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A Monoclonal Antibody Enhances Ligand Binding of Fibronectin MSCRAMM (Adhesin) from *Streptococcus dysgalactiae**

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A monoclonal antibody 3A10, generated from a mouse immunized with the *Streptococcus dysgalactiae* fibronectin (Fn) binding protein FnbA, was isolated, and its effect on ligand binding by the antigen was examined. The epitope for 3A10 was localized to a previously unidentified Fn binding motif (designated Au) just N-terminal of the repeat domain which represents the primary ligand binding site on FnbA. Fn binding to Au was enhanced by 3A10 rather than inhibited. This effect was demonstrated in two different assays. First, in the presence of 3A10 the Au-containing proteins and synthetic peptide more effectively competed with bacterial cells for binding to Fn. Second, 3A10 dramatically increased the binding of biotin-labeled forms of the Au-containing proteins to Fn immobilized on a blotting membrane. Pure 3A10 IgG did not recognize the antigen by itself, and Fn was required for the immunological interaction between the antibody and the epitope. This induction effect of Fn was shown in both Western blot and enzyme-linked immunosorbent assay in which immobilized Au-containing molecules were probed with 3A10 in the presence of varying concentrations of Fn. Specificity analyses of 3A10 revealed that the monoclonal also recognized a ligand binding motif in a *Streptococcus pyogenes* Fn binding MSCRAMM but not the corresponding motifs in two related adhesins from *Staphylococcus aureus* and *S. dysgalactiae*. Furthermore, 3A10 stimulated Fn binding by *S. pyogenes* cells. These results together with subsequent biophysical studies presented in the accompanying paper (House-Pomepeo, K., Xu, Y., Joh, D., Speziale, P., and Höök, M. (1996) *J. Biol. Chem.* 271, 1379–1384) indicate that the ligand binding sites of Fn binding MSCRAMMs have little or no secondary structure. However, on binding to Fn, they appear to undergo a structural rearrangement resulting in a defined structure rich in β sheet and expressing a ligand-induced binding site for antibodies such as 3A10.

Bacterial adherence to the host tissue is recognized as the first step in the pathogenesis of most infections. Adherence is mediated by bacterial surface components called adhesins which recognize and bind to specific structures in the host tissue. One family of adhesins recognize extracellular matrix components, and members of this adhesin family have been collectively called MSCRAMMs[1] (microbial surface components recognizing adhesive matrix molecules; for reviews, see Refs. 1 and 2). Several fibronectin (Fn) binding MSCRAMMs have been isolated and characterized from different Gram-positive bacteria. Genes encoding Fn binding MSCRAMMs from *Staphylococcus aureus* (3), *Streptococcus pyogenes* (4, 5), and *Streptococcus dysgalactiae* (6) have been cloned and sequenced. The deduced amino acid sequences revealed 60–100-kDa proteins with very similar structural organization. The N-terminal signal sequence is followed by a long stretch of unique sequence which in some cases is interrupted by two copies of a ~30-amino acid long segment. The ligand binding site is located just N-terminal of a proline-rich domain, which is believed to anchor the proteins in the cell wall. This domain is followed by the sequence LP*X*TG*X* which is a cell wall targeting signal (7), a stretch of hydrophobic residues representing a transmembrane unit and a short C-terminal cytoplasmic domain containing a cluster of positively charged residues. The primary Fn-binding sites on these MSCRAMMs consist of 30–42 amino acid long motifs repeated 3–4 times, and most of the repeated units contain a consensus sequence (6, 8). In a recent communication (9), we reported that recombinant proteins corresponding to the repeat regions from the different Fn binding MSCRAMMs are all capable of inhibiting binding of Fn to different Gram-positive bacteria, including *S. aureus*, *S. dysgalactiae*, and *S. pyogenes*. Furthermore, studies using individual synthetic peptides revealed that a number of the repeat units retain Fn-binding activity, and interfere with binding of Fn by all of the Gram-positive species tested. These data suggest that the binding sites in Fn for the different MSCRAMMs are either overlapping or closely spaced on the matrix protein.

The *S. aureus* Fn binding MSCRAMM contains an additional ligand binding site in a ~30-amino acid long segment which encompasses the consensus sequence and is located N-terminal of the repeat region. This segment can also interact with Fn and its N-terminal domain (designated N29) (10). The present study identifies a new ligand binding sequence called Au located just N-terminal of the A repeats of the *S. dysgalactiae* MSCRAMM, FnbA.

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[1] The abbreviations used are: MSCRAMM, microbial surface components recognizing adhesive matrix molecules; aa, amino acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; 3A10 ascites fluid, ascites fluid obtained from mice injected with cloned hybridoma cells expressing the monoclonal 3A10; 3A10 ascites IgG, IgG purified from 3A10 ascites fluid; 3A10 IgG, IgG purified from cultured cloned hybridoma cells expressing 3A10; LIBS, ligand-induced binding sites.

Because of the pivotal role that tissue adherence plays in the pathogenic process, bacterial adherence has been identified as a target in new strategies to prevent and treat infections. It is possible that adhesins can be used as vaccines to generate antibodies which in addition to participating in the conventional host defense, also can block tissue adherence. Previous studies using Fn binding MSCRAMMs as an antigen have given inconclusive results (11, 12), and it is unclear if generated antibodies can block ligand binding and adherence. In this study, we report on a monoclonal antibody (designated 3A10) which recognizes the newly identified ligand binding site Au in the *S. dysgalactiae* MSCRAMM FnbA only when Au is bound to Fn. Furthermore, the monoclonal antibody 3A10 appears to enhance ligand binding to recombinant proteins or synthetic peptides containing the Au sequence. This activity is similar to those of anti-LIBS (ligand-induced binding sites) antibodies described for the platelet integrin $\alpha_{IIb}\beta_3$ (13, 14). This family of antibodies appear to recognize a conformation of a receptor induced by ligand binding.

EXPERIMENTAL PROCEDURES

Bacteria and Growth Conditions

Escherichia coli strain JM101 (*supE thi* Δ(*lac-proAB*) (*F′tra*D36 *proAB*$^+$ *lacI*^{q} ZΔM15)) was used as the host strain to express the PAQ proteins. The strain TG1 (*supE hsd*Δ5 *thi* Δ(*lac-proAB*$^+$) (*F′tra*D36 *proAB lacI*^{q} *lacZ*ΔM15))(15) was used as host for the plasmids pSDF100, pSDF102, pSDF102c14, and pSDF102c18. *E. coli* cells were grown in Luria broth (Difco) at 37 °C. *S. dysgalactiae* strain S2 (6), *S. pyogenes* strain 64/14 (obtained from Richard Lottenberg, Department of Medicine, University of Florida, Gainesville) and *S. aureus* strains 8325–4 (16) and Cowan 1 (obtained from Instituto Sieraterapico Milanese, Milan, Italy) were grown as described previously (9).

