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Identification of D Motif Epitopes in *Staphylococcus aureus* Fibronectin-Binding Protein for the Production of Antibody Inhibitors of Fibronectin Binding

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A fibronectin-binding protein (FnBP) adhesin of *Staphylococcus aureus* possesses three 37- or 38-amino-acid motifs (D1, D2, and D3) that can each bind fibronectin (Fn) with low affinity and that in tandem comprise D1-3, a high-affinity Fn-binding domain. To identify epitopes for the generation of adhesion-blocking antibodies, rabbits were immunized with recombinant D1-3 or with a glutathione *S*-transferase fusion protein, GSTD1-3. Affinity-purified antibodies from the D1-3 immunization were poor inhibitors of Fn binding to *S. aureus* and recognized several different epitopes, with a preference for clusters of acidic amino acids that do not contribute to Fn binding. Antibodies generated with GSTD1-3 as an immunogen were more effective inhibitors, but concentrations in excess of 20 $\mu g \cdot ml^{-1}$ did not promote more than 50% inhibition. These antibodies were highly specific for amino acids 21 to 34 of D1 ($D1_{21–34}$), which contain a sequence that is essential for Fn binding and are identical to D2 at 12 of 14 residues. Neither antibody preparation recognized $D3_{20–33}$ of the D3 motif, where the only homology to $D1_{21–34}$ and $D2_{21–34}$ comprises a sequence motif, GG(X3,4)(I/V)DF, that is critical to Fn binding. However, antibodies specific for both $D1_{21–34}$ and $D3_{20–33}$ could be obtained by using synthetic peptides corresponding to these sequences as immunogens. $F(ab')_2$ fragments derived from these antibodies each caused 40 to 50% inhibition of Fn binding to *S. aureus*, and their ability to bind to purified FnBP was eliminated by competing Fn. However, mixtures of the two $F(ab')_2$ preparations did not provide additive or synergistic inhibition of Fn binding. Therefore, inhibition of Fn binding to *S. aureus* requires antibodies specific for $D1_{21–34}$ and $D3_{20–33}$, but a mixture of antibodies specific for both sequences did not provide complete inhibition.

Staphylococcus aureus is a major cause of nosocomial bacteremia and can infect virtually every tissue and organ system of the body (29, 30). Historically, *S. aureus* infection has been successfully treated with antibiotics. However, the recent widespread emergence of methicillin resistance and the threat of acquisition of resistance to vancomycin (22) have promoted renewed interest in a vaccine for *S. aureus* (5). Although preliminary studies suggest that a capsular polysaccharide-based vaccine may be effective (6), such a strategy may be limited by poor efficacy in the elderly and in young children, as observed with a *Streptococcus pneumoniae* capsular vaccine (7, 28). Consequently, identification of protein antigens as candidate vaccine components is an active area of research (31). The interaction of *S. aureus* with fibronectin (Fn) is of particular interest in this respect. *S. aureus* cells express a cell surface Fn-binding protein (FnBP) belonging to a family of microbial adhesins that mediate adherence of bacteria to extracellular matrix proteins (25, 26). Adhesive interactions attributed to FnBPs include colonization of implanted medical devices and traumatized heart valves for *S. aureus* (9, 18, 36); adhesion of *Streptococcus pyogenes* to epithelial cells, cutaneous tissues, and the extracellular matrix (11, 23, 24, 35); and adherence of the group G streptococcus to skin fibroblasts (17). Binding of soluble plasma Fn can also confer resistance to complement-mediated cell lysis (10), and binding of plasma proteins has been proposed as a means of sensing the external environment (2, 4). Therefore, FnBPs may possess multiple functions contributing to host-microbe interactions, and interfering with this receptor-ligand interaction represents a potential means of reducing the incidence and spread of *S. aureus* infections.

S. aureus possesses two tandem *fnb* genes, encoding FnBPs FnBPA and FnBPB (16, 32), each of which possesses three consecutive 37- or 38-amino-acid D motifs, designated D1, D2, and D3. In tandem, these motifs comprise a high-affinity Fn-binding domain, D1-3. Synthetic peptides representing each motif are also individually capable of low-affinity Fn binding and can competitively inhibit Fn binding to *S. aureus* (14, 32). These observations indicate a potential for the development of therapeutic agents that interfere with Fn binding, and the use of specific adhesion-blocking antibodies is one means by which this might be accomplished. However, attempts to generate blocking antibodies by employing both synthetic peptides and different forms of the D1-3 immunogen have been largely unsuccessful (3, 27, 34). While this suggests that the immunogens failed to elicit antibodies of the appropriate specificity, this issue has not been addressed with respect to knowledge of the mechanism of Fn binding. The ability to bind Fn is located exclusively within the C-terminal 20 amino acids of each D motif (14, 19, 20). These amino acids contain the sequence GG(X3,4)(I/V)DF, which is present in repeated motifs of other Fn-binding adhesins, and within the Fn-binding A2 motif of *Streptococcus dysgalactiae* FnBA, changes to either of the GG or IDF sequences resulted in loss of Fn binding (19, 25, 26). Here we present an analysis of the immunological response towards two different forms of recombinant D1-3 immunogen and demonstrate that inhibition of Fn binding requires antibodies specific for epitopes containing this conserved sequence. Furthermore, antibodies specific for this conserved sequence in the C-terminal half of the D3 motif

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could be obtained only by immunizing with a synthetic peptide containing the desired epitope.

