binding
assays, we report experimental evidence that, as with previously characterized streptococcal FNBPs, the FN-binding protein BBK32 from *B. burgdorferi* binds to FN by β-strand addition to an extended binding site comprising 2-FNI and 8-FNI. This finding is remarkable given that *B. burgdorferi* is a pathogen that is distinct from streptococci in its morphology, lifecycle, 

![Figure 5](image-url)  
**FIGURE 5. ITC of interactions between FN and Bbk32 (A), FN70K and Bbk32 (B), FN and FUD (C), and FN70K and FUD (D).** The experiments were performed in sodium phosphate buffer with 100 mM NaCl, pH 7.4, at 25 °C. The raw titration data and the integrated heats, with experimental data (dots) and best fit (lines), are shown in the top and bottom of each panel, respectively. Different volumes were injected during the course of the experiment (1, 4, and 8 μL). The concentrations of proteins and the thermodynamic parameters are shown in Table 2.

![Figure 6](image-url)  
**FIGURE 6. Enhancement of mAbIII-10 binding to FN by Bbk32 in comparison to FUD.** A, effect of increasing peptide concentration on the exposure of the mAbIII-10 epitope in FN determined by competitive enzyme-linked assay. FN, 10 nM, was preincubated with the indicated concentration of FUD or Bbk32. After mAbIII-10 was added, competition of the mixture for mAbIII-10 binding to a surface coated with a solution of 20 nM FN was determined. Data are expressed as the percentage of mAbIII-10 binding in the presence of 10 nM soluble FN without peptide. In controls not shown, the indicated concentration of FUD or Bbk32 did not inhibit binding of mAbIII-10 in the absence of soluble FN. B, enzyme-linked assay of competition of binding of mAbIII-10 to coated 20 nM FN by increasing concentrations of FN alone or FN-peptide complex. FN was preincubated with FUD or Bbk32 in a fixed molar ratio of 1:2.5 FN monomer to polypeptide. Values are expressed as the percentage of mAbIII-10 binding alone to a surface coated with a solution of 20 nM FN. Error bars represent S.D. about the mean of triplicate experiments.
and mode of infection (20). Further, BBK32 is distinct from the streptococcal FN-binding proteins in that it lacks additional FNBRs and attaches to the bacterial cell surface by its N rather than its C terminus (22).

BBK32 contains an LXGESGE sequence, which in FnZ from S. equi subsp. zooepidemicus binds to 8-FNI of FN (17). In collagen the 8-FNI-binding region contains a similar sequence, LXGXGXE (18). NMR spectroscopy and ITC show that the BBK32EN peptide (Fig. 1B) most likely binds to 8-FNI by β-strand addition. Using x-ray crystallography, the BBK32TwL peptide was shown to bind to 2-FNI through interstrand backbone hydrogen bonds. Further, comparison of the crystal structure of BBK32TwL in complex with 2–3-FNI with previously determined structures of the module pair complexed to SfbI or FNBPAs peptides (38, 40) shows that salt bridge interactions between Glu-183 and Arg-83 (2-FNI C-strand), and between Glu-184 and Arg-101 (2-FNI E-strand) (Fig. 4B), are conserved. Gram-positive FNBPAs typically contain an ED(T/S) motif (42) in the 2-FNI-binding sequence, which is not present in the 2-FNI-binding region of BBK32. It was demonstrated previously (23) that different sequence alignments of this region of BBK32 to other FNBPAs were possible; thus, the 2-FNI-binding region of BBK32 could not be identified on the basis of sequence alone. The structure of the 2–3-FNI-BBK32TwL complex resolves this ambiguity and shows that the 2-FNI-binding sequence of BBK32 instead has a non-canonical EEE motif. Thus, the results with BBK32EN and BBK32TwL support the sequence alignment in Fig. 1B and indicate that the major binding region of Bbk32 on FN extends across 2–5-FNI and 3-FNI.

The model is compatible with results using epitope-mapped monoclonal FN antibodies (16). FN antibodies binding to the D-E loop of 4-FNI (Ser-189 recognized by 7D5), the A-B loop adjacent to the E-strand of 3-FNI (Gly-536 recognized by 5C3), and near the presumed interface of 3-FNI and 4-FNIL (Gln-659 recognized by L8) all inhibited Bbk32 binding to FN, whereas an antibody to an epitope on the opposite side of 2-FNI from its E-strand (Arg-76 recognized by 4D1) did not. The ability of both 7D5 and 5C3 to inhibit Bbk32 binding to FN further supports a model in which BBK32 interacts with multiple modules on FN via a tandem β-zipper. In addition, the results with L8, which recognizes a conformationally sensitive epitope requiring both 3-FNI and 4-FNIL, suggest that the binding site may extend to the 4-FNIL module.

