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The Streptococcal Binding Site in the Gelatin-binding Domain of Fibronectin Is Consistent with a Non-linear Arrangement of Modules*

Received for publication, June 22, 2010, and in revised form, August 13, 2010. Published, JBC Papers in Press, September 15, 2010, DOI 10.1074/jbc.M110.156935

Kate E. Atkin1,2, Andrew S. Brentnall1,2, Gemma Harris1,2, Richard J. Bingham3, Michele C. Erat4, Christopher J. Millard5, Ulrich Schwarz-Linek5, David Staunton5, Ioannis Vakonakis6, Iain D. Campbell1, and Jennifer R. Potts1,2,6

From the Departments of 1,4 Biology and 5 Chemistry, University of York, Heslington, York, YO10 5DD, United Kingdom, the 2Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom, and the 3Centre for Biomolecular Sciences, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, Scotland, United Kingdom

Fibronectin-binding proteins (FnBPs) of *Staphylococcus aureus* and *Streptococcus pyogenes* mediate invasion of human endothelial and epithelial cells in a process likely to aid the persistence and/or dissemination of infection. In addition to binding sites for the N-terminal domain (NTD) of fibronectin (Fn), a number of streptococcal FnBPs also contain an upstream region (UR) that is closely associated with an NTD-binding region; UR binds to the adjacent gelatin-binding domain (GBD) of Fn. Previously, UR was shown to be required for efficient streptococcal invasion of epithelial cells. Here we show, using a *Streptococcus zooepidemicus* FnBP, that the UR-binding site in GBD resides largely in the 8F19F1 module pair. We also show that UR inhibits binding of a peptide from the α1 chain of type I collagen to 8F19F1 and that UR binding to 8F1 is likely to occur through anti-parallel β-zipper formation. Thus, we propose that streptococcal proteins that contain adjacent NTD- and GBD-binding sites form a highly unusual extended tandem β-zipper that spans the two domains and mediates high affinity binding to Fn through a large intermolecular interface. The proximity of the UR- and NTD-binding sequences in streptococcal FnBPs is consistent with a non-linear arrangement of modules in the tertiary structure of the GBD of Fn.

Many cell surface-anchored bacterial proteins bind extracellular matrix proteins in the host. These proteins, also known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (1, 2), are likely to play roles in the establishment, dissemination, and/or persistence of infection by mediating interactions with host tissues. Fibronectin (Fn)7

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*Author’s Choice—Final version full access.

1 Both authors contributed equally to this work.
2 Supported by the British Heart Foundation.
3 Present address: Dept. of Chemical and Biological Sciences, School of Applied Sciences, University of Huddersfield, Queensgate, Huddersfield HD1 3DH, United Kingdom.
4 Supported by a FP7 Marie Curie Fellowship.
5 Supported by the Wellcome Trust.
6 To whom correspondence should be addressed: Dept. of Biology, University of York, Heslington, York YO10 5DD, United Kingdom. Tel.: 44-1904-328679; Fax: 44-1904-328825; E-mail: jps16@york.ac.uk.
7 The abbreviations used are: Fn, fibronectin; FnBP, fibronectin-binding protein; NTD, N-terminal domain; GBD, gelatin-binding domain; FnBR, fibronectin binding repeat; UR, upstream region; ESL, electro spray ionization; ITC, isothermal calorimetry; HSQC, heteronuclear single-quantum coherence; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.
are both required for efficient invasion of streptococci into the eukaryotic cell (18).

It is likely that bacterial peptides, in binding Fn, harness (and probably modify) its normal physiological activity. Thus, understanding the mechanism of interaction of FnBPs with Fn will lead to a better understanding not only of the role of FnBPs in infection but also of the activities of Fn itself and how they might be controlled. For example, an S. pyogenes UR-containing peptide has been shown to reduce Fn matrix polymerization (19) and to increase turnover of Fn (20) and collagen I (21), suggesting possible applications in reducing pathological matrix remodeling (22).

Streptococcus zooepidemicus (Streptococcus equi subsp. zooepidemicus) is a frequent cause of opportunistic pyogenic infections in horses and also, although rarely, can cause human infections (23). An FnBP from S. zooepidemicus, FnZ (Fig. 1B), has a sequence organization similar to SfbI from S. pyogenes (2, 24), but the UR-like sequence lies between the first and second of five putative FnBRs (2, 24) (Fig. 1C). Because a similar motif is also present in the α1(I) collagen peptide (Fig. 1C), we hypothesized that bacterial peptides might bind to the GBD via the same mechanism as collagen and compete with collagen for Fn binding.