Preparation of Monoclonal Antibody

Recombinant Fn-binding MSCRAMM FnbA was purified from lysate of *E. coli* harboring the plasmid pSDF100 (6). The purified FnbA was used as an immunogen for BALB/c mice. The mice were injected intraperitoneally five times at 1-week intervals with 50 μg of the protein.

The antigen was mixed with complete Freund's adjuvant (Sigma) for the primary immunization, incomplete adjuvant for the next three injections, and in saline only for the final immunization. Three days after the last injection, the splenic lymphocytes were isolated and fused with Spe/0 Ag.14 mouse myeloma cells at the ratio 5:1 using 50% polyethyleneglycol 4000. Supernatants of the resulting hybridomas grown in selective media, (hypoxantine/aminopterin/thymidine) were first tested in an ELISA for the presence of antibodies reactive with FnbA-coated microtiter wells (Costar, Cambridge, MA). Positive hybridomas were then subcloned by limited phase dilution into 96-well plates. A clone designated 3A10 was subcloned and grown to high density in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and antibiotics.

To produce ascites fluid, cells were harvested from late-log phase cultures, washed with sterile phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.3) and 5×10^6 cells were injected intraperitoneally into Pristane-primed mice. The collected ascites fluid was clarified by centrifugation and when so indicated antibodies were purified on protein G-Sepharose as recommended by the manufacturer (Pharmacia, Uppsala, Sweden). Using a Mouse Isotyping Kit (Bio-Rad), the purified IgG was determined to be of the IgG_1 isotype.

Preparation and Iodination of Ligands, Synthetic Peptides, and Oligonucleotides

Porcine Fn was prepared as described previously (8). The N-terminal fibronectin fragment (N29) was isolated using the procedure of Houser-Pompeo *et al.* (17).

Labeling of Fn N29 with [^{125}I]iodine was performed according to the chloramine-T method of Hunter *et al.* (18).

Peptides were synthesized on an Advanced ChemTech multisynthesizer using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry as described (8). The amino acid sequence of the synthetic peptide Au is STPEGPTEGENNLGGQSEEITITEDSQSGM and corresponds to aa residues 806–835 of FnbA. The amino acid numbering of this and other constructs are based on the sequence of FnbA reported by Lindgren *et al.* (6).

Oligonucleotides were obtained from the Advanced DNA Technologies Laboratory, Texas A&M University (College Station, TX).

Construction of Expression Plasmids and Purification of Recombinant Proteins

The different recombinant constructs that were expressed are schematically presented in Fig. 1*A*. The plasmids pSDF100, pSDF102, pSDF102c14, and pSDF102c18 were described earlier (19). The recombinant proteins PAQ5 (aa 760–840), PAQ8 (aa 760–969), and PAQ14 (aa 844-1001) were expressed from plasmids derived from the vector pQE30 (Quiagen Inc., Chatsworth, CA) and purified as described (9). The inserts in the plasmids expressing the PAQ proteins were obtained by polymerase chain reaction using pSDF100 as template. The amplification was performed by using *Taq* polymerase (Life Technologies, Inc.). The polymerase chain reaction product was treated with proteinase K (20) digested with *Bam*HI and *Hin*dIII, and cloned into the *Bam*HI/*Hin*dIII site of pQE30 as described for other pQE30-based plasmids produced in our laboratory (9). The PAQ5-expressing plasmids contains the insert amplified with the oligonucleotide primers UF2 (5′-CAGTAAAGGATCCGTTCGTAAGCCACAGG-3′) and UR2 (5′-TTCCAGAAGCTTGATTTTGACCAGACATCC-3′). Cloning of the fragments amplified with the primers UF2/AR2 (9) and UF3 (5′-ACCGTCAGGATCCTCAGTTCCAGAGACATC-3′)/UR3 (5′-TCCACAACAGAAGCTTCATTAGAGCCAGAC-3′) resulted in the PAQ8- and PAQ14-expressing plasmids, respectively. The underlined nucleotides indicate the restriction sites introduced in the oligonucleotides to facilitate cloning of the polymerase chain reaction fragments.

Construction and purification of rFNBD-A, rFNBD-B, rFNBD-D, rFNBD-P, and CBD (151–318) was described earlier (9).

Labeling of Proteins with Biotin

Proteins (0.5 mg) dissolved in 0.5 ml of PBS were mixed with 0.5 ml of 0.2 M sodium borate buffer (pH 8.0) and 7.5 mg of NHS-LC-Biotin (Pierce) dissolved in 0.1 ml of dimethyl sulfoxide, and incubated at room temperature overnight on an end-over-end rotating mixer. The labeling mixture was dialyzed against PBS and stored at −20 °C in small aliquots.

Fibronectin-binding Assays

The Fn binding activity of recombinant proteins and synthetic peptides was determined in two different assays.

(i) Bacterial Binding Assay—The relative Fn binding activity of proteins or peptides was determined by measuring their ability to inhibit the binding of ^{125}I-labeled Fn or N29 to bacterial cells as described (19). For each binding assay, 10^9 bacterial cells were used except when otherwise indicated.

(ii) Affinity Western Blot Assay—Proteins (the PAQ proteins or Fn ligands) were separated by electrophoresis on 10 or 12% SDS-polyacrylamide gels. Reducing conditions were used for the PAQ proteins and nonreducing conditions were used for the Fn ligand. Proteins in the gel were electroblotted onto Immobilon P (Millipore) membranes for 2 h at 100 volts in transfer buffer (20 mM Tris-HCl, 150 mM glycine, 20% (v/v) methanol, pH 8.0). The membranes were then treated for 1 h in a solution containing 100 mg of dried skim milk/ml of PBS, washed 3 × 10 min with PBST (0.1% Tween 80 in PBS), followed by overnight incubation at 4 °C in PBSTB (0.1% Tween 80 and 0.1% bovine serum albumin in PBS) containing labeled protein (1000 cpm/μl ^{125}I-N29 or 1000-fold diluted biotin-labeled PAQ protein). Membranes probed with the ^{125}I-labeled proteins were washed 3 × 10 min in PBST and subjected to autoradiography. Membranes probed with biotin-labeled proteins were washed 3 × 10 min in PBST and incubated for 1 h in PBSTB containing 1000-fold diluted horseradish peroxidase-avidin D (Vector Laboratories, Burlingame, CA). After washing 3 × 10 min in PBST, the membrane was treated with ECL detection reagents 1 and 2 (Amersham Corp.) according to the procedure recommended by the manufacturer and exposed to an x-ray film for 2–10 s.

