Interaction of \textit{Staphylococcus aureus} Fibronectin-binding Protein with Fibronectin

AFFINITY, STOICHIOMETRY, AND MODULAR REQUIREMENTS*

From the \textsuperscript{a}Department of Biochemistry, American Red Cross Holland Laboratory, Rockville, Maryland 20855, \textsuperscript{b}Department of Dermatology, The Johns Hopkins University, Baltimore, Maryland 21287-0900, and \textsuperscript{c}Department of Laboratory Medicine and Pathobiology, University of Toronto and \textsuperscript{d}Department of Microbiology, Sunnybrook and Women’s College Health Science Centre, 2075 Bayview Avenue, Toronto, Ontario M4N 3M5, Canada

Kenneth C. Ingham\textsuperscript{a}, Shelesa Brew\textsuperscript{a}, Daryel Vaz\textsuperscript{a}, Daniel N. Sauder, and Martin J. McGavin\textsuperscript{a,b,c,d}

The repetitive D1, D2, and D3 elements of \textit{Staphylococcus aureus} fibronectin-binding protein FnBPA each bind the N-terminal 29-kDa fragment (N29) of fibronectin with low micromolar dissociation constants (K\textsubscript{d}), but in tandem they compose a high affinity domain (D1–3). An additional seven Fn-binding segments have been predicted in FnBPA in a region N-terminal of the D-repeats (Schwarz-Linek, U., Werner, J. M., Pickford, A. R., Gurusiddappa, S., Kim, J. H., Pilka, E. S., Briggs, J. A., Gough, T. S., Höök, M., Campbell, I. D., and Potts, J. R. (2003) Nature 423, 177–181). We have evaluated the requirements for high affinity binding of N29 to the D-repeat domain and determined the affinity and stoichiometry of N29 binding to segments that are N-terminal of the D-repeats in the related FnBPB adhesin. We confirmed that D1–3 has two equivalent high affinity sites (K\textsubscript{d}, \textsuperscript{1}N 1 nM) and provided evidence for one or more lower affinity sites (K\textsubscript{d}, \textsuperscript{2}N 0.5–10 nM). Bimodal D1–2 and D2–3 exhibit intermediate affinity sites with respective K\textsubscript{d} values of 0.25 and 0.044 \mu M, as well as a low affinity site with a K\textsubscript{d} value of 2.2–2.5 \mu M. We also identified two binding domains that are N-terminal of the D-repeats, designated DuB and DuA. Segments internal to these domains individually bound N29 with similar K\textsubscript{d} values of \textasciitilde 2 \mu M, whereas the DuBA polypeptide possessing both segments and other intervening sites bound four molecules of N29 with much higher affinity (K\textsubscript{d}, \textsuperscript{1}N 10 nM). DuBAD, a larger polypeptide harboring all of the known or predicted binding motifs in FnBPB, bound seven to eight molecules of N29, with a K\textsubscript{d} of \textasciitilde 7 nM. Because most of the isolated binding segments display low affinity for N29 and lack motifs for binding of one or both of the \textsuperscript{1}F1 and \textsuperscript{5}F1 modules in the N-terminal domain of Fn, we propose that high affinity is achieved in part as a consequence of self-interaction between bound molecules of N29.

\textit{Staphylococcus aureus} is the leading overall cause of nosocomial infections and is able to infect virtually every tissue and organ system of the body (1, 2). The increasing incidence of multiply antibiotic-resistant \textit{S. aureus} strains and the emergence of vancomycin-resistant, multiply antibiotic-resistant \textit{S. aureus} (3) have placed renewed interest on alternative means of prevention and control of infection. In this regard, members of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM)\textsuperscript{1} family of adhesion proteins have come under intensive scrutiny due to their ability to promote adhesion to the extracellular matrix that surrounds and anchors cells in tissue (4, 5), thus representing attractive targets for therapeutic and vaccination strategies aimed at interfering with colonization. The fibronectin-binding proteins FnBPA and FnBPB are archetypal members of the MSCRAMM family and are encoded by tandem genes fnbA and fnbB (6, 7).

As exemplified by FnBPA, members of the MSCRAMM family display a modular architecture (Fig. 1A). Following the traditional domain nomenclature first used to describe FnBPA (7), the N terminus is a region of \textasciitilde 500 amino acids designated the A domain, which is followed by the B-region composed of two 30-amino acid repeats, a short spacer designated C, and then the D-repeat domain composed of three complete 37- or 38-amino acid repeats and part of a fourth repeat. The C terminus of FnBPA and other MSCRAMMs is dedicated to anchoring the proteins within the peptidoglycan layer of the Gram-positive cell wall. Initial studies established that the D-repeat domain of FnBPA engaged a 29-kDa N-terminal domain (N29) of Fn, with an affinity in the low nanomolar range (7). We subsequently found that the individual D1 and D2 repeats each bound N29 with K\textsubscript{d} values of \textasciitilde 11–14 \mu M, compared with a K\textsubscript{d} of \textasciitilde 2 \mu M for the D3 repeat, and a recombinant polypeptide possessing the three D-repeats in tandem (D1–3) displayed a high affinity of 1.5 nM (8). Whereas this suggested that the three tandem D-repeats were required to form a structure that is favorable for high affinity ligand binding, circular dichroism analyses indicated that the D1–3 domain was primarily unstructured in solution, acquiring extensive \beta structure only when bound to N29 (9).