The aim of this work is to determine the mechanism of binding of streptococcal FnBPs to the GBD. First, we show that a S. zooepidemicus FnZ peptide binds both 8F19F1 and GBD. Second, data are presented that support an anti-parallel β-zipper mode of binding for FnZ to 8F19F1. Last, we show that the S. zooepidemicus FnZ peptide inhibits binding of the collagen peptide to 8F19F1, presumably due to overlap of the binding sites on 8F19F1. The role of conserved residues in 8F19F1-binding motifs and the consequences of the proximity of the NTD- and GBD-binding sites in the streptococcal proteins for the structure of Fn are also discussed.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant 8F19F1—Unlabeled and uniformly 15N-labeled 8F19F1 module pair (Fn precursor residues 516–608) was expressed in Pichia pastoris using a procedure similar to that described previously (25). Secreted protein was concentrated from fermentation media by cation exchange chromatography on a 5-ml SP-Sepharose Fast Flow column (GE Healthcare) at pH 3.0, eluting with a gradient from 0 to 1 M NaCl in 20 mM citric acid over 50 column volumes. Fractions containing protein were incubated with Endo Hf (New England Biolabs) at pH 5.5 to trim the sugars attached to glycosylation sites at Asn528 and Asn542 back to a single GlcNAc. 8F19F1 was further purified by gelatin affinity chromatography on an XK16 column (GE Healthcare) packed with 20 ml of gelatin-Sepharose 4B (GE Healthcare). Protein was loaded in phosphate-buffered saline (PBS) at pH 7.3 at 4 °C and eluted with a gradient from 0 to 4 M urea. The protein was finally purified by size exclusion chromatography on a HiLoad Superdex 75 gel filtration column (GE Healthcare) equilibrated in 40 mM NaH2PO4/K2HPO4, pH 7.3, 150 mM NaCl. The 8F19F1 concentration was determined from absorbance at 280 nm, and the molecular weight was confirmed by electrospray ionization mass spectrometry (ESI-MS). SDS-PAGE analysis of the purified protein showed a single band.

**Expression and Purification of FnZ—DNA for residues 370–428 of FnZ from S. zooepidemicus (24) was produced synthetically (Entelechon) and cloned into a pGEX-6P-1 vector (GE Healthcare). The protein was expressed recombinantly in E. coli BL21 (DE3) as a GST-tagged fusion protein using autoinduction. The fusion was isolated from soluble bacterial cell lysates using a 5-ml GSTrap high performance column (GE Healthcare) equilibrated in PBS, pH 7.3, and eluted using 20 mM Tris-HCl, 50 mM NaCl, 10 mM reduced glutathione, pH 7.0. Appropriate fractions were pooled, and the glutathione was removed using dialysis. After cleavage of the affinity tag using 3C protease (Novagen) (leaving five vector residues, GPLGS, at the N terminus), the FnZ construct was further purified using reversed phase high performance liquid chromatography on a C4 Jupiter column (Phenomenex). Buffer A was 0.15% trifluoroacetic acid (TFA), and buffer B was 100% acetonitrile, 0.1% TFA. Protein was loaded onto the column equilibrated in 10% buffer B and eluted on a continuous linear gradient of 10–45% buffer B in 50 ml, and appropriate fractions were lyophilized. Concentration measurements were made based on mass, and the molecular weight was confirmed by ESI-MS.

**Preparation of Fn Fragments and Synthetic Peptides—The GBD of Fn was obtained as a lyophilized proteolytic fragment (Sigma) and dialyzed into 10 mM NaH2PO4/K2HPO4 (pH 7.3) before use. A recombinant Fn 100-kDa N-terminal fragment (Fig. 1A) was expressed and purified as described elsewhere (26) and dialyzed against PBS (pH 7.3) prior to use. The S. zooepidemicus FnZ synthetic peptides (sequences RNPPLMGGGLAGESGETTPK and LAGESGET) were obtained from Alta Bioscience UK. The N and C termini of the shorter peptide were capped by acetylation and amidation, respectively; the termini of the longer peptide were uncapped. Peptide concentration measurements were made based on mass.

