Fibronectin Receptors from Gram-Positive Bacteria: Comparison of Active Sites†

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ABSTRACT: Many parasitic bacteria express fibronectin binding proteins that are located on the cell surface. These proteins may act as adhesins and mediate the adherence of the microorganisms to fibronectin-containing host tissues. The ligand binding sites in the fibronectin receptor proteins from Gram-positive bacteria are composed of unique 37–48 amino acid long motifs that are repeated 3–4 times. We have now expressed the ligand binding sites of fibronectin receptors from *Staphylococcus aureus*, *Streptococcus dysgalactiae* (two receptors), and *Streptococcus pyogenes* as recombinant proteins. The purified recombinant proteins have the expected molecular weights as indicated by electrospray mass spectroscopy although they migrate abnormally on SDS–PAGE. Each recombinant protein effectively inhibited the binding of ^{125}I-labeled intact fibronectin or the N-terminal fibronectin domain to *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus pyogenes*. The relative inhibitory potency of the different recombinant proteins was similar for all target bacteria and is reflected in their relative affinities for fibronectin. Synthetic peptides corresponding to the repeat units of the ligand binding site of the fibronectin receptor proteins were shown to inhibit the binding of the N-terminal fibronectin fragment to *Streptococcus pyogenes* cells. Together with amino acid sequence comparison, these data demonstrate that the repeat motif of the fibronectin receptor of *Streptococcus pyogenes* conforms to the consensus sequence previously reported for the *Staphylococcus aureus* receptor and to one of the *Streptococcus dysgalactiae* receptors (McGavin et al., 1993).

Fibronectin (Fn) is a dimeric glycoprotein found in high concentrations in the extracellular matrix and body fluids of vertebrates. The primary biological role of Fn appears to be related to the ability of the matrix form of the protein to serve as a substratum for the adhesion of animal cells [for a review, see Yamada (1989)]. Parasitic microorganisms have developed mechanisms that allow them to adhere to and colonize tissues in a host animal, a situation that may provide easier access to nutrients supplied by the host. Some bacteria express surface proteins which serve as Fn adhesins and can mediate the adherence of the bacteria to Fn-containing matrices.

The primary amino acid sequences of Fn receptors from *Staphylococcus aureus* (Signäs et al., 1989; Jönsson et al., 1991), *Streptococcus dysgalactiae* (Lindgren et al., 1993), and *Streptococcus pyogenes* (Talay et al., 1992; Sela et al., 1993) have been deduced by analysis of the respective genes. The different receptor proteins all share characteristic features (Figure 1). A signal sequence composed of 23–38 amino acids is located at the N-terminus. This segment is followed by a unique sequence which in some cases is interrupted by a once-repeated 20–35 amino acid long motif of unknown function. The primary Fn binding site is composed of a 37–48 amino acid long motif which is repeated 3–5 times and located upstream of a proline-rich, putative cell wall spanning domain. A motif, LPXTGX, characteristic for cell-surface proteins in Gram-positive bacteria is found just upstream of a segment rich in hydrophobic residues that may span the cell membrane. A short stretch of positively charged amino acid residues is located at the extreme C-terminus and may represent a cytoplasmic anchor (Schneewind et al., 1993). It should be noted that to date only part of the gene encoding the

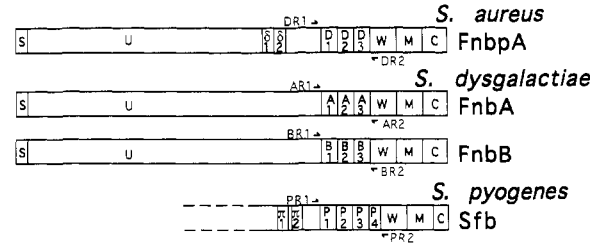

FIGURE 1: Domain organization of Fibronectin receptors from *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus pyogenes*. Fn binding repeat units are represented by A, B, D, and P. FnbpA and Sfb possess upstream repeat sequences indicated by δ and π, respectively. The small arrows indicate the primers (Table 1) used in construction of the plasmid expressing Fn binding repeat motifs. The sequence of the N-terminal portion of Sfb is not available. S, signal sequences; U, sequence unique to the Fn receptor; W, cell wall-spanning region; M, membrane-spanning region; C, intracellular sequence.

Streptococcus pyogenes receptor (Sfb) has been published (Talay et al., 1992); however, the deduced amino acid sequence conforms to the model described above.

We have previously shown that synthetic peptides mimicking the different repeat units in the *Staphylococus aureus* receptor and one of the receptors from *Streptococcus dysgalactiae* all bind Fn and inhibit the binding of Fn to bacterial cells (Lindgren et al., 1993; McGavin et al., 1993). Comparisons of the amino acid sequences of the repeat units in these two receptors revealed a pattern of conserved amino acid residues. Some of these conserved residues were found to be essential for Fn binding activity (McGavin et al., 1993). The repeat motif of the second receptor (FnbB) from *Streptococcus dysgalactiae* did not fit this pattern. Only one of the three repeat units was demonstrated to have Fn binding activity, and this unit does not contain the conserved amino acid residues (Lindgren et al., 1993).