MSCRAMMs from Groups A and C streptococci also possess repetitive motifs that bind N29 (10–13), which, together with the D-repeats of \textit{S. aureus} MSCRAMMs, shares a conserved pattern of amino acids defined as ED(T/S)(3,4)(I/V)(D/E), where acidic and hydrophobic amino acids in the C-terminal segment are essential for binding (14). The N29 fragment is itself composed of five tandem type I modules, \textsuperscript{1}F1–\textsuperscript{5}F1.

\textsuperscript{1}The abbreviations used are: MSCRAMM, microbial surface components recognizing adhesive matrix molecules; Fn, fibronectin; PBB, phosphate-buffered saline; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; ECM, extracellular matrix.

Received for publication, June 22, 2004

Published, JBC Papers in Press, August 4, 2004, DOI 10.1074/jbc.M406984200

This paper is available on line at http://www.jbc.org

42945
Affinity, Stoichiometry, and Modular Requirements of FnBP

Each module is a β-sandwich composed of two antiparallel β-sheets, with β-strands A and B forming a two-stranded sheet, and β-strands C, D, and E comprising a three-stranded sheet (15). This three-stranded β-sheet in each of the 1–F1 and 2–F1 modules of N29 is the ligand for a 25-mer synthetic peptide B3T derived from the Streptococcus dysgalactiae FnB MSCRAMM, which bound the 1–F1 module pair with a K of 1.0 μM (16). The B3T peptide displayed an extended linear conformation containing two β-strands, each of which contributed a fourth strand to the triple-stranded β-sheets in 1–F1 and 2–F1, forming an antiparallel β-zipper. Within the B3T peptide, a cluster of alternating hydrophobic amino acids (LISHPt-NEWP) formed contacts with tyrosine and leucine residues in 1–F1, and this was preceded by an acidic motif. EDS, that engaged a basic patch in 2–F1. Knowledge of these binding specificities, together with the previous identification of additional acidic and hydrophobic amino acids that are involved in ligand binding (14), also enabled the identification of putative 3–F1- and 4–F1-binding motifs in several different MSCRAMM-derived peptides.

Based on these known and predicted interactions, the ability of a 50-mer synthetic peptide derived from the R-repeats of the Streptococcus pyogenes SbB adhesin to bind N29 with high affinity was proposed to involve an extended antiparallel β-zipper spanning all five F1 modules (16). This was supported by showing that three consecutive overlapping synthetic peptides derived from the larger 50-mer could each bind the respective 1–F1, 2–3–F1, or 4–5–F1 bimodular F1 constructs with low affinity (K of 0.4–113 μM), whereas the intact 50-mer bound N29 (1–F1) with high affinity (K of 2 nM), and a peptide lacking only the C-terminal 1–F1-binding motif was of intermediate affinity (K of 0.062 μM). These findings collectively suggested that (i) conserved motifs promote the binding of specific F1 modules, (ii) short peptide segments with putative motifs specific for only two F1 modules will display low affinity ligand binding, (iii) high affinity binding requires that all five F1 modules are engaged in a tandem β-zipper, and (iv) the absence of a single F1-binding motif causes a significant reduction in affinity for N29.

Although this model is well suited to the S. pyogenes fibronecstin-binding MSCRAMM, it leaves a number of issues unresolved for the FnBPA and FnBPB adhesins of S. aureus. Although FnBPA was predicted to possess 11 distinct Fn-binding segments based on the occurrence of appropriately spaced putative F1-binding motifs, the first 7 of these segments are N-terminal of the D-repeats and were either previously not known to bind Fn or not well characterized in terms of affinity and specificity. Additionally, although the recombinant D1–3 polypeptide binds N29 with high affinity, an obvious 1–F1-binding motif does not occur anywhere in the FnBPA adhesin, and a putative 2–F1-binding motif does not occur outside of the D-repeat domain (16). Hence, the majority of the predicted binding segments lack putative motifs for binding of 1–F1 and 2–F1, and, according to the model that was proposed, should not support high affinity ligand binding.