**Isothermal Titration Calorimetry (ITC)—ITC experiments were carried out using a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA). In a typical experiment, the cell contained 1.4 ml of Fn fragment (8F19F1, GBD, or 100 kDa), and the syringe contained 277 µl of FnZ (synthetic peptide or recombinant protein) at a concentration 10–20 times higher than that of the protein in the cell. If possible, the cell concentration was chosen to correspond to a c value of 100–1000, where c = [protein]/predicted Kd (27). Titrations with 8F19F1 and GBD were carried out in 10 mM NaH2PO4/K2HPO4, pH 7.4, at 25 °C. Both the cell and syringe solutions were degassed at 20 °C for 15 min before use. Both sets of titrations were carried out as follows. One preinjection of 2 µl of syringe solution was followed by 39 injections of 7 µl at an injection speed of 0.5 µl/s. Stirring speed was 307 rpm, and there was a delay of 360 s between injections. For both titrations, a separate heat of dilution experiment was performed by injecting peptide into buffer. The averaged heats of dilution were subtracted from the main experiments. The titration with the 100-kDa Fn frag-
NMR Spectroscopy—All experiments were carried out at 25 °C on a Bruker Avance 700-MHz spectrometer. Samples were prepared by dissolving uniformly labeled $^{15}$N-$^{8}$F1$^{9}$F1 to a concentration of 0.2 and 1.0 mM, for binding studies and confirmation of previous $^{8}$F1$^{9}$F1 assignments (25), respectively, in 90% H$_2$O, 10% D$_2$O containing 150 mM NaCl, 20 mM KH$_2$PO$_4$/Na$_2$HPO$_4$, 0.02% sodium azide with pH adjusted to 7.2. A series of heteronuclear single-quantum coherence (HSQC) experiments with excitation sculpting for solvent suppression and with sensitivity enhancement (28) were carried out with processing and referencing were performed using NMRPipe (29), and spectral assignment was performed with CCPNMR Analysis version 1.0.15 (30).

RESULTS

FmZ Binds the 100-kDa N-terminal Region of Fn with High Affinity—Residues 370–428 of FmZ contains a site with high affinity to $^{8}$F1$^{9}$F1 was demonstrated by fluorescence anisotropy measurements. Samples contained a 50 mM concentration (determined by absorbance at 280 nm) of an N-terminally 5-carboxyfluorescein-labeled α1(I) collagen peptide spanning residues 778–799, which has been shown to bind $^{8}$F1$^{9}$F1 with an affinity ($K_a$) of 4.5 μM (17); unlabeled $^{8}$F1$^{9}$F1 at concentrations of 10, 20, or 30 mM; and unlabeled FmZ peptide at increasing concentrations in a 20 mM Tris-HCl (pH 7.4), 150 mM NaCl buffer. Samples were excited at 485 nm with a 515 nm cut-off, and fluorescence was observed at 538 nm using an M5 fluorometer (Molecular Devices) at 25 °C. Differences in fluorescence anisotropy ($F_A$) were simultaneously fit for all

$^{8}$F1$^{9}$F1 concentrations using the formula,

$$F_A = F_{A,\text{final}} + dF_A \left( \frac{[P] - 0.5a}{K_{\text{BL}} + [P] - 0.5a} \right)$$

(Eq. 1)

where

$$a = [P] + [CL] + K_{\text{BL}} - \sqrt{([P] + [CL] + K_{\text{BL}})^2 - 4[P][CL]}$$

(Eq. 2)

where $F_A$ is the fluorescence anisotropy measured at each point, $F_{A,\text{final}}$ is the fluorescence anisotropy at saturation of competing ligand (FmZ peptide), [P] is the protein ($^{8}$F1$^{9}$F1) concentration, [CL] is the concentration of competing ligand, $K_{\text{BL}}$ is the affinity of the competing ligand, and $K_{\text{BL}}$ is the affinity of bound ligand (collagen peptide, 4.5 μM).
The UR/spacer of FnZ binds $^8$F1$^9$F1—A sequence alignment of the UR sequences of FnZ and SfbI with the collagen α1 peptide (residues 778–799; Fig. 1C) shows that the LAGESGET motif is conserved between FnZ and SfbI and has some homology with the collagen peptide. ITC experiments (Fig. 2 and Table 1) show that the peptide spanning residues 370–391 from FnZ (Fig. 1C) binds with micromolar affinity to both the GBD ($K_d$ of 0.54 ± 0.04 μM; Fig. 2B) and $^8$F1$^9$F1 ($K_d$ of 10.9 ± 0.3 μM; Fig. 2C). The relative affinities suggest that the majority of the FnZ peptide binding site is within $^8$F1$^9$F1, with some limited involvement (through direct binding or through stabilizing interdomain interfaces) of other residues in GBD.