MATERIALS AND METHODS

Bacterial strains, plasmids, and protein purification. FnBP was purified from *S. aureus* Newman as described previously (8). Plasmid pGEXD1-3, for expression in *Escherichia coli* of the glutathione *S*-transferase fusion protein GSTD1-3, has been described elsewhere (14). Recombinant D1-3 was purified by treatment of GSTD1-3 with thrombin followed by ion-exchange chromatography (14). Molar extinction coefficients of GSTD1-3 and recombinant D1-3 were used to establish that an absorbance at 280 nm of 1.0 is equivalent to protein concentrations of 0.95 and 3.6 mg · ml^{-1}, respectively. DNA encoding D1-3 was also cloned in the vector pMAL-c2 (New England BioLabs), creating pMALD1-3 for expression in *E. coli* TB1 of a maltose-binding protein fusion protein, MBPD1-3. The MBPD1-3 fusion protein was purified from cell lysates by selective ammonium sulfate precipitation followed by Q-Sepharose and phenyl-Sepharose (Pharmacia) chromatography with a Pharmacia Gradi-Frac chromatography system. For purification of antibodies, an affinity matrix was prepared by dissolving the lyophilized fusion protein in 0.5 M sodium bicarbonate (2 mg · ml^{-1}) and adding an equal volume of carbonyldiimidazole-activated (1) Sepharose CL4B (Pharmacia). After coupling for 48 h at 4°C with end-over-end mixing, assay of residual protein by the bicinchoninic acid protocol (33) established a coupling efficiency of 70%.

Preparation of synthetic peptide antigens. Amino acids 21 to 34 of the D1 motif ($D1_{21–34}$) (QGGNIVDIDFDSVP) and amino acids 20 to 33 of the D3 motif ($D3_{20–33}$) (QFGGHNSVDFEEDT) were synthesized with an N-terminal cysteine by the University of Calgary Peptide Synthesis Core Facility, utilizing an Applied Biosystems model 431A peptide synthesizer. The peptides were coupled via the N-terminal cysteine to maleimide-activated keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) according to the protocols provided with the Imject Immunogen Conjugation kit (Pierce, Rockford, Ill.).

Production of antisera and purification of antibodies. Six male New Zealand White rabbits were immunized subcutaneously with 1 mg of GSTD1-3 in Freund's complete adjuvant (Sigma). Groups of three rabbits were boosted at 2-week intervals by intramuscular injection of 80 μg of either GSTD1-3 or D1-3 peptide in incomplete adjuvant. The animals were bled 10 days after each immunization and terminally bled by heart puncture after 128 days. For purification of antibodies, antiserum was depleted of Fn by chromatography on gelatin-Sepharose (21) and then passed over an affinity matrix consisting of MBPD1-3 fusion protein coupled to Sepharose CL4B. After sequential washes with phosphate-buffered saline (PBS) and PBS containing 0.5 M sodium chloride, bound antibodies were eluted in 3.5 M $MgCl_2$, dialyzed in 20 mM ammonium bicarbonate, and lyophilized. For antipeptide antibodies, each of two male New Zealand White rabbits was immunized subcutaneously with either 100 μg of $D1_{21–34}$ coupled to BSA ($D1_{21–34}$BSA) or $D3_{20–33}$ coupled to KLH ($D3_{20–33}$ KLH), each emulsified in Freund's complete adjuvant. Booster doses of 100 μg were administered at 2-week intervals until a plateau in the antibody titer was observed. Immunoglobulin G (IgG) from immune or preimmune sera was purified on protein A-agarose (12). To prepare $F(ab')_2$ fragments, 20 mg of lyophilized IgG was dissolved in 2 ml of 70 mM sodium acetate–50 mM NaCl (pH 4.0), and pepsin (Boehringer Mannheim catalog no. 108 057) was added at a ratio of 30 μg per mg of IgG. After overnight incubation in a 37°C water bath, Tris buffer (pH 8.0) was added to 0.1 M, and $F(ab')_2$ fragments were separated from the pepsin-digested Fc fragments by chromatography on a 1.5- by 90-cm column of Sephacryl S100 (Pharmacia) equilibrated in 50 mM Tris–150 mM NaCl–0.02% sodium azide (pH 7.4).