Antibody Binding Assay

The ability of recombinant proteins to bind 3A10 was assayed in ELISA and Western blot.

(i) ELISA—96-well Immunlon I (Dynatech Laboratories, Chantilly, VA) plates were used. Each well was incubated overnight at 4 °C with 100 μl of PBS containing 5–10 μg/ml protein. Additional protein binding sites in the wells were blocked by incubation for 1 h with 200 μl of PBS containing 3% (w/v) bovine serum albumin. The wells were then washed 5 times with PBST and incubated for 1 h with 100 μl of PBSTB

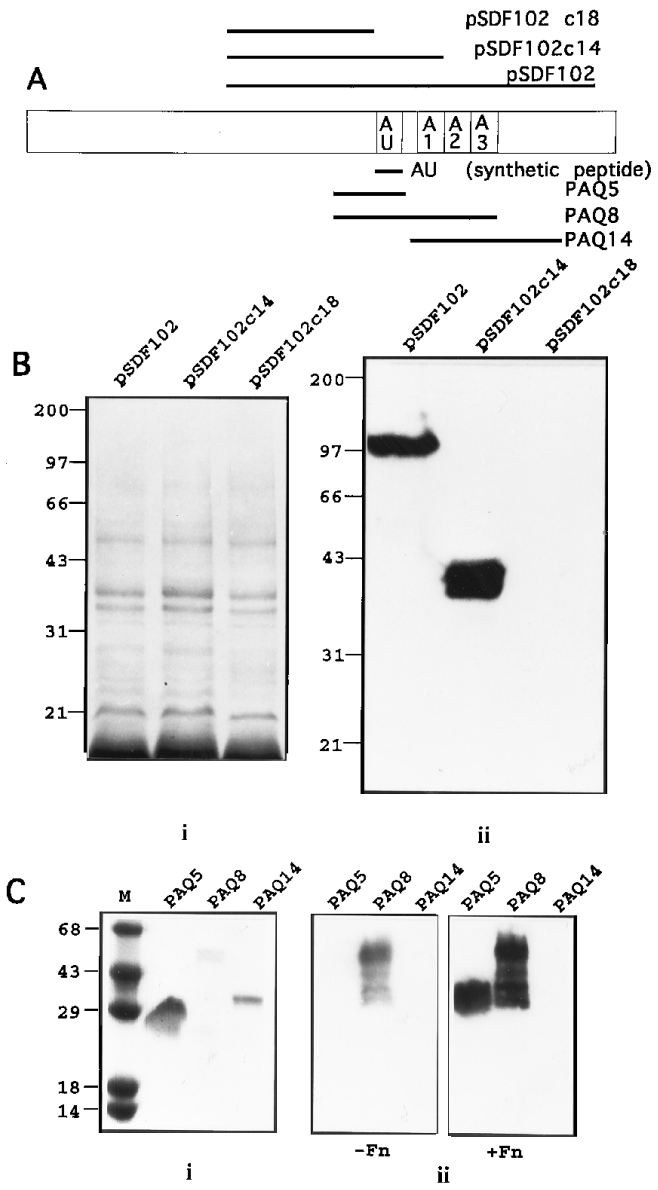

FIG. 1. **Mapping of the 3A10 epitope.** *A*, domain organization of FnbA and its truncated constructs. A1, A2, and A3 are the Fn-binding motifs of the primary ligand-binding site. Au is the newly-identified ligand-binding motif described in the text. The plasmids pSDF102, pSDF102c14, and pSDF102c18 are pUC18-based constructs containing DNA encoding the indicated region of the protein (6). PAQ5, PAQ8, and PAQ14 are polyhistidine fusion proteins expressed from pQE30-based plasmids containing the corresponding DNA fragments. Peptide Au is a synthetic 30-mer peptide (aa 806–835 of FnbA according to Lindgren *et al.* (6)) mimicking the Au motif. *B*, Coomassie Blue-stained gel (*i*) and Western blot (*ii*) of the lysates of *E. coli* harboring the indicated plasmids. The membrane containing the transferred proteins was probed with 3A10 ascites fluid. *C*, Coomassie Blue-stained gel (*i*) and Western blots (*ii*) of the PAQ proteins. The Western blots were probed with 3A10 ascites fluid in the absence (−*Fn*) and presence (+*Fn*) of added 10 μg/ml Fn.

containing antibody. Unbound antibody was removed by washing the wells 5 times with PBST. The conjugated enzyme bound to the well was detected by incubation with 100 μl of PBST containing 1000 fold-diluted goat anti-mouse IgG alkaline phosphatase conjugate (Bio-Rad). After 5 washes with PBST, bound alkaline phosphatase was allowed to act on 100 μl of 1 mg/ml phosphatase substrate (*p*-nitrophenyl phosphate disodium, Sigma) in 1 M diethanolamine and 0.5 mM $MgCl_2$ (pH 9.8). Absorbance at 405 nm was quantitated in a ThermoMax microplate reader (Molecular Device, Sunnyvale, CA). Alternatively, bound antibody was incubated 1 h with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Gostrup, Denmark) diluted 1:500. After washing, the conjugated enzyme was reacted with *o*-phenylenediamine dihydrochloride (Sigma) and absorbance at 492 nm was monitored.

(ii) Western Blot—Proteins were separated by electrophoresis on 10 or 12% SDS-polyacrylamide gels, and subsequently transferred to an Immobilon P membrane as described above. The membrane was treated with a solution containing 100 mg/ml dried skim milk, washed with PBST, and then incubated at 4 °C overnight in PBSTB containing ascites fluid diluted to 1:1000 or IgG as specified. The membrane was washed 3 × 10 min with PBST, and incubated for 1 h in PBSTB containing 0.1% (v/v) goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad). Bound peroxidase was detected as described above.

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P1  vetEdTkEpEvlMGGQSEsVeFTkDTQTGM
        * * * *  ****** * ** ******
Au  STPEGPTEGENNLGGQSEEITITEDSQSGM
          * *  ****   * *********
A2  edskpsqEdEviIGGQgqvIdFTEDTQSGM
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FIG. 2. **Sequence alignment of the P1, Au, and A2.** The Au sequence corresponds to aa 806–835 of FnbA from *S. dysgalactiae*. P1 and A2 are Fn-binding motifs present in Sfb from *S. pyogenes* and FnbA (9). The *bold-faced letters* in P1 and A2 represent residues identical with residues found in Au. *Asterisk* (*) represent homologous residues.