FnZ Residues 370–391 Bind to $^8$F1 via an Anti-parallel β-Zipper—The interaction between $^8$F1$^9$F1 and the FnZ peptide was explored using NMR spectroscopy; a series of $^1$H$^15$N-HSQC spectra were acquired for uniformly $^15$N-labeled $^8$F1$^9$F1 with increasing concentrations of peptide (Fig. 3A). The $^1$H$^15$N-HSQC spectrum of $^8$F1$^9$F1 when bound to the FnZ peptide was assigned using three-dimensional $^1$H$^15$N-HSQC-NOESY and $^1$H$^15$N-HSQC-TOSCY experiments. A plot of chemical shift changes between the free and bound forms of $^8$F1$^9$F1 (Fig. 3B)

shows that the most significant differences occur in the E-strand of $^8$F1 (Fig. 3C). Chemical shift changes were also observed in the A-strand of $^8$F1. In addition, four residues in the D-E loop of $^8$F1 could be assigned in the bound form, due to a sharpening of resonances compared with spectra of the unbound form of $^8$F1$^9$F1, where their assignment was not possible. This suggests that in the unbound form, this loop undergoes conformational exchange, resulting in line broadening that is reduced upon peptide binding. $S$. aureus and $S$. pyogenes FnBRs and the collagen α1(I) peptide bind along the E-strand of their respective F1 modules in an anti-parallel orientation. Fig. 3, A and B, shows that a shorter peptide (LAGESGET), from the C terminus of the FnZ $^8$F1$^9$F1-binding peptide, has no affect on $^8$F1 residues (e.g. Tyr$^{585}$ and Cys$^{587}$) that underwent chemical shift changes upon the addition of the longer peptide. Because $^8$F1 residues (e.g. Asn$^{542}$ and Glu$^{536}$) are affected by binding of both peptides, it is clear that FnZ also binds $^8$F1$^9$F1 in an anti-parallel orientation.

The FnZ Peptide Binds Competitively to the Collagen-binding Site of $^8$F1$^9$F1—The ability of the FnZ peptide to compete with the collagen peptide for $^8$F1$^9$F1 binding was tested by fluorescence anisotropy. Fig. 4 shows that increasing concentrations of unla- beled FnZ peptide added to $^8$F1$^9$F1 plus fluorescently labeled collagen peptide led to a steady decrease in anisotropy, suggesting dissociation of the collagen peptide-$^8$F1$^9$F1 complex. The $K_d$ of FnZ peptide binding was determined from this experiment to be 1.9 ± 0.1 μM, indicating tighter binding to $^8$F1$^9$F1 than observed for the collagen peptide under similar conditions by fluorescence anisotropy (4.5 μM) (17). Together, these results suggest that residues 370–391 from FnZ bind to the same region of $^8$F1$^9$F1 as the collagen peptide and through a similar anti-parallel β-zipper mechanism.

**DISCUSSION**

UR sequences are found in FnBPs from pathogenic streptococci (2, 18) and in BBK32, an FnBP from B. burgdorferi (2, 31). The UR binding site in SfbI from $S$. pyogenes had

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**TABLE 1**

<table>
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<th>Fn domain</th>
<th>[FnZ peptide]</th>
<th>[Fn domain]</th>
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<th>$\Delta S$</th>
<th>$K_d$ (μM)</th>
<th>$n$</th>
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<td>0.24</td>
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<td>-77.2</td>
<td>10.9±0.3</td>
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<td>GBD</td>
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<td>0.01</td>
<td>-17.7</td>
<td>-30.8</td>
<td>0.54±0.04</td>
<td>0.96</td>
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</table>

<table>
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<th>[FnZ(370–428)]</th>
<th>[Fn domain]</th>
<th>$\Delta H$</th>
<th>$\Delta S$</th>
<th>$K_d$ (μM)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
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<td>0.001</td>
<td>-54.6</td>
<td>-134.0</td>
<td>0.0008±0.0001</td>
<td>1.06</td>
</tr>
</tbody>
</table>
Streptococcal Binding Site in the GBD of Fn

been located to the GBD (18). Here we localized the FnZ UR binding site within GBD by showing that it binds primarily to the 8F1-5F1 module pair.