ELISA and epitope mapping. Enzyme-linked immunosorbent assay (ELISA) was performed in Corning 96-well microtiter plates with wash buffer consisting of PBS containing 0.05% (vol · vol^{-1}) Tween 20, a blocking solution consisting of 3% (wt · vol^{-1}) BSA in PBS, and antibody dilution buffer consisting of PBS containing 0.05% Tween 20 and 0.1% BSA. Incubation with primary and secondary antibodies was for 60 min at 20°C on an orbital mixer. The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG or alkaline phosphatase-conjugated $F(ab')_2$ fragment of $F(ab')_2$-specific goat anti-rabbit IgG (Jackson Immuno Research Laboratories, West Grove, Pa.) diluted 5,000-fold in antibody buffer. ELISA plates were developed for 60 min at 20°C with 1 mg of *para*-nitrophenyl phosphate (Sigma) · ml^{-1} in 0.1 M diethanolamine buffer (pH 9.8) and quantified on a Titertek Multiskan Plus ELISA plate reader equipped with a 405-nm filter. Titers are defined as the dilution of antiserum or concentration of antibody yielding one-half of the maximal ELISA response (A_{405}). For epitope mapping, overlapping decapeptides spanning the D1 motif and the N-terminal 18 residues of D2 were synthesized by Chiron Mimitopes (Clayton, Victoria, Australia), with each consecutive peptide sequence being offset by two amino acids from the previous sequence. A separate set of 15 overlapping decapeptides spanning the D3 motif was also synthesized. Each peptide contained an N-terminal biotin and a 4-amino-acid spacer sequence consisting of SGSG. Wells of Corning microtiter plates were coated with 100 μl of 5 μg of streptavidin (Gibco/BRL, Gaithersburg, Md.) · ml^{-1} in distilled, deionized H_2O and allowed to dry on the plates during overnight incubation at 37°C. Blocking and washing procedures were as described for the general ELISA protocol. Biotinylated peptides (2 mg · ml^{-1}) in 6 M guanidinium hydrochloride were diluted 1,000-fold in PBS, and 100-μl aliquots were incubated in the streptavidin-coated microtiter plates for 60 min at 20°C. The plates were then washed and incubated for 60 min with 100 μl of affinity-purified antibody (50 ng · ml^{-1}) diluted in PBS–0.1% BSA–0.05% Tween 20. The plates were then developed as described for the general ELISA protocol.

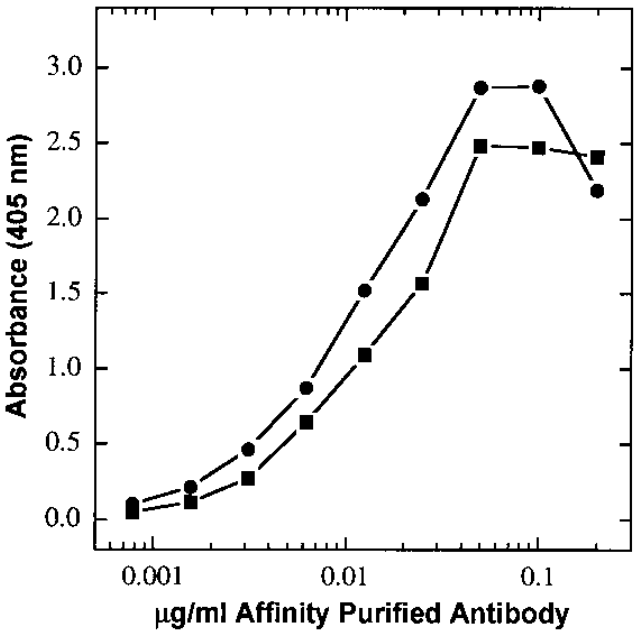

FIG. 1. Titration of affinity-purified antibodies obtained by immunizing with D1-3 (●) or GSTD1-3 (■). Microtiter plates were coated with 1 μg of D1-3 · ml^{-1} and incubated with the indicated concentrations of affinity-purified antibodies.

Assay for inhibition of Fn binding. The different antibody preparations were assayed for the ability to inhibit binding of Fn to *S. aureus* cells applied to Corning polystyrene ELISA well strips. Human plasma Fn was obtained from Gibco/BRL and labelled with ^{125}I to a specific activity of 27 MBq · $nmol^{-1}$, using the chloramine T procedure (15). *S. aureus* L857, a blood culture isolate obtained from the University of Manitoba Health Sciences Centre Clinical Microbiology Laboratory, was grown to mid-exponential phase in brain heart infusion broth (Difco, Detroit, Mich.) at 37°C. Suspensions of 10^{10} heat-killed bacteria · ml^{-1} were prepared as described previously (20), diluted 10-fold in carbonate-bicarbonate buffer, and allowed to coat Corning polystyrene ELISA well strips during overnight incubation at 4°C with orbital mixing. The wells were washed with PBS containing 0.05% (vol · vol^{-1}) Tween 20 and blocked by incubation with 200 μl of 3% (wt · vol^{-1}) BSA in PBS for 60 min at 37°C. After washing with PBS-Tween 20, 50 μl of antibody or $F(ab')_2$ inhibitor diluted in PBS containing 0.05% Tween 80 and 0.1% BSA was added. The cells were preincubated with inhibitor for 30 min on an orbital mixer, followed by addition of 50 μl of ^{125}I-Fn (50,000 cpm) diluted in the same buffer. After an additional 60 min of incubation, the wells were washed extensively with PBS–0.1% Tween 80 and then transferred to Sarstedt 75- by 12-mm polystyrene centrifuge tubes. The amount of ^{125}I-Fn bound was then quantified in an LKB Wallac model 1272 automatic gamma counter.