In this regard, the goal of our present study was to define the minimal requirement for high affinity ligand binding by the repetitive D-repeat domain and to determine whether binding segments that are N-terminal of the D-repeats are capable of high affinity ligand binding. Using bimodular D1–2 or D2–3 polypeptides, we show that the affinity is comparable with that observed for a previously described peptide derived from the SbB adhesin of S. pyogenes that engages only four F1 modules (2–F1) but is still an order of magnitude less than that of the D1–3 domain. Furthermore, using either soluble Fn or keratinocyte ECM as a selection method to screen a phage-display library of S. aureus genomic DNA, we isolated two distinct binding regions that are N-terminal of the D-repeats. These regions designated DaA and DaB harbored minimal binding segments DaARI and DaBRI that individually bind N29 with low affinity but, when combined as a recombinant DaBA polypeptide, display an affinity that is comparable with the D-repeats, despite their apparent lack of 1–F1 and 2–F1-binding domains. Combining DaBA with the previously described polypeptide, DaBAD, that had seven or eight high affinity binding segments for N29, close to the number of predicted binding segments. Thus, our data are consistent with multiple functional ligand-binding segments in the FnBP adhesins, located in two separate high affinity domains. However, we cannot completely rationalize the observed high affinity ligand binding with the previously proposed model, and we suggest that interaction between bound molecules of N29 might contribute to high affinity binding.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Keratinocyte Cell Culture—Multidrug-resistant S. aureus strain CMRSA-1B (isolate-317) has been described elsewhere (17, 18). Escherichia coli strains TG1 or MG1655 were used for maintenance of plasmids and expression of fusion peptides. Stock cultures of S. aureus and E. coli were maintained at 37 °C in 15% glycerol and grown in brain-heart infusion (Difco Laboratories, Detroit, MI) or 2YT (Difco Laboratories) broth, respectively. 2YT was supplemented with 25 μg/ml kanamycin (2YTK) when required for infection of E. coli. TG1 with M13K07 helper phage and with 50 μg/ml ampicillin (2TAA) for selection of plasmid vectors. Media were supplemented with 15 μg of agar liter−1 for preparation of solid media. All cultures were grown at 37 °C, in a static incubator for agar cultures, and a shaker-incubator with agitation at 250 rpm for liquid cultures. When grown for specific assays, the optical density of overnight cultures was measured at 600 nm (S. aureus) or 540 nm (E. coli), followed by subculturing into appropriate pre-warmed medium to achieve an initial absorbance of 0.1.

Normal human keratinocytes were obtained from neonatal foreskin and maintained in serum-free keratinocyte growth medium supplemented with bovine pituitary extract and recombinant epidermal growth factor (Invitrogen) (19). Trypsinized cells were adjusted to a density of 2 × 10⁴ cells/ml⁻¹, and 100-μl aliquots were transferred into wells of 96-well flat-bottom cell culture plates (Costar Corning, Corning, NY). When the cells had achieved confluence, ECM was exposed by treating the monolayers with 0.5% Triton X-100 for 30 min at 37 °C, followed by 25 mM ammonium hydroxide for 10 min as described elsewhere (20). After rinsing with phosphate-buffered saline (PBS), excess protein binding sites were blocked by incubation with 0.1% bovine serum albumin in PBS before use for selection of ECM-binding phage.

Phage-display Library Construction and Screening—Phagemid vector pGSSAET (21) was kindly provided by Dr. Lars Frykberg and is designed such that blunt end ligation of randomly sonicated genomic DNA fragments into a unique SnaBI site of the vector will promote expression of cloned fragments as an N-terminal fusion to the M13 phage coat protein gpVIII. A library of ~0.6–0.7-kb fragments of sonicated genomic DNA from S. aureus CMRSA-1B strain 317 was constructed in pGSSAET following established methods (22, 23), producing ~1 × 10¹³ ampicillin-resistant transformants, which was then amplified in E. coli TG1 to yield a phage titer of 3.3 × 10¹⁴ ampicillin transducing units/ml⁻¹.

For binding of soluble Fn, an aliquot of phagemid library containing ~10¹⁰ ampicillin transducing units was incubated for 1 h with 10 μg μl⁻¹ of biotinylated Fn in 500 μl PBS. Phage particles containing bound Fn where then captured with streptavidin-coated magnetic beads (Magnalab Streptavidin; Pierce), and after extensive washing with PBS containing 0.05% Tween 20, bound phage were eluted in 0.1 M glycine-HCl (pH 2.3) and then neutralized with 2 M Tris-HCl, pH 8.6, and used to reinfect E. coli TG1 cells. Amplified phage were then subjected to a second round of selection using an identical protocol. For selection on keratinocyte ECM, ~10¹⁰ ampicillin transducing units of phage diluted in PBS containing 0.1% bovine serum albumin were added to triplicate wells of 96-well cell culture plates containing keratinocyte ECM. After a 1-h incubation at room temperature with gentle agitation, the wells were washed with PBS containing 0.05% Tween 20, followed by elution of bound phage in low pH buffer and amplification in E. coli TG1 for a second round of screening. For both protocols, phage recov-
ered from the second-round of selection were used to infect E. coli TG1 cells, and cells were plated on 2XTA. Clones expressing functional fusion proteins were identified by colony blots using horseradish peroxidase-conjugated E-tag monoclonal antibody (Amersham Biosciences), specific for an epitope incorporated into pG8SAET immediately before the fusion site with the M13 phage gene VIII sequence.

**DNA Sequence Analyses**—Sequencing of phagemid DNA was performed with the University of Toronto Hospital for Sick Children DNA sequencing facility. Nucleotide sequences were analyzed using the MacVector program (Oxford Molecular, Oxford, UK), and the translated protein sequences were subjected to BLAST homology searches using the search engine provided by the National Center for Biotechnology Information, including access to *S. aureus* genome sequences.2

**Protein Purification**—Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose as described previously (20). The resulting concentration-dependent increases in anisotropy at 524 nm with excitation at 493 nm as described previously (8). Data for the bimodular peptides, D1-D3 repeats or D2 and D3 repeats, respectively, of the FnBPA adhesin of *S. aureus* 8325-4, were fit to a two-site model using the following equation:

\[
\frac{L}{K_d L + [N29]} (Eq. 1)
\]

where \(K_1\) and \(K_2\) represent the respective dissociation constants of sites 1 and 2, \(L\) is concentration of free N29. Because the concentration of labeled peptide was small compared with \(K_0\), the concentration of free N29 was assumed equal to the total concentration. This assumption is not valid for titration of FITC-D1–3, FITC-DuBA, or FITC-DuBAD because of the much higher affinity. In this case, it was necessary to use a quadratic equation that takes into account the amount of tinate bond and the stoichiometry as described previously (8).