We were unable to determine the structure of the 8F1-5F1-FnZ peptide complex using either NMR spectroscopy or x-ray crystallography. However, by close comparison of the UR and collagen peptide sequences with previously identified F1-binding motifs, there is no equivalent negatively charged or polar residue to interact with the C-strand arginine, and in isolation, this motif binds 5F1 only very weakly (13). In 2F1 binding, the aspartate of the EDT makes polar contacts with an arginine in the loop between the D and E strands. The sequence of this loop is very similar in 8F1, and the glutamate in the collagen peptide GER makes a similar interaction (Fig. 5A) as we suggest, does the glutamate in the GET in UR. The 3F1-binding motif lacks the negatively charged residue that could interact with the lysine residue in the D-E loop of 5F1. We predict that the threonine in the GET UR motif interacts with backbone atoms in the B-strand and D-E loop of the F1 module (as observed for homologous hydroxyl-containing residues in 2F1- and 4F1-binding motifs from S. aureus FnBPA (14)). The leucine in the LAG (UR) sequence is likely to be involved in hydrophobic contacts similar to those previously observed for the leucine in the LPG sequence in the collagen peptide (17). The role of the glycine in these motifs is less clear and will require further investigation.

These findings have important implications for the structure of the GBD. Fig. 6 shows a model of the 70-kDa region of Fn that contains both the NTD and GBD and the sequence of UR FnZ-2 (Fig. 1C). The binding sites for F1 modules in the FnZ sequence were used to derive models of the 8F1-5F1-FnZ complex with the 5F1 binding residue and the most C-terminal 8F1-binding residue based on crystal structures of S. aureus FnBPA-1 and FnBPA-5 peptides in complex with 4F1-5F1 (14) and on the understanding the role of conserved residues in the 8F1-binding motif of UR. For example, the glycine residue in the GET (UR) or GER (collagen peptide) appears to be conserved to avoid a steric clash with Trp553 in the E-strand of 8F1 (Fig. 5A). This is similar to the role of a conserved glycine residue in S. aureus FnBR 3F1-binding motifs (Fig. 5A) (14) because the E-strand of 5F1 contains a tryptophan side chain in a similar orientation to the 8F1 E-strand tryptophan (Fig. 5). This E-strand tryptophan residue is not conserved in 2F1, so here the equivalent peptide residue in the FnBR is the glutamate of the EDT motif (Fig. 5B), which forms a salt bridge with an arginine residue in the C-strand of the F1 module (Fig. 5A). In the collagen peptide-8F1-F1 structure, due to the requirement for the glycine, this interaction is shifted two residues along the peptide and is formed by the glutamine in GQR (Fig. 5A) and thus in UR by the glutamate in GES. In 3F1-binding motifs, there is no equivalent negatively charged or polar residue to interact with the C-strand arginine, and in isolation, this motif binds 5F1 only very weakly (13). In 2F1 binding, the aspartate of the EDT makes polar contacts with an arginine in the loop between the D and E strands. The sequence of this loop is very similar in 8F1, and the glutamate in the collagen peptide GER makes a similar interaction (Fig. 5A) as we suggest, does the glutamate in the GET in UR. The 3F1-binding motif lacks the negatively charged residue that could interact with the lysine residue in the D-E loop of 5F1. We predict that the threonine in the GET UR motif interacts with backbone atoms in the B-strand and D-E loop of the F1 module (as observed for homologous hydroxyl-containing residues in 2F1- and 4F1-binding motifs from S. aureus FnBPA (14)). The leucine in the LAG (UR) sequence is likely to be involved in hydrophobic contacts similar to those previously observed for the leucine in the LPG sequence in the collagen peptide (17). The role of the glycine in these motifs is less clear and will require further investigation.