RESULTS

Affinity purification of antibodies and inhibition of Fn binding. As measured by ELISA with D1-3 as the test antigen, pooled antisera from animals boosted with GSTD1-3 or recombinant D1-3 yielded maximum titers of 110,000 and 7,000 to 9,000, respectively (data not shown). Affinity purification from 300 ml of pooled antisera yielded 35 and 7 mg of antibody from antisera generated by using the GSTD1-3 and D1-3 immunogens, respectively. The affinity-purified antibodies exhibited respective titer values of 20 and 10 ng · ml^{-1} (Fig. 1). Therefore, while GSTD1-3 was a better immunogen for eliciting high-titer antisera, there was not a dramatic difference in the titers of the affinity-purified antibodies. However, the

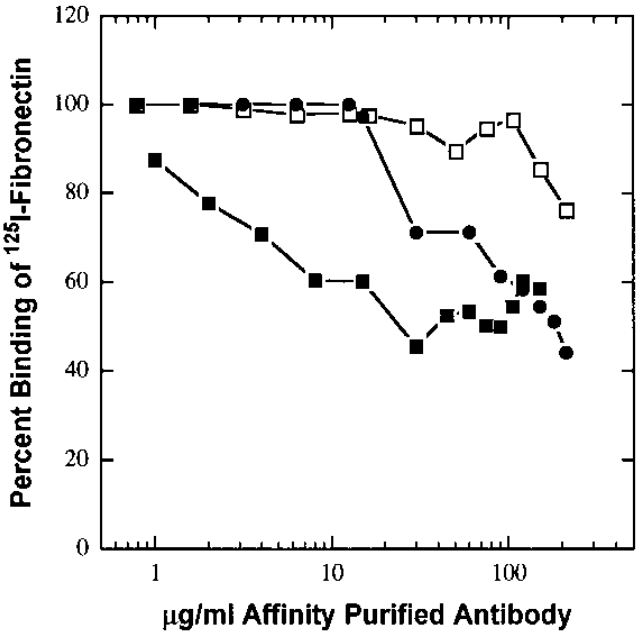

FIG. 2. Inhibition of ^{125}I-Fn binding to *S. aureus* L857 cells by preimmune antibodies (□) or affinity-purified antibodies obtained by immunizing with GSTD1-3 (■) or D1-3 (●). Results are expressed as the percentage of ^{125}I-Fn bound in the absence of added antibody. Each point represents the average from duplicate determinations.

GSTD1-3 fusion protein produced antibodies that were more effective inhibitors of Fn binding to *S. aureus* (Fig. 2). Dose-dependent inhibition was observed at antibody concentrations of 1 to 20 $\mu g \cdot ml^{-1}$, with 50% inhibition occurring at 20 $\mu g \cdot ml^{-1}$. However, higher concentrations did not promote more than 50% inhibition. For antibodies obtained with the D1-3 immunogen, a concentration of 200 $\mu g \cdot ml^{-1}$ was required to achieve 50% inhibition. Therefore, the D1-3 immunogen generated antibodies that were approximately 10-fold less effective as inhibitors of Fn binding than antibodies obtained by immunizing with the fusion protein.

Epitope specificity. Initially, 24 decapeptides were synthesized to span the entire D1 motif and the N-terminal 18 amino acids of D2, with each consecutive peptide overlapping the previous sequence by eight amino acids. The D1 and D2 motifs are highly homologous, differing at only 5 of 38 amino acids (32). Therefore, these decapeptides were designed to map dominant epitopes within the D1 motif and to identify epitopes spanning the C terminus of D1 and the N terminus of D2. The D1-3 immunogen produced antibodies with a broad spectrum of epitope specificities (Fig. 3A). Most notable was the recognition of epitopes with clusters of acidic amino acids, defined by SFEEDTEEDKPKYE and SFEEDTEKDKPK, spanning residues 7 to 20 of the D1 motif ($D1_{7–20}$) and $D2_{7–18}$, respectively. Previous work has shown that synthetic peptides $D1_{1–18}$ and $D2_{1–18}$ do not interact with the N-terminal fragment of Fn (14). Therefore, this antibody preparation contains a significant population of antibodies specific for amino acid sequences that do not contribute to Fn binding. There was also more variable recognition of several decapeptides spanning the sequence $D1_{17–38}$. In contrast, antibodies generated by using GSTD1-3 as the immunogen exhibited a restricted specificity, predominantly recognizing three consecutive decapeptides spanning the amino acid sequence $D1_{21–34}$, QGGNIVDIDFDS VP (Fig. 3B). In a previous study, synthetic peptides representing amino acids $D2_{18–38}$ and $D3_{16–36}$, respectively, bound the N-terminal fragment of Fn with an affinity comparable to that of the intact D2 and D3 motifs (14). Therefore, the major epitopes recognized by this antibody preparation occur within an amino acid sequence that is critical to Fn binding. Furthermore, this antibody preparation demonstrated little reactivity towards the clusters of acidic amino acids that were major epitopes for antibodies generated with D1-3 as an immunogen. Neither of the antibody preparations recognized epitopes within an amino acid sequence spanning the C-terminal eight amino acids of the D1 motif and the first eight amino acids of the D2 motif. Therefore, there appear to be no significant epitopes consisting of amino acid sequences that span two individual motifs.