**Analytical Size-exclusion Chromatography**—250-μl samples of N29 fraction, alone or premixed with various concentrations of recombi-
nant DuBA or DuBAD, were preincubated for 15 min and then injected onto a Superose-12 column using an Amersham Biosciences fast protein liquid chromatography system. The solvent was TBS, pH 7.4, at room temperature. The flow rate was 0.5 ml min⁻¹. Elution was monitored by absorbance at 280 nm.

**RESULTS**

**Identification of Ligand Binding Motifs by Phage-display**—A scale diagram of FnBPA and FnBPB and the location of gene segments recovered from phagemid clones are presented in Fig. 1, A and B. Overlapping DNA segments spanning the Fn-binding D-repeats were recovered from screening with both biotinylated Fn (three clones) and keratinocyte ECM (two clones), confirming the ability of the selection procedures to recover expected Fn-binding segments. Five clones isolated only on keratinocyte ECM possessed identical segments within the C domain of FnBPA, overlapping with a previously described binding domain designated Du (28). This domain spanning amino acids 593–655 of FnBPA is designated DuA. Surprisingly, both selection procedures identified a total of 15 clones possessing identical or overlapping sequences within a central segment of FnBPB that has not been characterized previously. This domain spanning amino acids 409–535 of FnBPB is designated DuB.

**Sequence Analysis of Binding Domains**—Due to the high homology between the C-terminal halves of FnBPA and FnBPB (6), the amino acid sequence of DuA (Fig. 1A) is nearly identical in the two proteins but was assigned as originating from fnbA on the basis of nucleotide sequence. The DuA sequence is

---

**Fig. 1.** Scale diagram of FnBPA (A) and FnBPB (B) proteins and amino acid sequence of the DuBA polypeptide derived from the FnBPB adhesin (C) that is characterized in this study. The domain architecture of FnBPA is labeled according to traditional nomenclature (7), with PRR and CWA indicating C-terminal proline-rich repeats and cell wall anchor domains. The solid lines underneath each diagram approximate the span of different polypeptides encoded by phagemid clones that were recovered after screening of the phage-display library with biotinylated Fn, and dashed lines indicate clones recovered by selection on keratinocyte ECM. Parentheses after each segment indicate the number of times each sequence was recovered, followed by the numbering of the N- and C-terminal amino acids of each segment, within the mature FnBP adhesins. Expansion of the DuA segment in A shows the amino acid sequence of this clone and its homology to the R-motifs from the SfbI/PrtF adhesins of *S. pyogenes*. An amino acid sequence within DuA that corresponds precisely to predicted Fn-binding segment 5 of the FnBPA adhesin (16) is underlined. Within the amino acid sequence of the DuB clone shown in B, the shaded amino acids at the N- and C termini indicate residues that were deleted in construction of GST-DuB1N and DuB1C, respectively. The YEEDTN sequence (in bold italic) at the C terminus represents the beginning of the adjacent DuA domain. The span of the DuBRI synthetic peptide is underlined with arrows, and underneath this is shown the alignment of this motif with the repetitive B1 and B2 motifs of FnBPA, in which putative FN-binding segments FnBPA 2 and FnBPA 3 (16) are underlined. In C, the amino acid sequence of the recombinant DuBA polypeptide derived from the FnBPB adhesin is displayed. The YEEDTN sequence (in bold italic) marks the juncture of the DuB and DuA domains as defined above. The underlined sequences are homologous to putative FN-binding segments that have been predicted to occur in the FnBPA adhesin (16), as labeled in parentheses on the right.
within the C domain that separates the repetitive B- and D-elements of FnBPA. A 36-amino acid internal segment of DuB is 51% identical to the R-repeats of the SfbI/PrtF adhesins from S. pyogenes (Fig. 1A) and also corresponds to predicted Fn-binding segment 5 of FnBPA (16). The amino acid sequence of DuB from S. aureus strain CMRSA-1 (Fig. 1B) shared 88% identity with known sequences of FnBPB from different S. aureus genomes and 42% identity with FnBPA. A 26-amino acid N-terminal segment of DuB shaded gray in Fig. 1B is enriched in tyrosine and contains 7 additional amino acids that are not evident in FnBPB of S. aureus 8325–4 (data not shown). A 36-amino acid internal segment of DuB is 56% identical to amino acids 506–543 and 537–571 of the FnBPA adhesin, which correspond to the B1 and B2 repeats (Fig. 1B) according to the historical nomenclature (7) and are now known to harbor predicted Fn-binding segments 2 and 3 (16). The C-terminal 26 amino acids of DuB are identical to sequences present in both FnBPA and FnBPB of S. aureus 8325–4, and the C terminus of DuB (Fig. 1C, YEEDTN) overlaps with the N terminus of DuA, indicating that these domains are contiguous. This is clarified in Fig. 1C, showing the amino acid sequence of the recombinant DuBA polypeptide derived from the FnBPB adhesin that is used later in this study, together with its complement of putative Fn-binding segments, based on comparison with the predicted Fn-binding segments of FnBPA.