RESULTS

Mapping of the Epitope for the Monoclonal Antibody 3A10—A panel of mouse monoclonal antibodies were generated against the full-length recombinant FnbA, one of the two Fn binding MSCRAMMs from *S. dysgalactiae*. One of the generated monoclonal antibodies, 3A10, was further characterized. The epitope for 3A10 was localized by Western blot analyses using various truncated recombinant forms of FnbA as targets. We initially examined a set of constructs with C-terminal serial deletions described by Lindgren *et al.* (6) (Fig. 1*A*). *E. coli* lysates containing the recombinant proteins were fractionated by SDS-PAGE. The separated proteins were transferred to a supporting membrane and probed with ascites fluid obtained from mice injected with cloned hybridoma cells expressing 3A10 ("3A10 ascites fluid"). The *E. coli* lysate expressing pSDF102 and pSDF102c14 reacted with 3A10 ascites fluid (Fig. 1*B*). Recombinant protein expressed from pSDF102c18 which contain ~240 nucleotides fewer than pSDF102c14 did not react with the antibody, suggesting that the 3A10 epitope is located in the ~80 amino acid long deleted segment.

To further identify the epitope recognized by 3A10, additional pQE30-based plasmids encoding the polypeptides corresponding to the segments described in Fig. 1*A* were constructed. In the pQE-based constructs the insert DNA is expressed as a fusion protein with a short N-terminal segment containing a stretch of histidine residues which allows purification by Ni^{2+}-chelating chromatography (21, 22). PAQ5 contains a 81-amino acid long fragment of the MSCRAMM located N-terminal of the previously identified ligand binding A repeats. PAQ8 corresponds to the A repeats and an additional 90-amino acid long N-terminal sequence. PAQ14 contains the A repeats but lacks the N-terminal sequence. When analyzed by SDS-PAGE, the purified PAQ proteins migrated slower than expected according to their respected mass (Fig. 1*C*, *i*); however, this behavior is consistent with our previous observations of similar constructs (9). The PAQ proteins are very acidic with isoelectric points of 4.6 (PAQ5), 3.8 (PAQ8), and 3.8 (PAQ14), which results in a relatively poor binding of SDS and abnormal migration in SDS-PAGE (23). The molecular masses of PAQ5, PAQ8, and PAQ14 calculated from the amino acid sequences are 10,112, 23,682, and 18,401 daltons, respectively. Electrospray mass spectroscopy analysis of the purified PAQ proteins produced spectra containing single major peaks. The electrospray mass spectroscopy determined molecular masses of the recombinant proteins correspond exactly to those deduced from

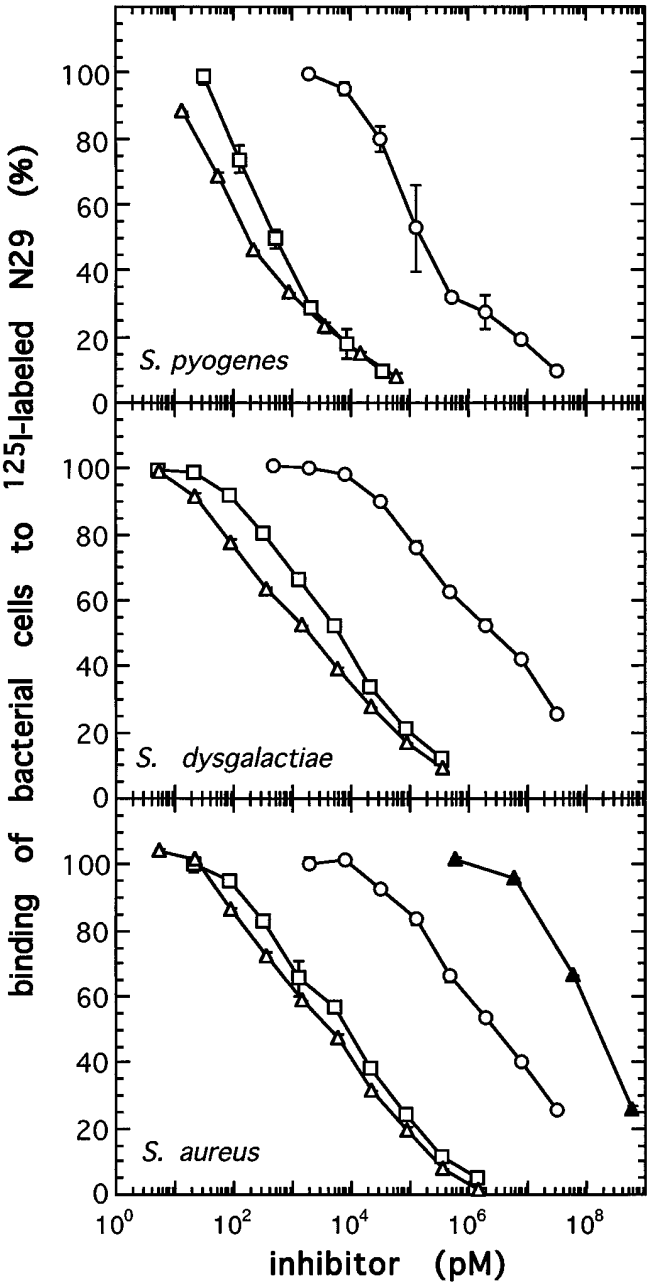

FIG. 3. **Inhibition of bacterial binding of ^{125}I-N29 by the PAQ proteins and peptide Au.** Binding of ^{125}I-labeled N29 to *S. pyogenes* strain 64/14, *S. dysgalactiae* strain S2, and *S. aureus* strain 8325–4 in the presence of varying concentrations of the potential inhibitors, PAQ5 (*circles*), PAQ8 (*squares*), PAQ14 (*open triangles*), and peptide Au (*filled triangles*). Bacterial binding in the absence of inhibitor was taken as 100%. *Error bars* indicate the variations between duplicate data points.

their respective amino acid sequences.

When recombinant PAQ proteins were fractionated by SDS-PAGE, transferred to a supporting membrane, and probed with 3A10 ascites fluid, only PAQ8 reacted with the antibody (Fig. 1*C*, *ii*). However, when 65 μg of porcine Fn was added to the 5-ml antibody solution in which the membrane was incubated, also PAQ5 but not PAQ14 reacted with the antibody. Furthermore, the antibody signal from the PAQ8 protein was stronger when Fn was added. Thus, these experiments demonstrated that the 3A10 epitope is located within the 81-amino acid long segment present in PAQ5 and PAQ8 but not in PAQ14. The effect of Fn on the antibody binding is discussed in detail below.