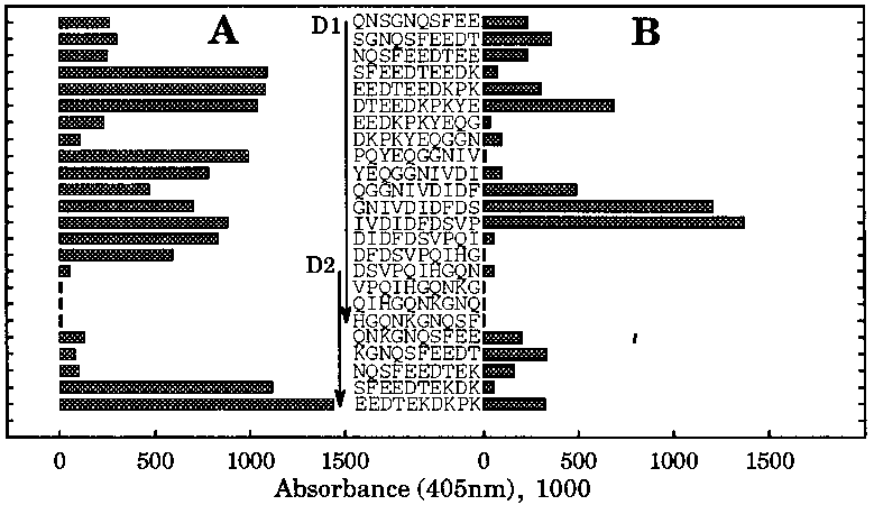

FIG. 3. Epitope mapping of affinity-purified antibodies obtained by immunizing with D1-3 (A) or GSTD1-3 (B). Peptide sequences are listed in descending order from the N terminus of D1 and ending with EEDTEKDKPK, in which the C terminus represents residue 18 of the D2 motif. Arrows labelled D1 or D2 indicate the span of peptides in which one or more amino acids are contributed by either of the D1 or D2 motifs. Biotinylated peptides were captured in wells of streptavidin-coated microtiter plates and incubated for 60 min with 100-μl aliquots of affinity-purified antibody (50 $ng \cdot ml^{-1}$). Each value represents the average from triplicate determinations.

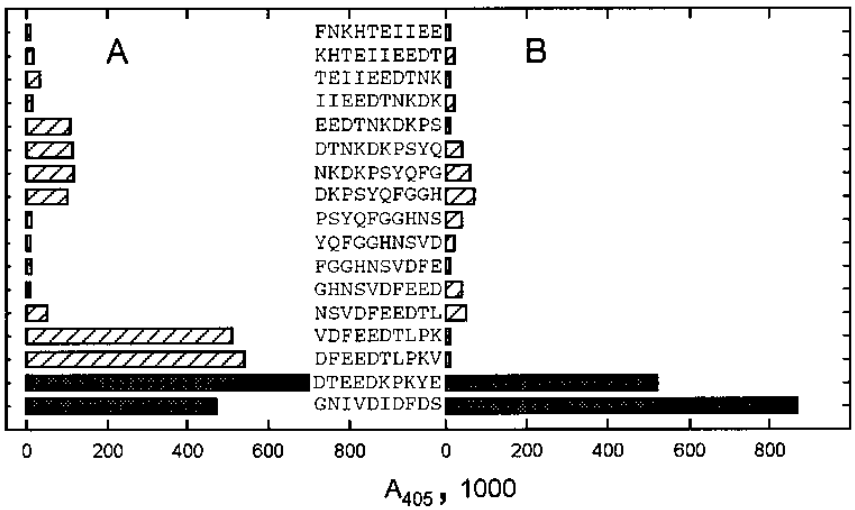

FIG. 4. Recognition of decapeptides spanning the D3 motif by affinity-purified antibodies generated with D1-3 (A) or GSTD1-3 (B) as the immunogen. Shaded bars represent the responses towards control peptides $D1_{11-20}$, and $D1_{23-32}$ of the D1 motif. The assay was conducted as described for Fig. 3.

When assayed with a series of 15 decapeptides spanning the D3 motif (Fig. 4), antibodies obtained with the GSTD1-3 immunogen exhibited no appreciable recognition of these peptides, while antibodies obtained with the D1-3 immunogen recognized VDFEEDTLPKV, representing the C-terminal 11 amino acids of D3. The sequence FEEDT is observed within this 11-amino-acid sequence and also in the two major epitopes within the D1 and D2 motifs that are recognized by this antibody preparation. Therefore, the D1-3 immunogen appears to have generated antibodies with a high specificity for clusters of acidic amino acids, most notably in the N-terminal halves of the D1 and D2 motifs, which do not contribute to Fn binding.