These findings have important implications for the structure of the GBD. Fig. 6 shows a model of the 70-kDa region of Fn that contains both the NTD and GBD and the sequence of UR FnZ-2 (Fig. 1C). The binding sites for F1 modules in the FnZ sequence are indicated, based on our previous work studying homologous proteins from S. pyogenes (12) (13) and S. aureus (14) and on the work presented here. What is immediately striking is that there are only four residues between the most N-terminal 5F1-binding residue and the most C-terminal 8F1-binding residue based on crystal structures of S. aureus FnBPA-1 and FnBPA-5 peptides in complex with 4F1-5F1 (14) and on the...
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FIGURE 5. Comparison of F1-binding motifs and the role of conserved residues. A, structure of F1-F1 binding motif from FnBPA-5 of S. aureus FnBPA (14) (Protein Data Bank entry 3CAL) and of F1-F1 in complex with the peptide from the α1 chain of type I collagen (17) (Protein Data Bank entry 3EIH). Conserved side chains mentioned under "Results" are shown. B, sequence alignments of F1, "F1", and "F1" and of F1-binding motifs. Coloring of side chains is as in A. Sequence numbering is according to UniProtKB accession codes P02751 (fibronectin), P14738 (FnBPA), P72416 (FnZ), and P02452 (collagen α1(1) chain after subtraction of signal and propeptide sequences).

![Diagram showing the comparison of F1-binding motifs and the role of conserved residues.](Image)

FIGURE 6. Model of the 70-kDa (NTD + GBD) domain of Fn in complex with FnZ (370–428) highlighting the unusual extended tandem β-zipper proposed to form across NTD and GBD. The locations of F1-binding motifs in FnZ based on previous data from SfbI-Fn and FnBPA-Fn interactions and from data in the current work are indicated. The question marks indicate that the location of the binding site on F1 is uncertain, as is the conformation of the four residues (purple) between the F1 and F1-binding motifs. The model was assembled from Protein Data Bank entries 3CAL, 2RLO (14), and 3EIH (17).

![Diagram showing the model of the 70-kDa domain of Fn in complex with FnZ.](Image)

8F1–9F1 collagen peptide complex (17). Thus, as suggested previously by the hairpin arrangement of modules in the solution structure of F1-F2-F2 (32), the GBD modules appear to have a non-linear arrangement in the complex. In fact, the FnZ sequence suggests that the 70-kDa (NTD + GBD) fragment of Fn presents a surface that is largely composed of F1 modules from both the NTD and GBD and that streptococcal FnBPs bind across this surface through a highly extended tandem β-zipper, forming a large protein-protein interface.

The UR region in SfbI from S. pyogenes is essential for efficient invasion of bacteria into epithelial cells, resulting in a 10-fold increase in invasion compared with bacteria expressing constructs that lacked this region and contained only the NTD-binding FnBRs (18). The precise molecular mechanism of this effect is unknown. We and others (15, 19) have previously suggested that the role of bacterial FnBRs in adhesion and invasion might be to disrupt intramolecular Fn-Fn interactions that maintain the compact conformation that is observed in solution, thus exposing the integrin binding site in F3. The NTD has been implicated in such intramolecular interactions and was shown recently to interact with F3 modules (26, 33). It is less clear whether the GBD modules are involved in intramolecular interactions outside the GBD, but the high affinity and large intermolecular interface formed by FnBPs that bind both the NTD and GBD might be particularly efficient in disrupting intramolecular Fn-Fn interactions. In addition, the suggestion that IGD sequences within the GBD of Fn might bind to the αvβ3 integrin (34) means that UR binding to the GBD could have a more direct effect on the cell binding activity of Fn.

We showed (Fig. 4) that the FnZ peptide inhibited binding of a peptide from the α1 chain of type I collagen to 8F1–9F1. Further work would be required to determine if FnBPs can inhibit intact collagen-Fn interactions. Recently, it was shown that collagen I matrix turnover is regulated by Fn (21), suggesting that the interaction between polymerized Fn and collagen stabilizes collagen within the extracellular matrix and reduces endocytosis and degradation of collagen. pUR4, a GBD/NTD-binding region from SfbI with homology to FnZ (Fig. 1C), inhibits Fn polymerization and enhances collagen endocytosis and degradation (21). The identification of the UR binding site within GBD and of the role of conserved residues will aid further studies of the function of the GBD in both physiological and pathological processes involving Fn and how bacterial FnBPs might modify or exploit these functions during infection.

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