The 3A10 Epitope Is Located within a Fn Binding Segment—

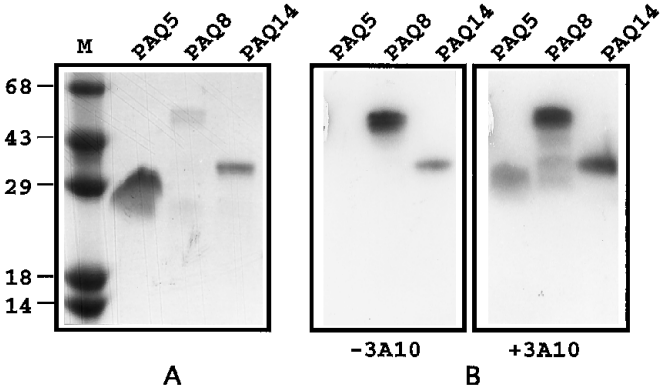

FIG. 4. **Affinity Western blots of PAQ proteins.** The PAQ proteins (PAQ5, PAQ8, and PAQ14) were fractionated by SDS-PAGE on a 12% polyacrylamide gels and stained with Coomassie Blue (*A*) or transferred to a supporting membrane. The membrane was probed with ^{125}I-labeled N29 (*B*) in the presence (*+3A10*) or absence (*−3A10*) of 10 μg/ml 3A10 ascites IgG.

Examination of the amino acid sequence containing the 3A10 epitope region revealed a 30-residue long stretch (corresponding to aa 806–835 in the original sequence) (6), that conforms to the consensus sequences of Fn-binding motifs from Gram-positive bacteria described in earlier studies (6, 8). This sequence, designated Au, was compared with the other Fn-binding motifs. The Au sequence revealed a high homology with the P motif of the Fn-binding MSCRAMM Sfb from *S. pyogenes* and the A2 motif of FnbA from *S. dysgalactiae* (Fig. 2). Because it contained the consensus sequence, Au was tested for Fn binding activity. The binding of ^{125}I-labeled N29 to cells of *S. aureus*, *S. dysgalactiae*, and *S. pyogenes* was inhibited in a dose-dependent manner by PAQ5 as well as by PAQ8 and PAQ14 (Fig. 3). Fifty to 100-fold higher concentrations of PAQ5 compared to PAQ8 or PAQ14 were required for similar levels of inhibition of ^{125}I-Fn binding to bacteria. This may reflect the presence of one Fn-binding motif in PAQ5 compared to 4 and 3 copies in PAQ8 and PAQ14, respectively. A synthetic peptide mimicking the newly identified consensus sequence (designated peptide Au; aa 806–835 of FnbA) also inhibited binding of ^{125}I-labeled N29 to *S. aureus* cells (Fig. 3).

The Fn binding activity of the PAQ proteins was also demonstrated by a Western blot type assay in which ^{125}I-labeled N29 was used as a probe and allowed to bind to the PAQ proteins that had been fractionated by SDS-PAGE and transferred to a supporting membrane. N29 bound strongly to PAQ8 and PAQ14 (Fig. 4*B*), but not enough ^{125}I-N29 bound to the immobilized PAQ5 to generate a detectable signal. However, when 75 μg of IgG purified from 3A10 ascites fluid ("3A10 ascites IgG") was added to the 5 ml of ^{125}I-N29 solution in which the membrane was incubated, binding of the labeled ligand to PAQ5 was markedly increased and readily detectable (Fig. 4*B*). Taken together, these data suggest that the epitope for 3A10 is located in the 81-residue long upstream segment which contains the newly-identified Fn binding motif Au.

Monoclonal Antibody 3A10 Enhances Fn Binding Activity of the Au Sequence—The data described above indicate that 3A10 does not interfere with Fn binding of the epitope-containing recombinant proteins such as PAQ5 but does in fact stimulate their ligand binding activity. To test this hypothesis further, we examined the Fn binding activities of the PAQ constructs in the presence or absence of 3A10 ascites IgG. As shown in Fig. 5, *A* and *B*, the ability of PAQ5 and PAQ8 to inhibit Fn binding by *S. aureus* increased dramatically in the presence of the antibody, over 250- and 10-fold, respectively. The inhibitory activity of PAQ14 which lacks the 3A10 epitope was not en-

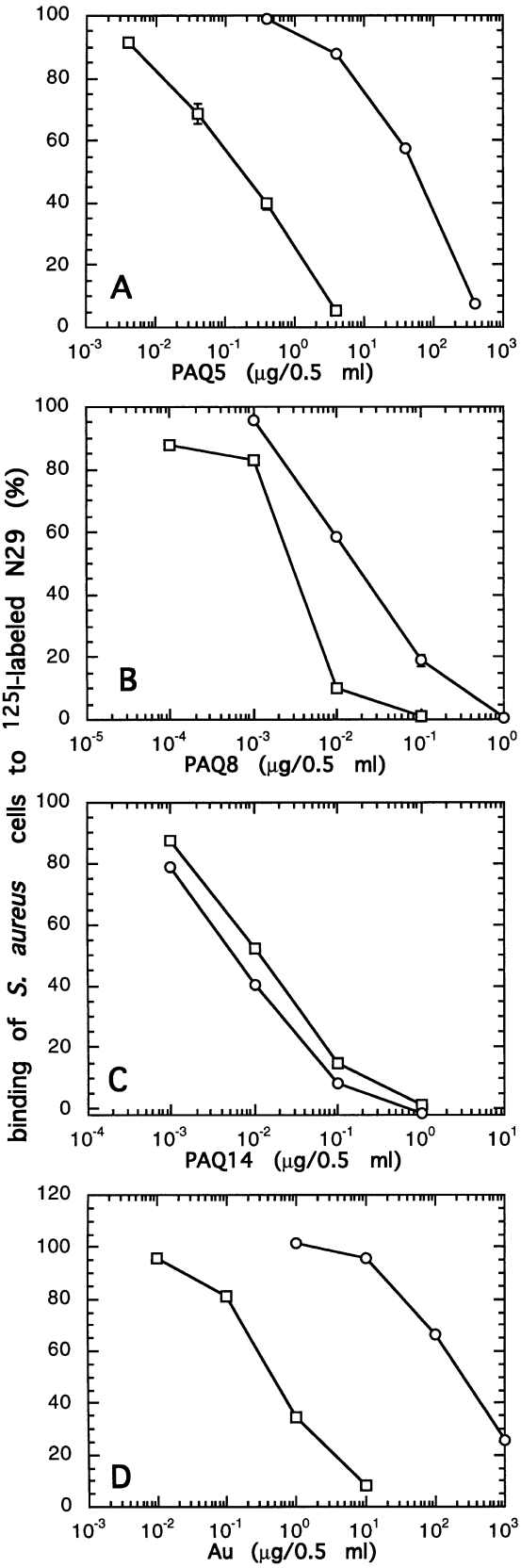

FIG. 5. **Monoclonal antibody 3A10 enhances the Fn binding activity of protein/peptides containing the Au sequence.** *S. aureus* cells (strain 8325-4) suspended in 0.5 ml of PBSTB were incubated with ^{125}I-labeled N29 and varying concentrations of PAQ5 (*A*), PAQ8 (*B*), PAQ14 (*C*), and peptide Au (*D*) were in the presence (*square*) and absence (*circle*) of 100 μg/ml 3A10 ascites IgG. Bacterial binding in the absence of the inhibitors and 3A10 was taken as 100%. *Error bars* indicate the difference between duplicate data points.