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Table 1: Primers and Templates Used in the Construction of Plasmids Expressing Fibronectin Binding Domains[a]

primer	target gene	PCR template	expression construct	expression product
AR1: 5′CCTGGATCCGGAAATGAAACAGTGGTT3′ AR2: 5′GGTGAAGCTTGGACTCTCTACTTGTCCAGC3′	*fnbA* from *S. dysgalactiae*	pSDF100 (Lindgren et al., 1993)	pFNBD-A	rFNBD-A
BR1: 5′GATGGATCCAAGCCGATTACTGAGGCGAGT3′ BR2: 5′TTGAAGCTTGTCTTCCTTAGGCCACTCGTT3′	*fnbB* from *S. dysgalactiae*	pSDF200 (Lindgren et al., 1993)	pFNBD-B	rFNBD-B
DR1: 5′CCGGATCCGAAGGTGGCCAAAAT3′ DR2: 5′CCGAATTCATTTTGGCCGCTTACTT3′	*fnbpA* from *S. aureus*	pBTFN1 (McGavin et al., 1993)	pFNBD-D	rFNBD-D
PR1: 5′CCGGGATCCACAGGTTTTTCAGGAAATATG3′ PR2: 5′TTCAAGCTTAGTCGCTGTTTCACTGAAACC3	*sfb* from *S. pyogenes*	pKK6414	pFNBD-P	rFNBD-P

[a] The underlined sequences indicate restriction sites used for cloning of the amplified fragments.

Recently, the amino acid sequences of the ligand binding site of the *Streptococcus pyogenes* Fn receptors were reported (Talay et al., 1992; Sela et al., 1993) and found to be composed of a highly conserved motif repeated 4 or 5 times. The *Streptococcus pyogenes* repeat units contain the consensus sequences previously found in the *Staphylococcus aureus* receptor and one (FnbA) of the *Streptococcus dysgalactiae* receptors (Figure 2). The study reported in the present paper was undertaken to compare the specific activity and relative affinity of recombinant forms of the ligand binding domains (designated rFNBD[1] proteins) of different Fn receptors from Gram-positive bacteria.

MATERIALS AND METHODS

Bacteria and Growth Conditions. *Escherichia coli* strain XL1-Blue (Stratagene Cloning Systems, La Jolla, CA) was used as the bacterial host in plasmid cloning. Luria broth (LB; Difco, Detroit, MI) was used as the culture medium for this strain. *Streptococcus pyogenes* strain 6414, obtained from Richard Lottenberg (Department of Medicine, University of Florida, Gainesville), and *Streptococcus dysgalactiae* S2 (Lindgren et al., 1993) were grown in Todd–Hewitt broth (Difco) supplemented with 0.2% yeast extract (Difco). *Staphylococcus aureus* 8325-4 was cultured in brain heart infusion broth (Difco). All bacterial strains were cultured at 37 °C. For Fn binding assays, cells were suspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.3) to a cell density of 2×10^{10} cells/mL, killed by heating at 88 °C for 20 min, and stored at −20 °C.

PCR and Cloning of the Fibronectin Binding Motifs. To produce recombinant proteins, we constructed a series of pQE30-based (Qiagen Inc., Catsworth, CA) expression plasmids that contained DNA segments encoding the ligand binding repeat motifs of the different Fn receptors. The vector pQE30 contains an IPTG-inducible promoter and a segment encoding a polyhistidine leader sequence upstream of the multicloning site. The DNA segments of interest were amplified by polymerase chain reaction (PCR) using a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT) and the Gene Amp Reagent Kit (Perkin Elmer Cetus) according to the manufacturer's guideline. The thermal profile consisted of 30 cycles of 1-min denaturation at 94 °C, 2-min annealing at 55 °C, and 3-min polymerization at 72 °C, except that denaturation was extended to 5 min for the first cycle and polymerization was prolonged to 7 min for the last cycle.

Amplified fragments were treated with proteinase K, digested with restriction endonucleases as described below for each construct, and purified using EluQuick Kit (Schleicher & Schuell, Keene, NH).

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A1:                VVEDTQTSQED-IVLGGPGQVIDFTEDSQPGMSGNNSHT
A2:                ITEDSKPSQEDEVIIGGQGQVIDFTEDTQSGMSGDNSHTDGTV
A3:                LEEDSKPSQEDEVIIGGQGQVIDFTEDTQSGMSGAGQVESP
P1,3:            VETEDTKE--P-EVLMGGQSESVEFTKDTQTGMSGQTTPQ
P2:              VETEDTKE--P-GVLMGGQSESVEFTKDTQTGMSGQTTPQ
P4:              VETEDTKE--P-GVLMGGQSESVEFTKDTQTGMSG
D1:          QNSGNQSFEEDTEEDKP-KYEQGGNIVDIDFDSVPQIHG
D2:          QNKGNQSFEEDTEKDKP-KYEHGGNIIDIDFDSVPHIHG
D3:          FNKHTEIIEEDTNKDKP-SYQFGGHN-SVDFEEDTLPKV
B1: EETLPTEQGQSGSTTEVEDTKG--P-EVIIGGQGEIVDI
B2: EENLPTEQGQSGSTTEVEDTKG--P-EVIIGGQGEVVDI
B3: EESLPTEQGQSGSTTEVEDSKPKLSIHFDNEWPKED
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FIGURE 2: Aligned sequences of the Fn binding repeat units indicated by A, B, D, and P in Figure 1. Sequences of the synthetic peptides used in this study are underlined.