Ligand Binding of DuA and DuB—Although the above sequence analysis refers to the occurrence of predicted Fn-binding segments in the DuA and DuB domains, our initial characterization of these domains was completed before the prediction of multiple ligand binding segments in the FnBPA adhesin. A series of GST fusion proteins harboring different segments of DuB was constructed and assayed for inhibition of biotinylated Fn binding to wells of microtiter plates coated with the full-length GST-DuB (Fig. 2). Soluble GST-DuB inhibited binding of biotinylated Fn with an IC50 value of 0.8 nM. Constructs lacking either the tyrosine-rich N terminus (GST-DuBAN) or the C-terminal segment that is conserved in both FnBPA and FnBPB (GST-DuBAC) as defined in the legend to Fig. 1 provided similar IC50 values of 0.4 and 0.2 nM, whereas GST-DuBANC lacking both N- and C-terminal segments possessed an IC50 of ~1.0 nM. Furthermore, an IC50 of 40 nM was obtained with fusion protein GST-DuBR1, which possessed only the 37-amino acid internal segment of DuB that aligns to the B1 and B2 repeats of FnBPA. On this basis, a synthetic 38-mer peptide DuBRI was selected for more detailed characterization, whereas synthetic DuARI was selected on the basis of its homology to the Fn-binding R-motifs of S. pyogenes (Fig. 1A).

When thermolysin-digested Fn was passed over affinity matrices containing covalently coupled DuARI or DuBRI synthetic peptides, a single ~25-kDa polypeptide was retained on each column (Fig. 3), and an identical result was obtained with an affinity matrix containing the immobilized D3 peptide, which possesses the highest affinity of the D-repeats and an exclusive specificity for N29 (8). Therefore, each of the three major binding domains shares a common exclusive specificity for the N29 fragment of Fn. Titration of FITC-labeled DuARI or DuBRI by fluorescence anisotropy with purified N29 provided Kd values of 1.8 and 2.0 μM, respectively (data not shown), compared with 1.8 μM for the D3 synthetic peptide (8). The 2.0 μM Kd value for DuBRI binding to N29 was some 50-fold lower than the 40 nM Kd value of recombinant GST-DuBR1 polypeptide, when assayed as an inhibitor of biotinylated Fn binding to the full-length DuB domain. This may reflect the fact that GST fusion proteins are expressed as dimers, and this dimerization can promote binding affinities that are orders of magnitude higher than that of the isolated binding domain (29).

Comparison of Bimodular D-repeat Constructs—We showed previously that a recombinant polypeptide consisting of the tandem D1, D2, and D3 repeats exhibited a Kd of 1.5 nM for the N29 fragment of Fn, compared with ~1.8 μM for the individual D repeats alone (8). The titration data obtained with the FITC-labeled D1–3 peptide indicated the presence of two equivalent high affinity binding sites, even though the three individual peptides bind with low affinity. To further explore the basis of this observation, we conducted titrations of FITC-labeled polypeptides D1–2 and D2–3, each of which contained two repeats (Fig. 4). As with our previous study of D1–3, the data fit poorly to a single-site model but exhibited a good fit with a two-site model, such that the bimodular polypeptides each possessed low and intermediate affinity binding sites. D1–2 exhibited apparent Kd values of 0.25 and 2.5 μM, whereas D2–3 exhibited values of 0.044 and 2.2 μM. Because the D1 and D2 repeats are nearly identical in amino acid sequence and possess similar Kd values of 11.4–14.0 μM for binding to the N29 fragment when assayed individually (8), it is apparent that joining the two repeats together promotes an approximate 50-fold increase in affinity for N29. Likewise, in comparing the published Kd of 1.8 μM for the D3 repeat with the high affinity Kd value of 0.044 μM for the D2–3 construct, there is an approximate 40-fold increase in affinity.

Because the higher affinity Kd value of the bimodular constructs did not approach our previously reported value of 1.5 nM for the entire D1–3 domain (8), we repeated the titration of FITC-D1–3 while extending beyond the range of our previous data to include higher concentrations of N29 (Fig. 5). When these new data were fit to a quadratic expression identical to that used previously but including an additional term for the weak site(s), there was evidence for a weaker site in this construct as well with a Kd value of 0.5 μM, whereas the higher affinity interaction of 0.52 nM was close to our previously pub-
lished value of 1.5 nM. The stoichiometry for the weak site could not be determined, but the number of high affinity sites was 1.7, close to the value of 1.9 obtained previously. We conclude that the D1–3 polypeptide possesses two equivalent high affinity binding sites (Kd ≈ 1 nM) and at least one lower affinity site (Kd ≈ 0.5 μM), whereas the bimodular constructs possess two sites, one with an affinity that is intermediate between D1–3 and single D-repeats, and a second whose affinity is comparable with those of the latter.