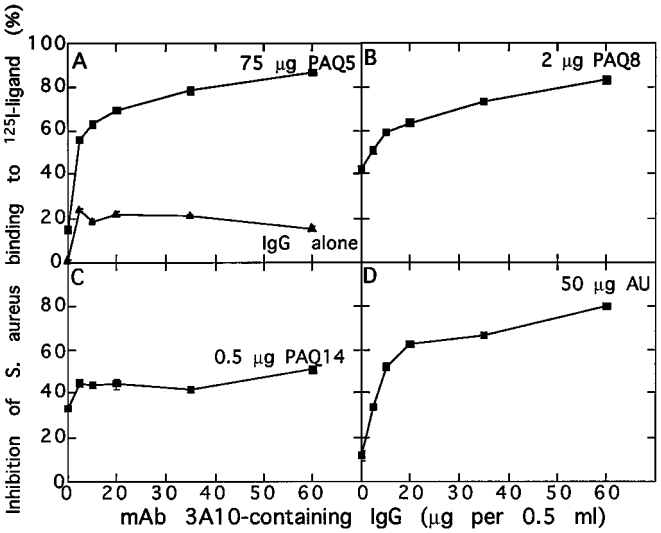

FIG. 6. **Dose-dependent effect of 3A10.** Cells (5×10^8) of *S. aureus* Cowan 1 suspended in 0.5 ml of PBSTB were incubated with indicated concentrations of PAQ5, PAQ8, PAQ14, and peptide Au and ^{125}I-labeled N29 (the PAQ proteins) or Fn (peptide Au) in the presence of varying concentrations of 3A10 ascites IgG. Inhibition (%) = {1 − (binding in the presence indicated inhibitor and 3A10)/(binding in the absence of inhibitor and 3A10)} × 100. *Error bars* indicate the difference between assays performed in duplicate.

hanced by the antibody (Fig. 5*C*). As predicted from the above results, the Fn binding activity of the synthetic peptide Au was dramatically enhanced by 3A10 ascites IgG (Fig. 5*D*). In the absence of antibody, ~200 μg of Au peptide was required for 50% inhibition of Fn binding to *S. aureus* cells; whereas, in the presence of 3A10 IgG, only 0.4 μg of peptide was needed for similar inhibition. Thus, the potency of the Au peptide was increased ~500-fold by the presence of the antibody. An irrelevant mouse IgG was without effect in this system (data not shown).

The potentiating effect of the 3A10 antibody on the Fn binding activity of Au-containing proteins/peptides was measured as a function of antibody concentration. *S. aureus* cells were incubated with ^{125}I-labeled ligand and either PAQ protein or Au peptide where the concentration of protein or peptide was set at a level which cause 10–40% inhibition of ligand binding in the absence of 3A10 IgG. As the concentration of 3A10 ascites IgG was increased from 0 to 120 μg/ml, the inhibitory activity of PAQ5 (at 0.75 μg/0.5 ml) was enhanced in a concentration-dependent manner from 10% up to 85% (Fig. 6*A*). The inhibition by 2 μg of PAQ8 was increased in a similar way from 40 to 80% by 3A10 ascites IgG (Fig. 6*B*), whereas the inhibitory activity of PAQ14, which lacks the 3A10 epitope, was only marginally affected by the antibody (Fig. 6*C*). The Fn binding activity of the synthetic peptide Au was also dramatically enhanced by 3A10 in a concentration dependent manner. In the absence of 3A10 antibody, the Au peptide at 100 μg/ml caused <10% inhibition of bacterial binding of ^{125}I-N29. This inhibition was enhanced up to 80% in the presence of the highest concentration of 3A10 ascites IgG tested (Fig. 6*D*). The 3A10 antibody alone had only a marginal effect on the binding of N29 to *S. aureus* (Fig. 6*A*). The response of peptide Au to 3A10 further demonstrates that the binding sites for both Fn and 3A10 reside in the 30-residue synthetic peptide.

The enhancement of Fn binding activity of PAQ5 by 3A10 was also demonstrated in a Western blot type assay. Fn and N29, separated on SDS-PAGE, were transferred to a membrane and allowed to interact with biotin-labeled PAQ proteins in the absence or presence of 3A10. Binding of PAQ5 to both intact Fn and N29 immobilized on the membrane was wholly

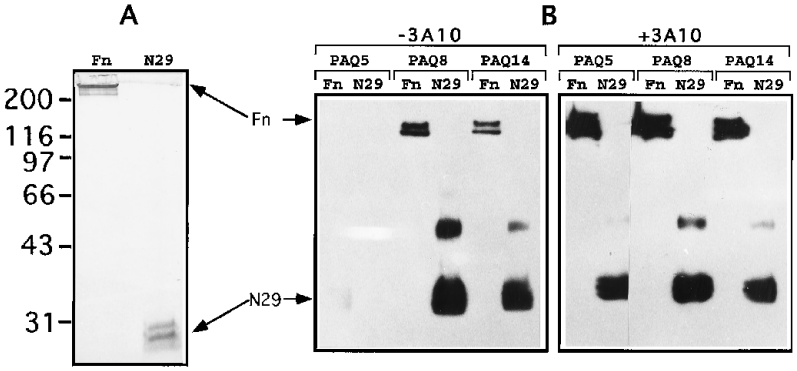

FIG. 7. **Effects of 3A10 on binding of the PAQ proteins to immobilized ligand.** Fn and N29 separated by nonreducing SDS-PAGE on 10% polyacrylamide gels were stained with Coomassie Blue (*A*) or electroblotted onto supporting membranes (*B*). The blots were probed with biotin-labeled PAQ proteins in the absence (*−3A10*) or presence (*+3A10*) of 10 μg/ml 3A10 ascites IgG. A high molecular weight (~60,000) band is observed in the lanes of N29. This fragment which is not observed in the stained gel may have resulted from incomplete digestion of Fn during the N29 preparation.