The primers and templates used for amplification of DNA encoding the Fn binding domains of different receptors are specified in Table 1. The primers were designed to contain suitable restriction sites, and their locations along the deduced protein structures are indicated in Figure 1. Oligonucleotide primers AR1, AR2 and BR1, BR2 were used to amplify DNA encoding the A repeats of FnbA and the B repeats of FnbB from *Streptococcus dysgalactiae*, respectively (Lindgren et al., 1993). After digestion with *Bam*HI and *Hin*dIII, the amplified DNAs of the A and B motifs were cloned directly into pQE30, resulting in pFNBD-A and pFNBD-B, respectively. Plasmid pBTFN-1 (McGavin et al., 1993) was used as template DNA to amplify the D repeats, using oligonucleotide primers DR1 and DR2. The amplified fragment was digested with *Bam*HI and *Eco*RI and inserted into the vector pBluescript (Stratagene Cloning Systems). The *Bam*HI/*Hin*dIII fragment of the resulting plasmid was isolated and introduced into pQE30. This construct was named pFNBD-D.

A clone encoding a Fn binding protein was isolated from a λgt11 chromosomal DNA library of *S. pyogenes* strain 6414 obtained from Richard Lottenberg. The sequence of the insert revealed near-complete identity with *sfb* (Talay et al., 1992). The insert was subcloned into the *Eco*RI site of the vector pKK223-3 (Pharmacia, Piscataway, NJ), resulting in pKK6414. Oligonucleotide primers PR1 and PR2 were used to amplify the insert of pKK6414 by PCR. The amplified DNA was digested with *Bam*HI and *Hin*dIII and cloned into pQE30 as described above. The resulting construct was named pFNBD-P.

Expression and Purification of Recombinant Fusion Proteins. Recombinant proteins expressed from the pFNBD plasmids are designated as rFNBD proteins; e.g., rFNBD-A is the expression product of pFNBD-A (Table 1).

Overnight cultures of *E. coli* XL1-Blue harboring the recombinant pFNBD plasmids were diluted 1:50 in 1 L of LB containing 50 μg/mL ampicillin. *E. coli* cells were grown until the culture reached an OD_{600} of 0.5–0.8. Expression of

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; OD, optical density; IPTG, isopropyl β-D-galactoside; PBS, phosphate-buffered saline; rFNBD, recombinant fibronectin binding domain.

the rFNBD proteins was then induced by adding IPTG to a final concentration of 0.2 mM. After a 3-h induction period, cells were collected by centrifugation, resuspended in 15 mL of buffer A (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9), and lysed by passage through a French press (SLM Instrument Inc., Urbana, IL) twice at 11 000 lb./in^2. Cell debris was removed by centrifugation at 50000*g* for 10 min, and the supernatant was passed through a 0.45-μm filter (Nalgene, Rochester, NY).

The recombinant proteins were purified by immobilized metal chelate chromatography, using a column of iminodiacetic acid/Sepharose 6B (Sigma, St. Louis, MO) charged with Ni^{2+} (Porath et al., 1975; Hochuli et al., 1988). Supernatants from cells producing rFNBD-A or rFNBD-B and rFNBD-D or rFNBD-P were mixed with 1.5 and 0.7 mL of charged resin, respectively. Preliminary experiments revealed that these amounts of resin were optimal for the concentration of recombinant proteins in the supernatant. The mixtures were incubated end-over-end for 15 min. The resins with bound protein were centrifuged (1000*g* for 5 min), washed twice with 10 mL of buffer A, and transferred to columns. Subsequently, the columns were washed with 10–20 mL of additional buffer A, and proteins were eluted with 40 mM imidazole in 20 mM Tris-HCl/0.5 M NaCl, pH 7.9. Eluted proteins were dialyzed against PBS to remove imidazole. The polyhistidine leader of these rFNBD proteins has a relatively low affinity for the Ni^{2+}-chelated column when compared to other polyhistidine fusion proteins produced in our laboratory. If excess charged resin is used, the recombinant proteins become contaminated by cellular proteins that have an affinity for the resin and are eluted with 10–50 mM imidazole. By design, the capacity of charged resins used was not quite enough to bind all of the recombinant protein present in the supernatant. This procedure yields ≈30 mg of rFNBD-A and rFNBD-B and ≈15 mg of rFNBD-D and rFNBD-P of pure material. The polyhistidine fusion protein corresponding to a segment of *Staphylococcus aureus* collagen adhesin, CBD-(151–318), was purified as described (Patti et al., 1993) and used as a control.

Molecular Weight Analysis of rFNBD Proteins. Molecular weights were determined by electrospray mass spectrometry and SDS–PAGE. The electrospray mass spectrometry was performed by The Analytical Chemistry Center at the University of Texas Medical School at Houston on a Finnigan TSQ70 upgraded to a TSQ700 equipped with a Vestec ESI source. Protein samples (100 μM) were diluted 1/10 in a 50/50/5 (v/v/v) methanol/water/acetic acid solution prior to analysis. Molecular weight values based on sequence information were also calculated by the UT Analytical Chemistry Center.

SDS–PAGE was carried out using stacking and running gels with 3 and 12% acrylamide, respectively, and the proteins were stained with Coomassie Brilliant Blue.

Preparation and Labeling of Ligands, Synthetic Peptides, and Binding Assay. Porcine Fn was prepared as described by McGavin et al. (1993). The N-terminal Fn fragment was isolated from chymotrypsin-digested Fn using the procedure outlined below. Fn dissolved (concentration 1 mg/mL) in chymotrypsin buffer (25 mM sodium phosphate, 50 mM NaCl, and 0.5 mM EDTA, pH 7.5) was digested with chymotrypsin (5 μg of enzyme for each milligram of Fn) for 20 min at room temperature. The reaction was stopped by addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM. The digest was applied to a gelatin–Sepharose (Vuento et al., 1979; Miekka et al., 1982) column equilibrated with chymotrypsin buffer, and nonbinding material was then applied to a heparin–Sepharose (Pharmacia) column equilibrated with buffer B (25 mM sodium phosphate/0.5 mM EDTA, pH 7.5). The heparin binding material was eluted with a linear gradient of up to 450 mM NaCl in buffer B, dialyzed into 20 mM Tris-HCl, pH 7.0, and applied to a DEAE-Sepharose column equilibrated with the same buffer. The flow-through, constituting the N-terminal fragment of Fn, was dialyzed into PBS.