Comparison of Multidomain Constructs—The assays described above clearly define a stepwise increase in affinity for the D-repeat domain, as single repeats are extended to two and three tandem repeats. We next turned our attention to a FITC-labeled DuBA polypeptide, derived from the FnBPB adhesin as shown in Fig. 1C. The best fit of the titration data to the quadratic expression gave a Kd of 10.7 nM with n = 3.5 (Fig. 6A). This affinity is 2 orders of magnitude (200-fold) higher than either module alone and substantially higher than either of the bimodular D-repeats. A longer construct containing DuB, DuA, and all three D-repeats produced a slightly lower Kd (7.0 nM) with higher stoichiometry (n = 7.3; Fig. 6B). The dashed and dotted curves together with the insets in each panel show how the quality of the fit is diminished by arbitrarily fixing n at values above and below the best fit value. From this analysis, we conclude that the stoichiometry for DuBA could be anywhere between 3 and 4, whereas that of DuBAD is between 6 and 8.

Given the importance of stoichiometry in validating the hypothesis of multiple Fn-binding segments, additional information was obtained by size-exclusion chromatography of N29 in complex with recombinant Fn-binding polypeptides. Because of the absence of tryptophan and the low amount of tyrosine in DuBA and DuBAD, their absorbance at 280 nm is trivial compared with the N29 fragment, allowing the elution behavior of N29 to be independently monitored in the presence of varying amounts of the FnBP constructs. Typical data are shown in Fig. 7, where the N29 fragment elutes in a sharp peak near 12.5 ml whose magnitude is progressively diminished upon pre-mixing with increasing concentrations of DuBAD. This was concomitant with the appearance of an earlier peak representing elution of the complexes formed. The concentration of N29 used in these experiments was constant at 4 μM, enough to saturate the lower concentrations of DuBA and DuBAD. When the decrease of the N29 peak height is plotted against the ratio of the concentrations of DuBA (or DuBAD) to that of N29 (Fig. 8), it is clear that the peak is 90% or more depleted at a ratio less than 0.3 for DuBA and 0.15 for DuBAD (Fig. 8). The data above a peak height of 10 were fit by linear regression to obtain
the anisotropy measurements. Elution was monitored by absorbance at 280 nm.

**FIG. 7.** Analytical size-exclusion chromatography of the N29 fragment of Fn alone and with various concentrations of DuBAD in TBS, pH 7.4, at room temperature. The concentration of N29 was 4.0 μM and that of DuBAD (in μM) is indicated next to the curves. Elution was monitored by absorbance at 280 nm.

the solid straight lines, whose intercepts on the abscissa occur at ratios of 0.254 and 0.122, corresponding to n values of 3.9 for DuBA and 8.2 for DuBAD, in good agreement with those obtained from the anisotropy measurements.

**DISCUSSION**

We have completed an analysis of the minimal requirements for high affinity binding of the 29-kDa N-terminal fragment of Fn (N29) to the FnBPA adhesin of *S. aureus*. In addition to the D-repeats, two prominent binding domains were identified, which we designated DuB and DuA. Our initial characterization of these domains led to the identification of two minimal binding segments, DuBRI and DuARI, which correspond to Fn-binding segments that were later predicted to occur in FnBPA (16). The precise amino acid sequence of these and most of the other Fn-binding segments that were predicted to occur within FnBPA are presented in Fig. 9, and our present work appears to have validated the functionality of most of these segments. As shown in Fig. 1C, the DuBA polypeptide does not possess an obvious equivalent of the predicted FnBPA binding segment 1. However, the DuBRI segment is homologous to the predicted FnBPA segments 2 and 3, and this is followed by complete equivalents of the predicted Fn-binding segments 4 and 5 and a partial copy of Fn-binding segment 6. The occurrence of three complete binding segments and a partial copy of a fourth segment is in excellent agreement with the stoichiometry values of 3.5 (anisotropy) and 3.9 (size-exclusion) obtained in our analysis of N29 binding to DuBA. In addition to these segments, the DuBAD polypeptide contains a complete FnBPA segment 6, followed by segments 7–10 and a portion of segment 11 containing the C terminus of the D3 repeat. Therefore, DuBAD should contain an additional five complete known or predicted binding segments that are not present in DuBA, for a total of 9, just slightly more than our observed stoichiometry of 7 to 8. These findings strongly corroborate the existence of multiple ligand binding segments that were predicted to occur in the FnBPA adhesin, based on the occurrence of appropriately spaced putative F1-binding motifs (16).

We have further shown that the individual DuARI and DuBRI segments each bind N29 with low affinity, whereas the larger DuBA polypeptide that harbors these and other functional segments displays an ~200-fold greater affinity than either DuARI or DuBRI alone. Similar traits are exhibited by the D-repeats, where bimodular constructs containing two tandem D-repeats display an affinity for N29 that is intermediate between individual repeats, and the trimodular D1–D3 polypeptide. The increased affinity of the bimodular D1–2 and D2–3 polypeptides compared with individual repeats can be explained by the model proposed by Schwarz-Linek *et al.* (16) to define the molecular basis of high affinity N29 binding (Kd, 2 nM) to 50-amino acid synthetic peptide Sbi493–542, derived from the R-repeats of the *S. pyogenes* PrtF/SfbI MSCRAMM. However, the greater affinity of D1–3 compared with the bimodular polypeptides and the high affinity of the DuBA domain are more difficult to reconcile with this model.