Use of synthetic peptides to generate antibodies of defined specificity. The GSTD1-3 immunogen produced antibodies that were effective inhibitors at low concentrations and exhibited a high specificity for QGGNIVDIDFDSVP, spanning the amino acid sequence $D1_{21-34}$. The sequence $D2_{21-34}$ of the D2 motif (HGGNIIDIDFDSVP) is nearly identical, suggesting that antibodies specific for these epitopes alone are responsible for the inhibition of Fn binding to *S. aureus*. However, the inhibition of Fn binding was incomplete, and these antibodies exhibited no reactivity towards the sequence QFGGHNSVD FEEDT, comprising amino acids 20 to 33 of the D3 motif ($D3_{20-33}$) and containing amino acids that are known to be critical for Fn binding (19, 20). To obtain antibodies specific for this sequence, rabbits were immunized with a synthetic peptide, $D3_{20-33}$, which was synthesized with an N-terminal cysteine for coupling to maleimide-activated KLH. Rabbits were also immunized with $D1_{21-34}$ coupled to maleimide-activated BSA, representing the major epitope recognized by antibodies generated with the GSTD1-3 immunogen. Both antipeptide antibody preparations recognized the recombinant D1-3 peptide (data not shown). Although the $D1_{21-34}$ immunogen was coupled to BSA, inclusion of 0.1% BSA in the antibody dilution buffers was sufficient to eliminate interference from recognition of the BSA blocking reagent that was employed in the ELISA assay. Subsequently, IgG was purified from immune sera by utilizing protein A and converted into $F(ab')_2$ fragments. The resulting $F(ab')_2$ fragments recognized FnBP purified from *S. aureus* Newman (Fig. 5), and a reduction in the ELISA response was observed in the presence of increasing concentrations of soluble competing Fn, such that recognition of FnBP was virtually eliminated at 50 μg of soluble Fn · ml^{-1}. Consistent with the recognition of epitopes containing amino acid sequences that are critical to Fn binding, both $F(ab')_2$ preparations exhibited concentration-dependent inhibition of Fn binding to *S. aureus* cells in the range of 5 to 2,000 μg · ml^{-1} (Fig. 6). At a concentration of 1 mg · ml^{-1}, the $F(ab')_2$ preparations obtained by immunizing with $D1_{21-34}$ and $D3_{20-33}$ elicited 48.5 and 45.6% inhibition of Fn binding, respectively. However, when mixtures of the two $F(ab')_2$ preparations were assayed for inhibition of Fn binding

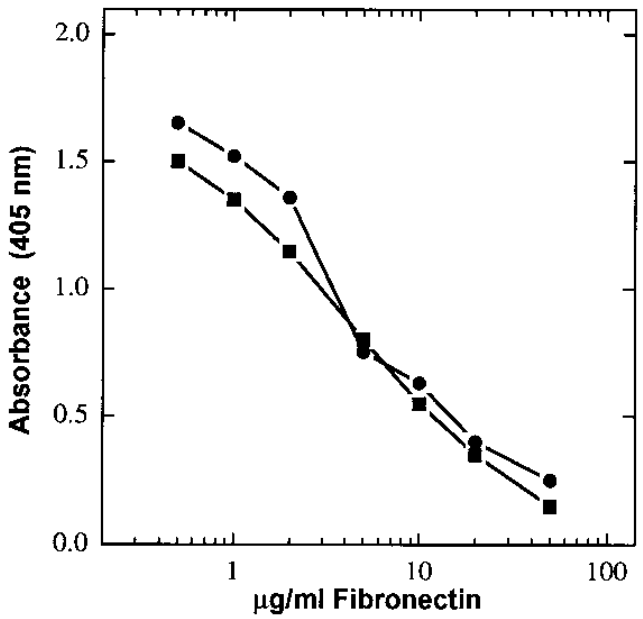

FIG. 5. Competition between soluble Fn and anti-$D1_{21-34}$ (●) or anti-$D3_{20-33}$ (■) $F(ab')_2$ fragments for binding to FnBP purified from *S. aureus* Newman. The $F(ab')_2$ fragments were diluted to 100 μg · ml^{-1} in antibody buffer containing the indicated concentrations of soluble Fn and then added to wells of Corning microtiter plates coated with 5 μg of FnBP · ml^{-1}. The secondary antibody was alkaline phosphatase-conjugated $F(ab')_2$ fragment of $F(ab')_2$-specific goat anti-rabbit IgG.

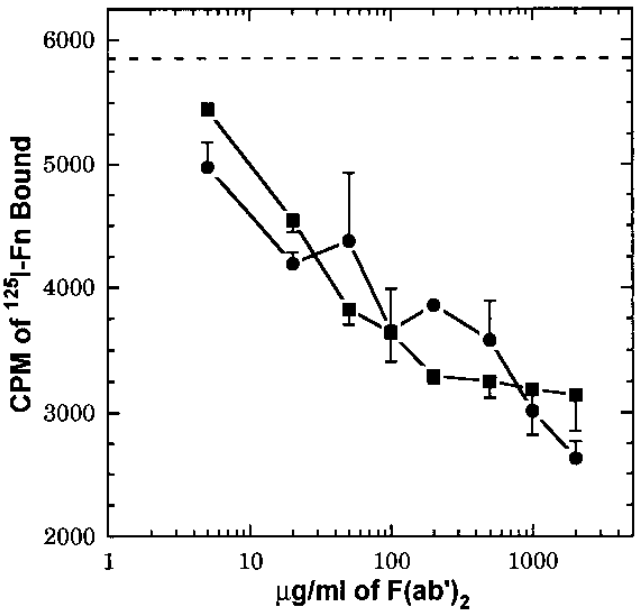

FIG. 6. Inhibition of ^{125}I-Fn binding to *S. aureus* L857 by anti-D1$_{21–34}$ (●) or anti-D3$_{20–33}$ (■) F(ab′)$_2$ fragments. Each point represents the average from triplicate determinations (with the standard deviation), and the dashed line indicates the amount of binding in the absence of added F(ab′)$_2$ fragments (100%). Preimmune F(ab′)$_2$ did not inhibit Fn binding at any of the concentrations assayed.