Labeling with ^{125}I of Fn and the N-terminal fragment was performed according to the chloramine T protocol of Hunter (1978).

Synthesis of peptides has been described in detail elsewhere (Lindgren et al., 1993). The synthetic peptides used in this study are designated as A2, B1, B3, D3, and P1 (Figure 2).

Binding of ^{125}I-labeled ligand to bacteria and the ability of rFNBD proteins and synthetic peptides to inhibit this interaction were quantitated as described by Signäs et al. (1989), except that 10^9 cells were used.

Analysis of Fn/rFNBD Interactions Using Biosensor Technology. The interactions of the various recombinant Fn receptor fragments with Fn were analyzed with a novel biosensor technology, the BIAcore system (Biosensor AB, La Jolla, CA). When an analyte in solution binds to a ligand immobilized on the sensor surface, the increase in mass is quantitated by the optical technique of surface plasmon resonance (Malmqvist, 1993). Individual rFNBD proteins were covalently coupled to the carboxymethylated dextran matrix of the CM5 chip via primary amine groups using the following conditions: The surface was activated by derivatization with 100 mM *N*-hydroxysuccinimide, mediated by treatment with 400 mM *N*-ethyl-*N'*-[(dimethylamino)propyl]-carbodiimide for 4.0 min (flow rate 5 μL/min). Less than 50% of the reactive carboxyl groups on the dextran surface should be converted to reactive *N*-hydroxylsuccinimide (NHS) esters during activation. The NHS ester reacts readily with uncharged primary amino groups on the rFNBD proteins. After activation, a solution of one of the four rFNBD proteins (≈10 μg/mL) in 10 mM formic acid was passed over the surface, resulting in 140–200 response units (RU) bound (approximately 0.2 ng/mm^2). Noncovalently associated protein was washed off with elution buffer (150 mM NaCl, 50 mM HEPES, and 0.05% P-20 surfactant, pH 7.4), and unreacted activated sites on the dextran surface were blocked by treatment with 1 M ethanolamine hydrochloride for 4 min.

Using a continuous flow process (flow rate 5 μL/min), 30 μL of Fn (concentration range 25–400 nM) was passed over the immobilized rFNBD, and the binding was monitored by the change in the response signal (reflecting the change in surface concentration) in real time. To monitor the dissociation of Fn from the immobilized ligand, buffer was passed over the surface containing the Fn/rFNBD complexes for 4 min at a flow rate of 20 μL/min. After each analysis, the biospecific surface was regenerated (residual bound Fn was removed) by passing 30 μL of 1.5 M guanidine hydrochloride and 1 M NaCl through the flow cell.

RESULTS AND DISCUSSION

Molecular Weight Analysis of Recombinant Fn Binding Proteins. The purity of the isolated rFNBD fusion proteins was analyzed by SDS–PAGE (Figure 3). The rFNBD proteins migrated essentially as single bands on acrylamide, indicating a high degree of purity. Occasionally, additional minor bands of lower apparent molecular weights could be detected. These components probably represent degradation

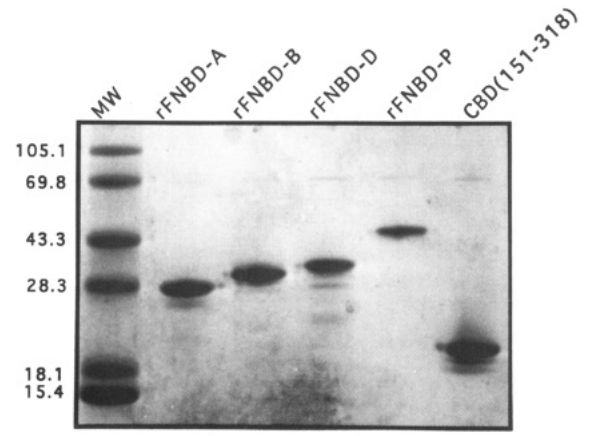

FIGURE 3: SDS–PAGE analysis of rFNBD proteins, the recombinant proteins corresponding to the Fn binding domains shown in Figure 1. CBD(151–318) is a recombinant form of the collagen binding domain of the *Staphylococcus aureus* collagen adhesin (see text). This protein serves as a negative control in the experiments shown in Figures 4 and 5. Molecular weight standards are also shown.

Table 2: Comparison of Calculated Isoelectric Points and Molecular Weights of rFNBD Protens and CBD(151–318) Estimated by Various Methods

	SDS–PAGE	Electrospray	aa sequence	p*I*
rFNBD-A	28K	14.990	14.990	3.9
rFNBD-B	31K	14.595	14.598	4.1
rFNBD-D	35K	15.772	15.769	4.6
rFNBD-P	44K	18.343	18.345	4.2
CBD(151–318)	20K	ND[a]	20.286	7.0

[a] Not determined.

products or premature termination of translation and were not removed.