Engagement of the extended binding site afforded to Bbk32 by having 2–5-FNI and 4-FNI in the same molecule increases binding affinity at least 1000-fold over binding to FN modules of the three smaller peptides derived from Bbk32. In addition, Bbk32 binding causes a conformational change in FN that is similar to that observed previously for FUD binding as seen via assays using mAbIII-10, which inhibits integrin-mediated cell adhesion and can be considered a surrogate for exposure of the integrin-binding site in 10-FNI (31). The ability of both Bbk32 and FUD to cause conformational changes in FN suggests the effect that these bacterial proteins have on the conformation of FN is critical to the role of FN binding in infection. It has been deduced that multiple FNs bind to tandem FNBRs of Sbl co-operatively, thus allowing a single adhesin to capture up to five FNs (43). Fig. 1B indicates residues of Bbk32 that, as a peptide, bind to 1–3-FNI and cause FN to aggregate (24). This sequence maps to the 3-FNI-binding region and the linker between the 3-FNI- and 4-FNI-binding regions. Although we found that Bbk32 at low concentration binds to the FN70K region with no indication of Bbk32 binding to 1–5-FNIL modules, the 1–3-FNIL-binding residues are largely outside of the presumptive 2-FNI- and 4-FNI-binding motifs. Thus, it is possible that high surface concentrations of Bbk32 cause FN to both change conformation and aggregate. A high density of conformationally changed FN may allow B. burgdorferi to engage host cell integrins or other FN-binding proteins on cells or in extracellular matrices.

For the peptides that have been crystallized in complex with tandem FN modules, Fig. 1B highlights the residues that participate in hydrogen bonds with E-strand atoms of the corresponding FN modules. In each structure, the peptide binds along a broad groove with alternate side chains extending perpendicularly to either side (18, 38, 40); such placement of side chains is demanded by the β-strand conformation. Of the 11 positions with backbone hydrogen bonding that can be compared in multiple structures, the identical residue is found at 4: asparagine and glutamate in peptides interacting with 4–5-FNI and glycine and glutamate in peptides interacting with 2–3-FNI (Fig. 1B). BBK32TwL, BBKFF, BBKTT, and full-length Bbk32 can be aligned with the conserved residue at three of these positions (Fig. 1B). The same three residues are conserved in the alignment shown for FUD. Interestingly, the (E/D)(T/S)

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**Figure 7.** Effect of complex formation with mAbIII-10 or heparin on binding of soluble FN with Bbk32 in comparison to FUD. A and B, competition for binding of 0.5 nM b-FUD to a surface coated with a solution of 20 nM FN70K by increasing concentrations of FN or FN plus mAbIII-10 (present at a ratio of 1 IgG per FN subunit) (A) and soluble FN or FN plus 0.25 mg/ml heparin (B). C and D, competition for binding of 0.5 nM b-BBK32 to FN70K in the presence of FN or FN plus mAbIII-10 (present at a ratio of 1 IgG per FN subunit) (C) and FN or FN plus 0.25 mg/ml heparin (D). Values are expressed relative to biotinylated polypeptide alone binding to coated 20 nM 2-FNI. Error bars represent S.D. about the mean of triplicate experiments.
residues at the C terminus of previously identified 4F1-binding motifs (13) is present in Bbk32 but not FUD.

FN is a vertebrate "invention" that arose 500 million years ago and the only protein with arrays of FNI modules (44). Despite their limited sequence identity, FUD and Bbk32 both bind to FN and to FN70K with low nm affinity. Thus, the "rules" that determine whether an unstructured protein segment can engage the extended binding site on FN by β-zipper formation are lenient. The finding that two fundamentally different bacteria have evolved different proteins that target the same extended binding site on FN raises questions of whether endogenous proteins bind to this site and for what reasons. It is estimated that up to 30% of sequences in the human proteome are intrinsically unstructured (45). A variety of proteins in blood plasma and platelet lysate interact with FN70K via a mechanism that is blocked by FUD (46). It has been proposed that proteins on surfaces of fibroblasts tether FN via the FN70K region, thus causing extension and exposure of its integrin-binding sites, recruitment of integrins, and assembly of FN (27). Our results suggest that identifying these proteins based on their sequence alone is likely to prove challenging.

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