at either 100 or 200 $\mu g \cdot ml^{-1}$, the extent of inhibition was not greater than that observed with the same concentrations of either F(ab′)$_2$ alone (Fig. 7). Therefore, although the F(ab′)$_2$ fragments were specific for epitopes within the D1 and D3 motifs that are critical for Fn binding, there did not appear to

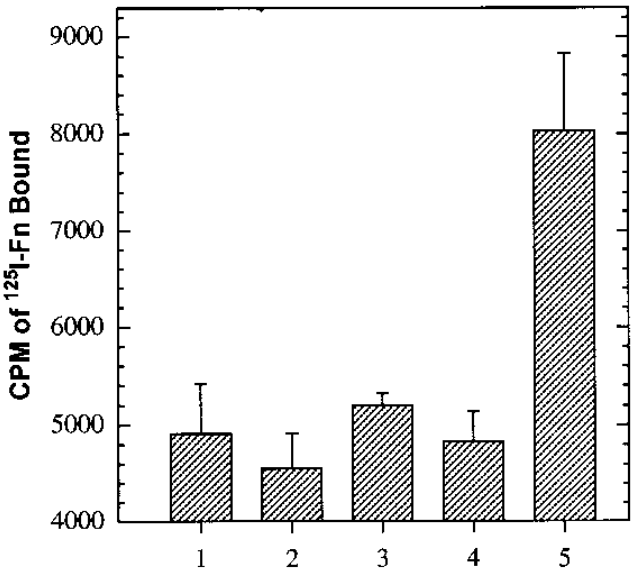

FIG. 7. Assay for additive or synergistic inhibition of Fn binding by mixtures of anti-D1$_{21–34}$ and anti-D3$_{20–33}$ F(ab′)$_2$ fragments at concentrations of 100 $\mu g \cdot ml^{-1}$ (bar 1) and 200 $\mu g \cdot ml^{-1}$ (bar 2) compared to inhibition by anti-D1$_{21–34}$ (bar 3) or anti-D3$_{20–33}$ (bar 4) F(ab′)$_2$ alone at 100 $\mu g \cdot ml^{-1}$. Bar 5 represents the binding in the absence of added F(ab′)$_2$ fragments, and each column represents the average from triplicate determinations (with the standard deviation).

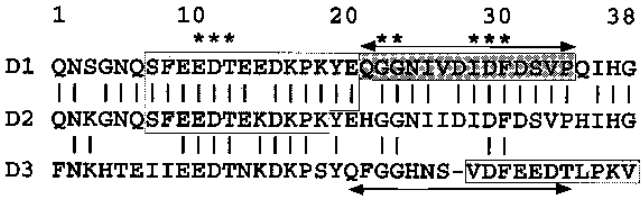

FIG. 8. Amino acid sequences of the D motifs. Asterisks indicate amino acids common to repeated motifs of adhesins from *S. aureus* and *Streptococcus* species (19, 25, 26), in which GG and (I/V)DF are essential for Fn binding. Sequences with light and heavy shading represent major epitopes recognized by antibodies from rabbits immunized with D1-3 and GSTD1-3, respectively. The sequences of D1$_{21–34}$ and D3$_{20–33}$ are indicated by arrows above and below the D1 and D3 motifs, respectively.

be either an additive or a synergistic effect when the preparations were assayed in combination.

DISCUSSION

This study provides new insight into the use of the Fn-binding D motifs of *S. aureus* FnBP as immunogens for the production of antibodies capable of blocking binding of Fn to *S. aureus*. Previous attempts to generate blocking polyclonal antibodies by using various recombinant forms of Fn-binding adhesins or synthetic peptides as immunogens have been largely unsuccessful (3, 27, 34). In the present study, we have identified specific amino acid sequences which constitute epitopes for antibodies that inhibit Fn binding. Figure 8 displays the amino acid sequences of the individual D motifs, the location within each motif of the major epitopes recognized by the different antibody preparations, and a pattern of amino acids which is essential for ligand binding and common to repeated motifs from several different Fn-binding adhesins (19, 25, 26). Antibodies generated with recombinant D1-3 as the immunogen were comparatively poor inhibitors of Fn binding, and a significant proportion of these antibodies were specific for epitopes in the N-terminal 18 amino acids of the D1 and D2 motifs, which have been shown to exhibit no affinity for Fn (14). In contrast, antibodies generated with GSTD1-3 as the immunogen provided dose-dependent inhibition at low concentrations and were specific primarily for epitopes within the amino acid sequence D1$_{21–34}$. This sequence contains the conserved amino acids GG and IDF, which are essential for Fn binding by the A2 motif of *S. dysgalactiae* and present in repeated motifs of several different Fn-binding adhesins from *Staphylococcus* and *Streptococcus* species (19, 25, 26). Furthermore, the comparable sequence D2$_{21–34}$ of the D2 motif is nearly identical (Fig. 8). Therefore, these data establish a correlation between inhibition of Fn binding and recognition of epitopes containing a conserved pattern of amino acids that is critical to ligand binding. However, a limitation associated with both immunogens was the failure to generate antibodies specific for an Fn-binding sequence in the C-terminal half of the D3 motif.

