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The extracellular matrix protein fibronectin (FN) mediates the adhesion of bacteria as well as T lymphocytes. Mammalian cells express integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$ as the major FN-binding cell surface receptors. Bacteria such as Staphylococcus aureus, also express FN-binding receptors that are important for adherence to host tissue and initiation of infection. The S. aureus FN-binding protein, FnbpA, has been previously identified, and recombinant proteins that correspond to distinct functional regions of this protein have been made. Three recombinant truncated forms of FnbpA, rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881), were examined for effects on in vitro adhesion and coactivation of human T lymphocytes. These proteins, when coimmobilized with anti-CD3 mAb, activated T lymphocyte proliferation. The coactivation signal generated by the rFnbpA proteins required medium containing serum with FN. Furthermore, the costimulatory signal could be restored in FN-depleted serum when the rFnbpAs were preloaded with soluble FN. Monoclonal Ab blocking studies revealed that integrin $\alpha_5\beta_1$ is the major receptor responsible for the rFnbpA costimulatory signal. Shear flow cell detachment assays confirmed that lymphocytes can bind to FN captured by the rFnbpA proteins. These results suggest that the S. aureus rFnbpA can interact with integrin $\alpha_5\beta_1$ via an FN bridge to mediate adhesion and costimulatory signals to T lymphocytes. The Journal of Immunology, 2001, 166: 5129–5138.

Control of lymphocyte function must be strictly regulated to protect against invading microorganisms and inappropriate lymphocyte activation that can potentially lead to pathological disease. The regulation of T lymphocyte activation is a carefully orchestrated process that involves signals derived from the extracellular milieu. T cell activation requires two signals; one signal is mediated through the TCR complex, which provides Ag specificity, and a second signal is generated through costimulatory molecules, such as integrins, resulting in T lymphocyte proliferation and differentiation (1, 2). Integrins are heterodimeric cell surface proteins composed of different pairs of noncovalently associated $\alpha$- and $\beta$-chains, which define specificities for various extracellular matrix (ECM)3 components and cell surface ligands (3). On human T cells, integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$ bind to fibronectin (FN) as well as function as costimulatory molecules for T cell activation (3–7).

A critical first step in the development of an infection is the adherence of pathogenic microbes to host tissue (8). For extracellular bacteria such as Staphylococcus aureus, this colonization is mediated by cell surface-expressed adhesins, called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), which specifically bind to host ECM components, including collagen, fibrinogen (Fg), and FN (9, 10).

Two FN-binding MSCRAMMs, FN-binding protein (Fnbp) A and FnbpB, have been identified in S. aureus and the corresponding genes sequenced (11–13). The Fnbps have structural features that are common to other surface proteins expressed by Gram-positive bacteria (see Fig. 1). A signal sequence (region S) is found at the N terminus of these proteins. At the C terminus are features responsible for anchoring the proteins to the cell wall. These include a hydrophobic membrane-spanning region (region M) and a LPXTG motif, the target of a specific transpeptidase (called “sortase”) that covalently links the proteins to the cell wall peptidoglycan (14). The FN-binding activity of the Fnbps has been localized to a ~40 aa residue long repeat unit (D repeat) in the C-terminal portion of these proteins. FnbpA and FnbpB of S. aureus strain 8325-4 contain four tandemly repeated units (D1-D4) and a fifth repeat (Du) is found ~100 aa residues N-terminal to D1 (11–13, 15–17). These repeats are highly conserved between the two proteins (~94% aa identity), and homologous repeats are also found in the FN-binding MSCRAMMs expressed by several streptococcal species (16, 17). The binding site in FN for the repeat units has been mapped to the N-terminal type I modules of this glycoprotein (reviewed in Ref. 18). The N-terminal A regions of the Fnbps share ~45% aa identity and have recently been shown to bind specifically to Fg (19). Two additional repeats (B repeats) of a ~30 aa residue long unit are located at the C-terminal end of the A region of FnbpA. However, the function of these repeats is currently unknown.

It is well documented that FN can serve as a bridging molecule between several different bacterial species and a variety of host cell types, including epithelial and endothelial cells and fibroblasts, and that this interaction can result in bacterial cellular invasion (reviewed in Ref. 18). In the case of S. aureus, it has been shown that...
FN-dependent invasion is mediated by the cell surface-expressed Fnbps and the host cell integrin α5β1 (20–24). Therefore, in this study, we investigated the possibility that FN may also serve as a bridging molecule between the *S. aureus* Fnbps and human T lymphocytes. We examined the effect of recombiant fragments of FnbpA on the activation and adhesion of human T lymphocytes. We show that, by binding to FN, immobilized rFnbpA can provide a costimulatory signal that results in T cell activation and can also mediate T lymphocyte adhesion under conditions of fluid shear stress. Furthermore, we reveal that integrin α5β1 is involved in the activation and adhesion of T lymphocytes in this system.

Materials and Methods

Cell culture

Human T lymphocytic cell lines HPB-ALL and Jurkat, and the human erythroleukemic cell line K562 were maintained in complete medium RPMI 1640 (Mediatech, Herndon, VA) supplemented with 2 mM L-glutamine and 10% FBS (complete medium) at 37°C in 5% CO2.