As shown in Fig. 9, the high affinity (Kd, 0.002 μM) of the Sbi493–542 peptide is attributed to a series of conserved motifs that promote formation of a β-zipper structure that spans the five F1 modules in the N29 domain of Fn. This high affinity peptide could be subdivided into smaller segments (Sbi491–512, Sbi511–528, and Sbi518–542) that bound specific pairs of F1 modules with much lower affinity (Kd, 0.4–113 μM). Although the specificity was not rigorously addressed, it was proposed that conserved motifs in each of these three segments are specific only for the indicated pairs of F1 modules. This was most evident with the Sbi518–542 segment, which bound 1–2F1 with a Kd of 0.4 μM. This was attributed to two motifs, EDT and FHFDNNEP, which closely resemble motifs in the B3T peptide derived from FnB of *S. dysgalactiae* and are known to engage 2F1 and 3F1, respectively, when bound in tandem to the 1–2F1 module pair with a Kd of 1.0 μM (16). The inference is that peptide segments that bind only two F1 modules are expected to display low micromolar Kd values, and high affinity binding requires that all five F1 modules be engaged in a tandem β-zipper. The reduced strength of a four-module β-zipper is evident from the 0.062 μM Kd of Sbi456–492, which lacks only the 1F1-binding motif and yet displayed a 30-fold loss of affinity for N29 compared with Sbi493–542 (Fig. 9). Because FnBPA of *S. aureus* lacks an obvious 2F1-
binding motif (16), the ability of the D1–3 polypeptide to bind N29 with an affinity comparable with that of Sbi493–542 must be accounted for by mechanisms that do not involve direct binding of \( ^{1}F1 \).

Our interpretation of how the complement of binding motifs that are either proven or predicted to engage specific F1 modules (16) to interact with the D1–3 polypeptide is displayed at the bottom of Fig. 9. The \( ^{1}F1 \) module, which is connected to \( ^{2}F1 \) by a flexible linker (15), is depicted as being displaced from the interface of N29 with D1–3. Thus, D1–3 is shown to possess an array of motifs sufficient to bind two molecules of N29 in an antiparallel \( \beta \)-zipper involving the \( ^{2}–^{3}F1 \) modules, as supported by our titration data indicating two equivalent high affinity sites (\( K_d \approx 1 \text{nM} \)). The C-terminus of D1–3 represented by KPSYPGGHNSVDFEEDTLPKV is shown to bind a third molecule of N29 by engaging a \( ^{4}–^{5}F1 \) module pair, which should account for the lower affinity binding site (two complete segments, segments 9 and 10) in close proximity to the \( ^{3}–^{4}F1 \) modules. In our previous study showing that synthetic D3 segment containing this same sequence could bind \( ^{4}–^{5}F1 \) with a \( K_d \) of 4.6 \( \mu \text{M} \) (8) but showed no detectable interaction with \( ^{1}–^{2}F1 \) or \( ^{3}–^{4}F1 \).

This same explanation applies to the common low affinity sites shared by the D1–2 and D2–3 polypeptides (\( K_d \approx 2.5 \text{ and } 2.2 \text{ \mu M} \), which we attribute to the respective C-terminal segments (GGNIDIDIFDS and GGHNSVDFEEDT) that possess putative motifs for binding of \( ^{4}–^{5}F1 \) (Fig. 9). The higher affinity sites, with \( K_d \) of 0.25 \( \mu \text{M} \) for D1–2 and 0.044 \( \mu \text{M} \) for D2–3, can be explained by the joining of the individual peptides to form intact segments 9 and 10, allowing formation of antiparallel \( \beta \)-zippers that engage four F1 modules, \( ^{2}F1–^{3}F1 \). In support of this notion, the \( K_d \) of 0.044 \( \mu \text{M} \) for D2–3 is very close to that of the Sbi456–492 peptide of \( S. pyogenes \), which has the same complement of four binding motifs and possesses a \( K_d \) of 0.062 \( \mu \text{M} \) for N29 (16). However, the additional 40–250-fold increase in affinity that occurs when all three D repeats are fused cannot be explained on this basis because D1–3 contains no new junctures beyond those already present in either D1–2 or D2–3. The only thing new is that D1–3 has two four-motif sites (two complete segments, segments 9 and 10) in close proximity to the \( ^{3}–^{4}F1 \) modules.
proximity, which prompts us to suggest that cooperative interactions between bound N29 molecules may lend additional stability to the complex. Recall in this regard that the N29 domain of Fn serves a critical role in the self-assembly of Fn fibrils in the extracellular matrix (30).