The relative migratory distance of the different rFNBD proteins on SDS–PAGE suggests that the proteins were of much higher molecular weights than predicted from the corresponding DNA inserts (Table 2). The relatively slow migration properties of the Fn receptor domains were not a consequence of the polyhistidine carrier of the fusion protein since CBD(151–318) (Patti et al., 1993), a fusion protein with the same carrier but with a segment of the *Staphylococcus aureus* collagen adhesin, migrated as predicted (20 kDa) based on the vector construct (Figure 3). The high content of acidic amino acid residues may be responsible for the slow migration of the rFNBD proteins (Table 2), probably due to a decrease in SDS binding capacity (Tung et al., 1972). Molecular weight determinations using electrospray mass spectroscopy indicated that the actual molecular weights of the rFNBD proteins were consistent with those predicted (Table 2). It is worth noting that when the native Fn receptor was isolated from *Staphylococcus aureus*, this protein migrated on SDS–PAGE as a protein of molecular mass 200 kDa, whereas analysis of the corresponding gene predicted a molecular mass of 110 kDa and a p*I* of 4.5 (Signäs et al., 1989).

rFNBD Proteins Inhibit Fn Binding to Bacterial Cells. The rFNBD proteins were compared with respect to their ability to inhibit the binding of ^{125}I-labeled Fn to *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus pyogenes*. All rFNBD proteins effectively inhibited the binding of Fn to each species of the Gram-positive bacteria tested. Similar relative activities were observed regardless of the species of the target bacteria (Figure 4). rFNBD-B was the most effective inhibitor overall, and rFNBD-P was consistently the least effective inhibitor while rFNBD-A and rFNBD-D had intermediate activities. The difference in the recombinant

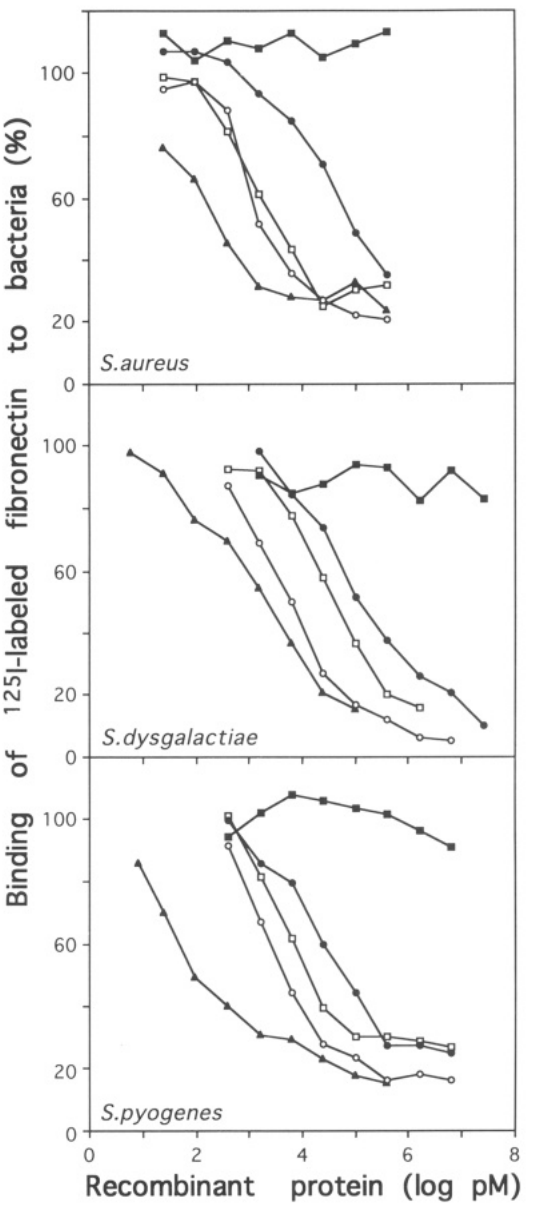

FIGURE 4: Inhibition of bacterial adherence to Fn by the rFNBD proteins. Binding in the absence of competitor was taken as 100%. The data points represent average values of duplicate assays. (□) rFNBD-A; (▲) rFNBD-B; (○) rFNBD-D; (●) rFNBD-P; (■) CBD-(151–318).

proteins' inhibitory activities was also reflected by a difference in their apparent affinities for Fn (see below). The rFNBD proteins also effectively inhibited the binding of the ^{125}I-labeled N-terminal domain of Fn (Figure 5). The relative inhibitory activity of the various recombinant proteins was very similar to those seen when intact Fn was used as a ligand, although small differences were observed (Figures 4 and 5). Most noticeable is that when *Streptococcus pyogenes* is used as a target cell, rFNBD-B has a higher relative inhibitory activity against binding of intact Fn compared to the N-term Fn fragment (bottom panels, Figures 4 and 5). The significance of this difference is unclear at present.

Taken together, these results suggest that the repeat motifs in the identified Fn receptors represent the major Fn binding site present on the various Gram-positive bacterial species. However, we cannot exclude that additional Fn binding sites, with properties similar to those of the repeat motifs, are located outside these domains on the receptor protein or on other bacterial surface molecules. In fact, recent studies of the *Staphylococcus aureus* Fn receptors have shown that an