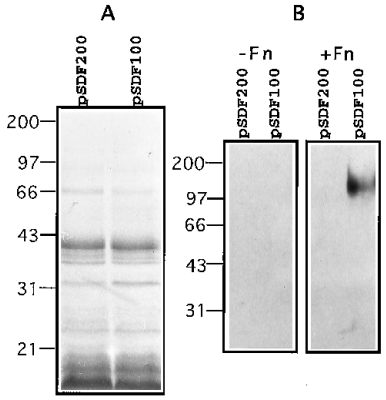

FIG. 8. **Fn-dependent interaction between 3A10 and FnbA.** Lysates of *E. coli* harboring pSDF100 or pSDF200 separated by SDS-PAGE on 10% polyacrylamide gels were stained with Coomassie Blue (*A*) or electroblotted onto supporting membranes (*B*). The membranes were probed with 10 μg/ml 3A10 ascites IgG in the absence (*−Fn*) or presence (*+Fn*) of added Fn (5 μg/ml). Bound antibody was detected as described under "Experimental Procedures."

dependent on the presence of 3A10 (Fig. 7*B*). Both PAQ8 and PAQ14 bind Fn and N29 also in the absence of 3A10 which is expected since both constructs contain the A1, A2, and A3 repeat domains of the primary ligand binding site.

The 3A10 Epitope Is Induced by Ligand Binding—In the epitope localization experiments described in Fig. 1, ascites fluid was used as a source of antibody. The ascites fluid contains, in addition to the IgG, several other components including Fn as previously reported (24) and also demonstrated for these samples in a Western blot assay using an anti-human Fn polyclonal antibody (data not shown). When the Western blot analyses were repeated with 3A10 ascites IgG as the probe instead of 3A10 ascites fluid, no bacterial proteins were recognized by 3A10 irrespective of the presence of the Au sequence. An example is shown in Fig. 8 where proteins in lysates of *E. coli* harboring pSDF100 or pSDF200 (6) were separated by SDS-PAGE, transferred to a membrane, and probed with 3A10 ascites IgG. The plasmid pSDF100 contains the gene encoding FnbA in which the Au sequence is present, and the plasmid pSDF200 contains the gene encoding a second *S. dysgalactiae* Fn binding MSCRAMM, FnbB. The 3A10 ascites IgG did not react with any component in either lysate in the absence of Fn. However, in the presence of 25 μg/ml Fn, 3A10 ascites IgG recognized a band corresponding to FnbA. The FnbB-containing lysate did not react with the antibody even in the presence of Fn, which is consistent with the absence of an Au sequence in FnbB.

The requirement of Fn for the interaction of 3A10 with the Au sequence was further demonstrated in an ELISA assay (Fig. 9). The 3A10 IgG failed to bind to PAQ proteins coated on microtiter wells in the absence of Fn. Binding of 3A10 IgG to

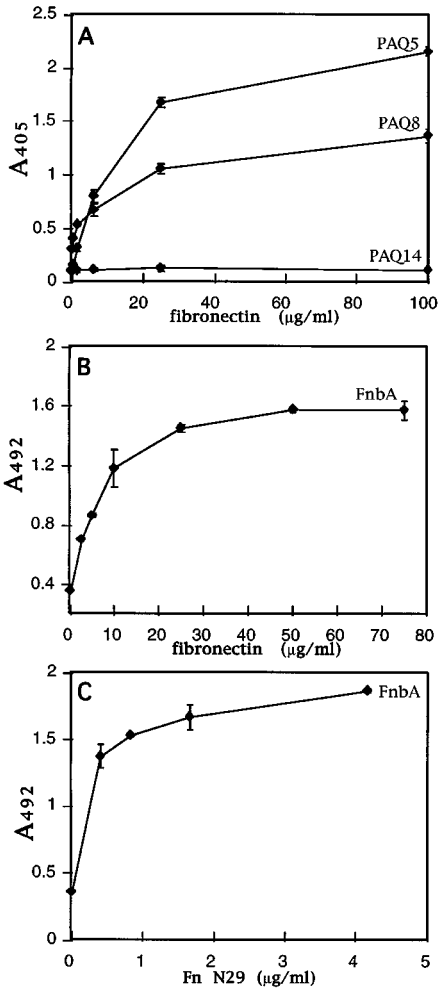

FIG. 9. **ELISA analyses of immobilized FnbA and its derivatives.** PAQ5, PAQ8, PAQ14 (*A*), or FnbA (*B* and *C*) was immobilized onto microtiter wells and probed with 100 μl of 180 pg/ml (*A*) or 10 μg/ml (*B* and *C*) monoclonal 3A10 IgG in the presence of varying concentrations of Fn (*A* and *B*) or N29 (*C*). Bound 3A10 was detected as described under "Experimental Procedures." *Error bars* indicate standard error in triplicate or quadruplicate assays.

PAQ5 and PAQ8 but not PAQ14 was obtained by the addition of soluble Fn in conjunction with the antibody (Fig. 9*A*). Full-length FnbA did not react with 3A10 IgG in the absence of Fn, but bound the antibody in the presence of Fn or its N-terminal fragment N29 (Fig. 9, *B* and *C*). These effects of Fn were dose-dependent. Thus the epitope recognized by 3A10 is formed by an interaction of the Au motif in FnbA with Fn regardless of if the Au motif is present in a synthetic peptide, a short recombinant protein, or the full-length MSCRAMM.

Specificities of 3A10—The specificity of 3A10 was examined

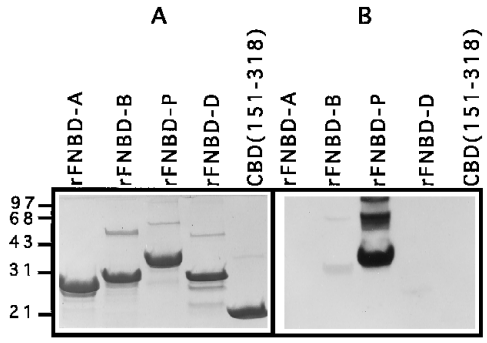

FIG. 10. **Specificity of 3A10.** The rFNBD proteins and CBD (151–318) (9) fractionated by SDS-PAGE on a 10% polyacrylamide gel were stained with Coomassie Blue (*A*) or blotted onto a supporting membrane (*B*). The blot was probed with 3A10 ascites fluid.