The seven predicted Fn-binding segments that are N-terminal of the D domain also lack a \(^3\)F1-binding motif and, judging by the alignment in Fig. 8, should also lack a \(^3\)F1 motif because the GG couples implicated in binding to this module are either absent or, in the case of DuARI, significantly out of register. Whereas this suggests binding of N29 to these segments involves up to three F1 modules (\(^2\)-\(^3\)F1; Fig. 9), the \(-2 \mu M K_d\) values obtained here for DuARI and DuBRI are of similar magnitude to those of other peptides (e.g. D3b, B3T, SbfI511–528, and SbfI518–542) that are either known or predicted to bind just two F1 modules. Nonetheless, when the DuB and DuA domains are expressed in tandem as the recombinant DuBA polypeptide, we observed strong binding of N29 with a \(K_d\) of \(-10 \text{ nM and stoichiometry of 4, in agreement with its comple-}

REFERENCES

1. Lowy, F. D. (1998) *N. Engl. J. Med.* **339**, 520–532
2. Sheagren, J. N. (1984) *N. Engl. J. Med.* **310**, 1368–1373
3. Weigil, L. M., Clewell, D. B., Gill, S. R., Clark, N. C., McDougal, L. K., Flinnagan, S. E., Kolarz, J. F., Shetty, J., Killgore, G. E., and Tenover, F. C. (2003) *Science* **300**, 1569–1571
4. Foster, T. J., and Hoik, M. (1998) *Trends Microbiol.* **6**, 484–488
5. Potts, J. M., Allen, B. L., McGavin, M. J., and Hoik, M. (1994) *Annu. Rev. Microbiol.* **48**, 555–617
6. Jonsson, K., Signas, C., Muller, H. P., and Lindberg, M. (1991) *Eur. J. Bio-chem.* **202**, 1041–1048
7. Signas, C., Raucchi, G., Jonsson, K., Lindgren, P. E., Anantharamaiah, G. M., Hoik, M., and Lindberg, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 699–703
8. Huff, S., Matsuoka, Y. V., McGavin, M. J., and Ingham, K. C. (1994) *J. Biol. Chem.* **269**, 15563–15579
9. House-Pompeo, K., Xu, Y., Joh, D., Speciale, P., and Hoik, M. (1996) *J. Biol. Chem.* **271**, 1379–1384
10. Lindmark, H., Jacobsson, K., Frykberg, L., and Guss, B. (1996) *Infect. Immun.* **64**, 3993–3999
11. Lindgren, P. E., McGavin, M. J., Signas, C., Guss, B., Gurusiddappa, S., Hoik, M., and Lindberg, M. (1993) *Eur. J. Biochem.* **214**, 819–827
12. Molinari, G., and Chhatwal, G. S. (1999) *J. Infect. Dis.* **179**, 1049–1050
13. Talay, S. R., Valentin-Weigand, P., Jerlstrom, P. G., Timmis, K. N., and Chhatwal, G. S. (1992) *Infect. Immun.* **60**, 3837–3844
14. McGavin, M. J., Gurusiddappa, S., Lindgren, P. E., Lindberg, M., Raucchi, G., and Hoik, M. (1993) *J. Biol. Chem.* **268**, 23946–23953
15. Potts, J. R., Bright, J. R., Bolton, D., Pickford, A. R., and Campbell, I. D. (1999) *Biochemistry* **38**, 8304–8312
16. Schwarz-Linek, U., Werner, J. M., Pickford, A. R., Gurussiddappa, S., Kim, J. H., Pilka, E. S., Briggs, J. A., Gough, T. S., Hoik, M., Campbell, I. D., and Potts, J. R. (2003) *Nature* **423**, 171–181
17. Huesca, M., Peralta, R., Sauder, D. N., Simon, A. E., and McGavin, M. J. (2002) *J. Infect. Dis.* **185**, 1285–1296
18. Papakriacou, H., Var, D., Simor, A., Louie, M., and McGavin, M. J. (2000) *J. Infect. Dis.* **181**, 990–1000
19. Kondo, S., Kono, T., Sauder, D. N., and McKenzie, R. C. (1993) *J. Invest. Dermatol.* **101**, 690–694
20. Huesca, M., Sun, G., Peralta, R., Shrivjy, G. M., Sauder, D. N., and McGavin, M. J. (2000) *Infect. Immun.* **68**, 1156–1163
21. Zhang, L., Jacobsson, K., Vasi, J., Lindberg, M., and Frykberg, L. (1998) *Microbiology* **144**, 985–991
22. Jacobsson, K., and Frykberg, L. (1995) *BioTechniques* **19**, 878–885
23. Jacobsson, K., and Frykberg, L. (1996) *BioTechniques* **20**, 1070–1078
24. Miekka, S. I., Ingham, K. C., and Menache, D. (1982) *Thromb. Res.* **27**, 1–14
25. Bors, L., Castellani, F., Balaz, E., Siri, A., Pellicchia, C., De Scalzi, F., and Zardi, L. (1986) *Anal. Biochem.* **155**, 335–345
26. McGavin, M. J., Zahradka, C., Rice, K., and Scott, J. E. (1997) *Infect. Immun.* **65**, 2621–2628
27. Ingham, K. C., and Brew, S. A. (1981) *Biochim. Biophys. Acta* **670**, 181–189
28. Joh, D., Speciale, P., Gurussiddappa, S., Manor, J., and Hoik, M. (1998) *Eur. J. Biochem.* **258**, 897–905
29. Mikhailenko, I., Kryst, D., Argyrova, K. M., Roberts, D. D., Liu, G., and Strickland, D. K. (1997) *J. Biol. Chem.* **272**, 6784–6791
30. Wierzchicka-Patynowski, I., and Schwarzbauer, J. E. (2003) *J. Cell Sci.* **116**, 3269–3276
31. Penkett, C. J., Dobson, C. M., Smith, L. J., Bright, J. R., Pickford, A. R., Campbell, I. D., and Potts, J. R. (2000) *Biochemistry* **39**, 2887–2893