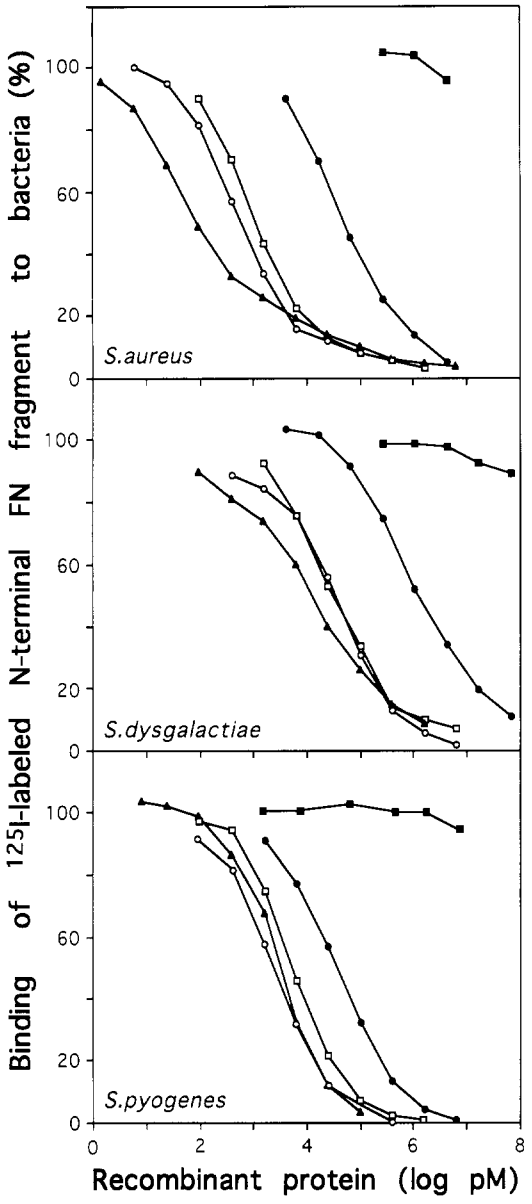

FIGURE 5: Inhibition of bacterial adherence to the N-terminal fragment of Fn. Binding in the absence of competitor was taken as 100%. The data points represent average values of duplicate assays. (□) rFNBD-A; (▲) rFNBD-B; (○) rFNBD-D; (●) rFNBD-P; (■) CBD(151–318).

additional Fn binding site is located just ≈50 amino acids upstream to the domain containing the repeat motif (Jönsson et al., 1992). The amino acid sequences of these additional binding sites contain the features which we have previously identified as essential for Fn binding (see Figure 2). Our recent examination of the amino acid sequence of FnbA of *Streptococcus dysgalactiae* also revealed a stretch of sequence located just upstream to the A repeat motif that shares homology with the A repeat units. A recombinant protein containing this upstream sequence possesses Fn binding activity and competes with the bacterial cells for binding to Fn (manuscript in preparation). Sela et al. (1993) also observed Fn binding ability in the region just upstream to the P repeats of the *Streptococcus pyogenes* Fn receptor.

The maximum extent of inhibition obtained with the rFNBD proteins differed when intact Fn or the N-terminal domain was used as the ligand. For both *Staphylococcus aureus* and *Streptococcus pyogenes*, residual Fn binding corresponding to ≈20% of total binding appears insensitive to the presence of high concentrations of any of the rFNBD proteins (Figure 4). On the other hand, the extent of inhibition approached 100% when the N-terminal Fn domain was used as a ligand (Figure 5). This difference may be explained by the presence of bacterial binding sites on Fn outside the N-terminal domain; such sites have been reported for both *Staphylococcus aureus* and *Streptococcus pyogenes* (Kuusela et al., 1984; Speziale et al., 1984; Bozzini et al., 1992). It is noteworthy that the binding of intact Fn to *Streptococcus dysgalactiae* cells was inhibited to almost 100% by the various rFNBD proteins. This may indicate that binding of Fn to this species of bacteria may only involve the N-terminal domain of the matrix protein.

Kinetic and Affinity Constants of rFNBD Protein/Fibronectin Binding. As described under Materials and Methods, the rFNBD proteins were immobilized, and the association and dissociation rates of Fn binding to the biospecific matrix were measured. A series of typical response curves are shown in Figure 6A where rFNBD-D is immobilized on the carboxymethylated dextran surface. Several Fn concentrations are passed over the immobilized ligand by continuous flow. From 0 to 360 s, the Fn solution is in contact with the biospecific matrix, and rFNBD-D/Fn binding is measured. At higher concentrations of Fn, variations of response (R) vs time become essentially zero, and an equilibrium between free and bound analyte is reached. The dissociation phase begins after 360 s when the Fn solution is replaced with buffer. The variable response changes in the interface between the association and dissociation phase are a result of bulk refractive index changes and were not a factor in the mathematical analysis.

Assuming pseudo-first-order kinetics, the rate equation for binding of soluble Fn to immobilized rFNBD proteins can be represented such that the rate of binding is a linear function of the amount of analyte bound:

$$\frac{dR}{dt} = k_{on}R_{max}C - (k_{on}C + k_{off})R \qquad (1)$$

where k_{on} is the association rate constant, R_{max} is the maximum binding capacity of the immobilized ligand, R is the amount of bound analyte (in resonance units), C is the concentration of analyte in solution, and k_{off} is the dissociation rate constant. Using eq 1, k_{on} can be derived from a plot of dR/dt vs R (Figure 6B). However, an alternative approach that eliminates the necessity of knowing R_{max} is to measure the association rate at several different analyte concentrations and to plot the slope of dR/dt vs R against the concentration:

$$\text{slope} = k_{on}C + k_{off} \qquad (2)$$

The slope of this graph is the measured association rate constant (Figure 6C). In theory, the dissociation rate constant is the intercept on the ordinate when the slope of dR/dt vs R is plotted against C (eq 2). However, dissociation rate constants can be determined more reliably by evaluation of the dissociation of Fn from the biospecific surface after the 400 nM Fn solution is replaced by buffer. The dissociation rate can be obtained directly from a plot of $\ln(R_0/R)$ vs elapsed time (t) using the following equation (derived by integration of the dissociation rate equation):

$$\ln\left(\frac{R_0}{R}\right) = k_{off}(t - t_0) \qquad (3)$$

where R_0 is the response at time = t_0 and R is the response at time t ($t > t_0$) (Figure 6D).