Another study reported that antisera of mice immunized with a D1-3–β-galactosidase fusion protein recognized synthetic peptides representing the D1 and D2 motifs but failed to recognize D3 (27). Therefore, similar difficulties have been reported in studies employing different animals and three different forms of the D1-3 immunogen. We have extended these findings by demonstrating that D1$_{21–34}$ was a major epitope for antibodies generated with the GSTD1-3 immunogen, but these antibodies exhibited no appreciable recognition of D3$_{20–33}$ in the D3 motif. The only identity between D1$_{21–34}$ and D3$_{20–33}$

comprises the conserved GG and (I/V)DF sequence motif (Fig. 8), which is conserved in repeated motifs of other Fn-binding adhesins and is essential for Fn binding. Therefore, although these two sequences are functionally identical, antibodies specific for $D3_{20–33}$ could not be obtained by immunizing with either D1-3 or GSTD1-3. One explanation for this observation could involve antigen presentation. When D3 is presented as an immunogen as a component of the D1-3 polypeptide, its amino acid sequence divergence from D1 and D2 (Fig. 8), combined with its 5- to 10-fold-greater affinity for Fn (14), may contribute to it being less immunogenic. Furthermore, D1-3 and tandem Fn-binding repeated motifs from other gram-positive adhesins are essentially unstructured in solution but undergo a significant rearrangement when complexed with the N-terminal fragment of Fn (13). Therefore, the interaction of D1-3 with Fn in vivo could either mask epitopes that are critical to ligand binding, interfere with antigen processing, or promote the generation of antibodies specific for amino acid sequences that are not essential for ligand binding.

The relevance of these considerations is supported by our finding that antibodies specific for $D3_{20–33}$ could be obtained only by immunizing with a synthetic peptide corresponding to this specific sequence. In a previous study, it was found that $D3_{20–33}$ could not inhibit binding of Fn to *S. aureus* but that addition of the tripeptide PSY to the N terminus ($D3_{17–33}$) or of LPK to the C terminus ($D3_{20–36}$) restored Fn-binding function (20). Therefore, although this sequence contains the conserved GG and (I/V)DF amino acids that are essential for Fn binding, it is by itself unable to bind Fn. It was postulated that $D3_{20–33}$ represents a core sequence that is critical to Fn binding but requires three or more N- or C-terminal amino acids to achieve a secondary structure that is favorable for Fn binding. Alternatively, it was also suggested that initial contact with Fn might trigger a conformational change necessary for the peptide to maintain contact with the ligand and that $D3_{20–33}$ was unable to achieve this conformational change. This hypothesis is favored by the observation that D1-3 undergoes a structural rearrangement on contact with the N-terminal fragment of Fn (13). This may be relevant to many receptor-ligand interactions and could influence the production of antibodies that inhibit receptor function, as demonstrated when the Fn-binding FnbA adhesin of *S. dysgalactiae* was used as an immunogen. This resulted in a monoclonal antibody that recognized a ligand-induced binding site and stimulated the ability of peptides containing this sequence to inhibit Fn binding to *S. aureus* cells (34). It was postulated that the ligand-bound conformation of the Fn-binding site was stabilized by the monoclonal antibody.

Since the D1-3 polypeptide is unstructured in solution, binding of polyclonal antibodies to different epitopes may result in a number of unpredictable effects, as evident from the use of synthetic peptides to produce antibodies specific for the catalytic γ subunit of phosphorylase kinase (37). Antibodies produced with amino acids 322 to 346 as an immunogen inhibited phosphorylase activity, whereas activity was stimulated by antibodies specific for the partially overlapping sequence of amino acids 342 to 366. It has been proposed that during a polyclonal antibody response towards immunogens containing Fn-binding repeated motifs, antibodies that stimulate Fn binding may compromise the effect of adhesion-blocking antibodies and that this may represent a process that has been evolved by bacteria to avoid immunological interference of adherence to Fn (34). In this situation, it would be beneficial to limit the diversity of epitope specificities. This was the rationale for immunizing with synthetic peptides $D1_{21–34}$ and $D3_{20–33}$. This strategy was successful in generating antibodies specific for amino acid sequences that are involved in ligand binding, as evident from the ability of soluble Fn to compete with the corresponding $F(ab')_2$ preparations for binding to FnBP purified from *S. aureus* and from the concentration-dependent inhibition of Fn binding to *S. aureus* cells. However, it is not clear why a mixture of the two $F(ab')_2$ preparations did not provide greater inhibition than either one alone. As the peptide sequences $D1_{21–34}$ and $D2_{21–34}$ are nearly identical (Fig. 8), a mixture of the two $F(ab')_2$ preparations should be sufficient to recognize epitopes that are critical to Fn binding within each of the three D motifs. Potential explanations for the failure to observe an additive or a synergistic effect include steric hindrance considerations, or alternatively, binding of antibody to $D3_{20–33}$ could elicit a conformational change in D1-3 that limits access of antibody to the $D1_{21–34}$ epitope (or vice versa). Work on the production of monoclonal antibodies specific for both sequences is currently in progress, which will allow these considerations to be addressed and will allow for a more detailed analysis of the ability of antibodies specific for these single epitopes to inhibit binding of Fn to *S. aureus.*

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