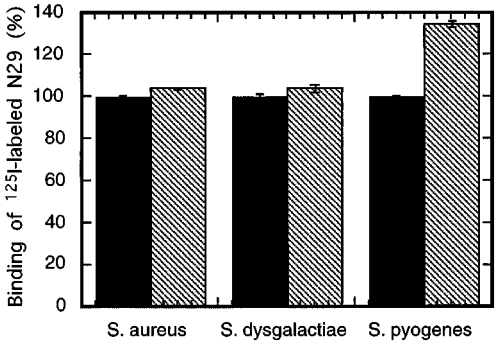

FIG. 11. **Effect of 3A10 on Fn binding to bacterial cells.** Binding of ^{125}I-labeled N29 to bacterial cells were measured in the absence (*solid bar*) and presence of 4.8 μg/ml 3A10 IgG (*hatched bar*). Binding in the absence of IgG was taken as 100%. *Error bars* indicate the difference between the assays performed in duplicate.

in a Western blot assay where recombinant truncated forms of Fn binding MSCRAMMs from different Gram-positive bacteria were subjected to SDS-PAGE. The fractionated proteins were transferred to a support membrane and probed with 3A10 ascites fluid. As shown in Fig. 10, 3A10 reacted strongly with rFNBD-P (9), the ligand binding domain from *S. pyogenes* Fn-binding MSCRAMM Sfb which contains repeat sequences similar to Au (Fig. 2). Weak reactivity was seen with rFNBD-B and rFNBD-D which represent the ligand binding domains of MSCRAMMs FnbB and FnbpA from *S. aureus* and *S. dysgalactiae*, respectively. The monoclonal 3A10 did not recognize rFNBD-A which included the Fn binding motifs A1, A2, and A3 (also found in PAQ14) despite the sequence similarity of the Au sequence with A2. The 3A10 antibody did not react with CBD(151–318), an unrelated collagen binding MSCRAMM (9) from *S. aureus*. These results indicate that the 3A10 epitope, formed when Au binds to Fn, depends on the primary structure and not only on higher order structure formed on ligand binding. The important residues are present in the Au and the P1 and/or P2 repeat units but not in the other known Fn binding repeat units. In support of this conclusion, we found that when Gly^{14} and Gly^{15} in the Au peptide were replaced by Val, the peptide retained Fn binding activity but failed to bind 3A10 even in the presence of Fn. This result indicates that one or both of the Gly residues are part of the epitope recognized by 3A10 (data not shown).

The effect of 3A10 on Fn binding by bacteria was examined by incubating cells of *S. aureus*, *S. dysgalactiae*, or *S. pyogenes* with ^{125}I-labeled N29 in the presence or absence of purified 3A10 IgG (Fig. 11). The monoclonal 3A10 dramatically enhanced the amount of ^{125}I-labeled N29 bound to the *S. pyogenes* cells, while only marginally stimulating N29 binding to *S. aureus* and *S. dysgalactiae*. It is important to note that FnbA is not expressed in the *S. dysgalactiae* strain grown under conventional culture conditions (6), which explains the low enhancement in Fn binding to the *S. dysgalactiae* cells. Therefore, the result shown in Fig. 11 is consistent with the difference in reactivity of 3A10 with the rFNBD proteins observed in the Western blot (Fig. 10). The results demonstrate that 3A10 can recognize an intact MSCRAMM expressed on the surface of a bacterial cell and enhance Fn binding to bacteria presumably by stabilizing the MSCRAMM-ligand complex.

DISCUSSION

The presence of a previously unidentified ligand binding site in the Fn binding MSCRAMM FnbA from *S. dysgalactiae* is demonstrated in this study. This ligand binding site, Au, is contained within a 30-amino acid residue segment which ends 8 residues N-terminal of the previously identified primary binding domain consisting of the three Fn binding motifs A1, A2, and A3. The Au sequence, present in a synthetic peptide or in a recombinant fusion protein also binds Fn as shown by its ability to inhibit Fn binding to *S. aureus* cells.

The epitope for the monoclonal antibody 3A10 has been located to the Au sequence. In the absence of Fn, however, 3A10 does not recognize the Au sequence in a recombinant full-length MSCRAMM, in truncated proteins, or in a synthetic peptide. The epitope recognized by 3A10 appears to be conformation dependent and formed by the binding of Au to Fn. Thus 3A10 can be said to recognize a LIBS in the Fn binding MSCRAMM FnbA. Recent biophysical analysis (17) has shown that the primary ligand binding domain present in the recombinant protein rFNBD-A (9) has little, if any, secondary structure and no tertiary structure, and occurs largely as a random coil. However, on binding to Fn, rFNBD-A appears to undergo a substantial structural change, adopting a defined conformation rich in β-sheet structure. Although the Au sequence has not been directly examined in similar studies, it seems reasonable to assume that, on ligand binding, this segment also undergoes a conformational change from "random coil" to a defined structure contains the epitope for 3A10.

The monoclonal antibody 3A10 also enhances the Fn binding activity of a synthetic peptide or recombinant proteins containing the Au sequence. Presumably, this is caused by 3A10 stabilizing the conformation induced in Au on binding to Fn. Although the A1, A2, and A3 sequences also presumably undergo "induced-fit" conformational changes on binding to Fn, 3A10 does not recognize these sequences nor does it stabilize their ligand complex. Furthermore, 3A10 ascites fluid reacts strongly with rFNBD-P and weakly with rFNBD-B and rFNBD-D. Taken together, these results suggest that the 3A10 epitope is composed of specific amino acid residues present in Au and the P1/P2 motif (Fig. 2) which form a specific conformation on binding to Fn.

Frelinger *et al.* (13, 14) described a number of monoclonal antibodies for LIBS expressed on the platelet integrin $\alpha_{IIb}\beta_3$ only when the integrin forms a complex with the ligand fibrinogen or ligand mimetics. These antibodies were isolated from mice immunized with a mixture of the integrin and a RGD-containing peptide, a ligand mimetic. The anti-LIBS antibodies recognizing $\alpha_{IIb}\beta_3$ or the Fn binding MSCRAMM appear to bind to cell surface "receptors" which undergo fairly extensive conformational changes on ligand binding. In the case of the platelets, this conformational change appears to be of physiological importance and is part of the activation process involved in platelet aggregation.

It is tempting to speculate that the conformational changes in the Au sequence induced by ligand binding and manifested by establishing the 3A10 epitope is a reflection of an important

process evolved in bacteria to avoid immunological interference of microbial adherence to Fn. Our attempts to generate blocking polyclonal antibodies using various recombinant forms of Fn binding MSCRAMMs, or synthetic peptides as antigens have been largely unsuccessful. We do not know if the antigens used generate blocking antibodies together with enhancing anti-LIBS antibodies which compromised the effect of the blocking antibodies, or if the animals are unable to generate any blocking antibodies at all. If the MSCRAMM did not undergo a conformational change on ligand binding, the generated antibodies to the MSCRAMM could interfere with or be indifferent to ligand binding. A conformational rearrangement in the MSCRAMM on Fn binding makes it possible to generate anti-LIBS antibodies which stabilize the ligand MSCRAMM complex resulting in enhanced Fn binding and substrate adhesion. This would clearly represent an advantage to the microbe since the generated antibodies could enhance adherence to host tissue rather than to inhibit this critical step in tissue colonization and the pathogenic process. Studies are in progress to further analyze the immunological response to Fn binding MSCRAMMs.

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A Monoclonal Antibody Enhances Ligand Binding of Fibronectin MSCRAMM (Adhesin) from *Streptococcus dysgalactiae*

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