A summary of the results obtained is presented in Table 3.

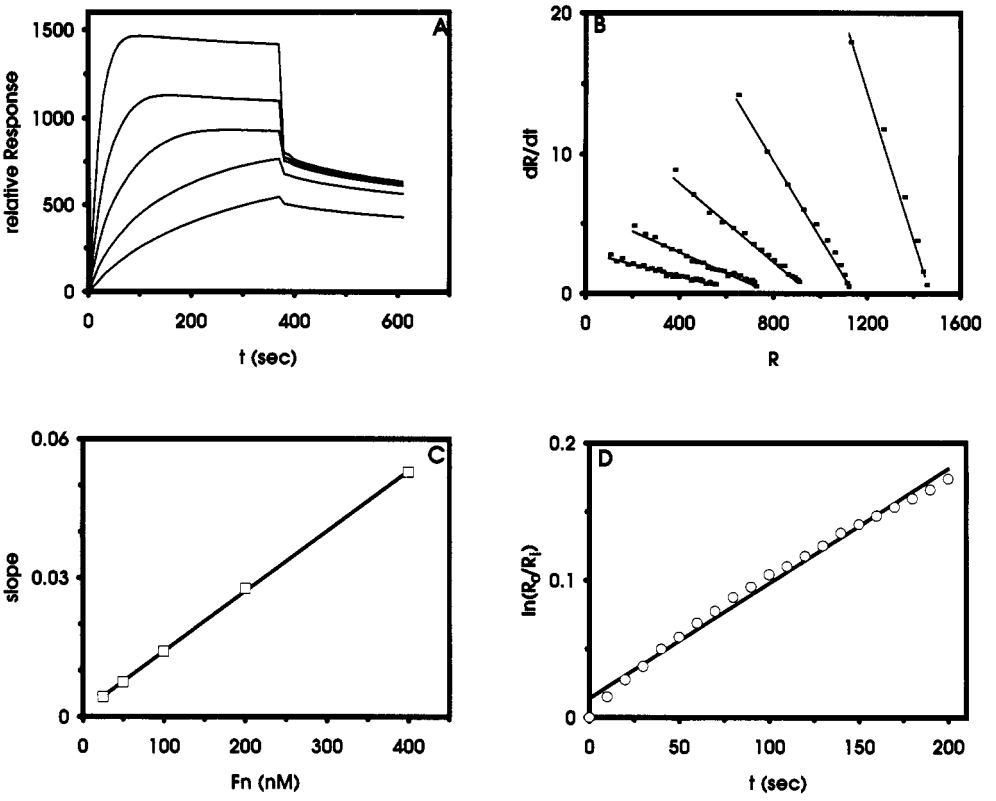

FIGURE 6: Representative data for the BIAcore analysis of the kinetic and binding constants of the rFNBD proteins. (A) Relative response vs time for the binding of Fn to immobilized rFNBD-D. Concentrations represented are (from bottom to top) 25, 50, 100, 200, and 400 nM. The linear portion of the association phase (from t = 0–360 s) is used to determine the slopes of the dR/dt vs R (response) lines shown in (B). Concentrations represented are (from left to right) 25, 50, 100, 200, and 400 nM. (C) The slope of each individual line in (B) is plotted vs each respective Fn concentration to give a line whose slope is the experimentally determined association rate (M^{-1} s^{-1}). (D) Determination of the dissociation rate of Fn bound to immobilized rFNBD-D (slope = dissociation rate in s^{-1}).

Table 3: Summary of Measured Kinetic Rates and Calculated Binding Constants Determined by BIAcore Analysis

Fn binding domain	k_{on} ($\times 10^4$) (M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$) (s^{-1})	K_A ($\times 10^7$) (M^{-1})	K_D (nM)
rFNBD-A	9.9 ± 1	12.0 ± 0.5	8.0	13
rFNBD-B	5.5 ± 0.4	1.8 ± 0.3	31	3.3
rFNBD-D	13.0 ± 0.6	7.8 ± 0.8	16	6.2
rFNBD-P	7.6 ± 0.5	12.0 ± 0.6	6.2	16

The apparent affinity constant (K_A) is equal to k_{on}/k_{off}, and the reciprocal of K_A is the dissociation equilibrium constant (K_D). As expected due to their high degree of homology, all of the rFNBD proteins exhibited similar kinetic and binding constants, and the apparent K_D values for all four were in the nanomolar range.

This linear least-squares analysis of rate data assumes pseudo-first-order kinetics. This may not be a valid assumption for the dissociation rate, and more accurate k_{off} values could possibly be calculated using a nonlinear curve-fitting approach (O'Shannessy et al., 1993). However, this change should only marginally affect the calculated K_D values.

Comparison of the BIAcore results with the analysis of rFNBD proteins inhibiting bacterial cell binding to ^{125}I-Fn (Figure 4) shows some interesting similarities. The calculated K_D values and the measured K_I at 50% inhibition are in the same range. Inhibition studies of rFNBD-B indicate that it is the most effective inhibitor of bacterial cell binding to Fn (Figure 4). This could be explained in part by the linear least-squares analysis of the dissociation data which indicates that rFNBD-B has a somewhat slower dissociation rate when compared to the other three rFNBD proteins. It is interesting that in spite of rFNBD-B's increased inhibitory activity, only

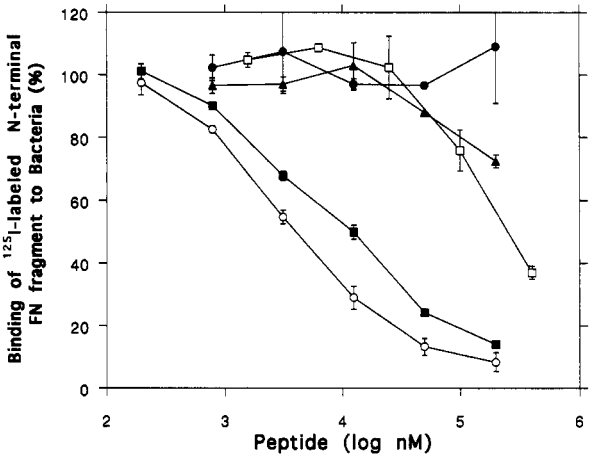

FIGURE 7: Inhibition of *S. pyogenes* adherence to the N-terminal Fn fragment by synthetic peptides. Sequences of the synthetic peptides are shown underlined in Figure 2. Bacterial binding without a peptide was taken as 100%. The data points represent average values of duplicate assays, with bars indicating differences between the values measured in duplicate. (○) A2; (●) B1; (■) B3; (□) D3; (▲) P1.

one of the repeat units, B3 (Figure 2), revealed inhibitory activity when each synthetic repeat was tested individually (Figure 7; Lindgren et al., 1993). Moreover, the amino acid sequence of B3 lacks some of the conserved amino acid residues thought to be essential for Fn binding in the other repeat units. That rFNBD-B may be binding Fn via a different mechanism is a question that will be addressed in future studies.

A Synthetic Peptide Mimicking the Repeat Unit in the Streptococcus pyogenes Fn Receptor Inhibits Fn Binding to Bacteria. In our previous studies, we have demonstrated that

synthetic peptides mimicking the individual repeat units of the Fn receptors from Gram-positive bacteria often inhibit the binding of Fn to the bacteria (Lindgren et al., 1993; McGavin et al., 1993). We therefore synthesized one of the repeat units (called P1) of the *Streptococcus pyogenes* Fn receptor (see Figure 2) which has not previously been examined, and compared its inhibitory activity with peptides mimicking repeat units in Fn receptors from various Gram-positive bacteria.

Peptide P1 was shown to inhibit binding of the ^{125}I-labeled N-terminal Fn domain to *Streptococcus pyogenes*, but P1 was substantially less effective than A2 and B3 and somewhat less effective than D3 peptide (Figure 7). As previously reported, peptide B1 had no inhibitory activity at the concentrations tested. The low activity of P1 may be predicted from the observation that rFNBD-P had the lowest Fn binding activity (Figures 4 and 5) and highest dissociation constant (Table 3).

In the cases of rFNBD-A, -D, and -P, multivalency or cooperativity in binding of intact repeat structures is strongly suggested by the finding that the entire repeat domains in the rFNBD proteins have by far more potent Fn binding activities than individual repeat units represented by synthetic peptides (Lindgren et al., 1993; McGavin et al., 1993; Figure 7). The interaction of rFNBD-B with Fn may be through a different mechanism because only one (B3) of the repeat units revealed Fn binding activity in our assays, and this sequence does not follow the pattern observed in the repeat motifs of the other Fn receptors (Lindgren et al., 1993; McGavin et al., 1993).

CONCLUDING REMARKS

In this paper, we report that the repetitive motif P of the *Streptococcus pyogenes* Fn receptor also conforms to the model described for the *Staphylococcus aureus* and *Streptococcus dysgalactiae* receptors. Furthermore, a synthetic peptide corresponding to the P1 repeat unit exhibits a low but significant inhibitory activity toward Fn binding of *Streptococcus pyogenes*.

The ability of rFNBD proteins to cross-inhibit different species of bacteria may suggest that although sequences of the ligand binding sites of the Gram-positive bacterial Fn receptors vary considerably, they bind Fn in a very similar manner. Alternatively, these recombinant proteins may interact with Fn in different ways and sterically block the access of the bacterial cells to the ligand. In either case, it seems possible to construct an inhibitor which could broadly affect Gram-positive bacterial adherence to Fn.

Some eucaryotic cells, e.g., rat and human macrophages, express a Fn receptor that binds the N-terminal domain of Fn (Blystone et al., 1992). However, the binding mechanism of these eucaryotic proteins has yet to be characterized.

Gram-negative bacteria may adhere to Fn by a mechanism similar to that of the Gram-positive bacteria. The E protein, a component of P fimbriae of *E. coli*, possesses an acidic sequence, DFTVD, analogous to the repeats of the Gram-positive bacterial Fn receptors (Westerlund et al., 1993). Although it is not clear whether the acidic sequence is involved in Fn binding of the protein, the E protein binds the N-terminal domain of Fn through a protein–protein interaction (Westerlund et al., 1991). Furthermore, we observed that a number of synthetic peptides corresponding to the Gram-positive bacterial Fn binding repeats inhibit adherence of an *E. coli* strain to Fn (unpublished data), suggesting that Gram-negative bacteria may express Fn receptors with a similar binding mechanism to those studied in this paper.

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