Purification of human T lymphocytes

Human peripheral blood T cells were isolated by negative selection to avoid activation via the purification protocol. Mononuclear cells were isolated from the buffy coats of healthy donors (Gulf Coast Regional Blood Center, Houston, TX) by density-dependent cell separation on Ficol (1.077 g/ml; Pharmacia Biotech, Uppsala, Sweden). Monocytes were removed by several rounds of plastic adherence on polystyrene petri dishes (Corning, Corning, NY) for 30–45 min at 37°C in 5% CO2. Residual monocytes, polymorphonuclear cells, and RBC were removed by density-dependent cell separation performed on discontinuous Percoll (295 mOsom; Sigma, St. Louis, MO) gradients 37, 44, 60% (v/v) Percoll in RPMI 1640. The remaining cells in the 60% layer were carefully collected, washed in RPMI 1640, resuspended in complete medium, and incubated on a nylon wool column for 30–45 min at 37°C in 5% CO2 to remove the B lymphocytes (25). The column was then washed with complete medium, and the purified T lymphocytes were isolated and used within 24 h. The resultant T lymphocyte population was routinely 95% CD3+ as determined by flow cytometric analysis (FACS) (Epics Profile; Coulter, Miami, FL). In experiments that used FN-depleted FBS, the cells were washed twice in 2 mM EDTA/PBS, to remove any FN bound to the cells, then resuspended either in complete medium or medium with FN-depleted FBS.

FN-depleted FBS

FN was removed from the FBS (Atlanta Biologicals, Norcross, GA) by passage over a gelatin-Sepharose 4B column (Amersham, Uppsala, Sweden) twice. Removal of FN was confirmed by Western blot analysis using rabbit anti-bovine FN polyclonal Abs (26).

Reagents

Most of the mAbs used in this study were made in this laboratory. Anti-β1 mAb 33B6, anti-α5β1 mAb 19H8, anti-α1β2 mAb 32E6, and anti-CD28 mAb 95F12 were used as purified IgG. Anti-α5 was purchased from Chemicon (Temecula, CA). Anti-CD3 mAb OKT3 was obtained from American Type Culture Collection (Manassas, VA) and purified from ascites fluid. Polys-l-lysine was obtained from Sigma. BSA was purchased from Intergen (Purchase, NY). Collagen I was purchased from Collagen Biomaterials (Palo Alto, CA), vitronectin was purchased from Sigma, and Fg was purchased from Calbiochem (La Jolla, CA) and was run over a gelatin column to remove contaminating FN. FN was affinity purified from 200 ml of human plasma (Gulf Coast Regional Blood Center) as follows. Purification was conducted at room temperature. Briefly, a gelatin-Sepharose 4B column was prewashed with PBS containing 2 mM EDTA and 0.1 mM PMSF (PBS/EDTA/PMSF). Plasma with 2 mM EDTA and 0.1 mM PMSF was passed over the column twice, then washed with four column volumes with PBS/EDTA/PMSF. To elute FN from the column, 4 M urea in 0.15 M NaCl, 2 mM EDTA, 0.1 mM PMSF, 20 mM Tris-HCL, pH 8.0, was used. Collected fractions were dialyzed into PBS. Purity of FN was determined by SDS-PAGE.

Expression and purification of recombinant FnbpA proteins

Cell lysates of *Escherichia coli* strain JM101 containing the recombinant FnbpA proteins were prepared as previously described (16, 19, 27). The 6xHis-tagged rFnbpA(37-881), rFnbpA(37-605), and cClfA(221-559) (previously called ClfA; DU1234 in Ref. 16) proteins were purified on a glutathione-Sepharose column, as previously described (16, 19, 27).

Endotoxin contamination was removed from the purified recombinant proteins by detergent extraction followed by affinity chromatography. The proteins (in TBS: 10 mM Tris-HCl, 150 mM NaCl, pH 7.5) were diluted with buffer A (4 mM Tris-HCl, 100 mM NaCl, pH 7.9). Triton X-114 (Sigma) was added to a final concentration of 1% (v/v), and the solution was mixed gently for 1 h at 4°C. The samples were then equilibrated to room temperature and centrifuged for 5 min at 3000 rpm using a Sorvall RC3B Plus (DuPont, Newton, CT). The top aqueous phase was collected and the extraction procedure was repeated. The collected protein extracts were then repurified by either metal chelate affinity chromatography or on a glutathione-Sepharose column, as described previously (16, 19). To remove any remaining endotoxin, polyoxin B-Sepharose (Pierce, Rockford, IL) columns were used. The columns were washed with 1% (v/v) sodium deoxycholate (Sigma), followed by 10 bed volumes of distilled water. The repurified proteins were applied to the columns, and the flow-throughs were collected. The flow-throughs were then reapplied to the columns and collected. After dialyzing the proteins into PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO4, 1.4 mM KH2PO4, pH 7.5), endotoxin contamination was quantified using a Limulus amebocyte lysate (LAL) endotoxin detection kit, according to the manufacturer’s instructions (BioWhittaker, Walkersville, MD). Using this assay, values as high as 3000 endotoxin U/ml have been reportedly obtained for Gram-negative bacterial lysates (28). After decontamination, values ≤3.75 were obtained for the proteins used in this study.

Immunizing mAbs and proteins

Immunization of mAbs or proteins to a 96-well Corning plate (Costar, Corning, NY) has been previously described (29). Briefly, mAbs or proteins were diluted in 0.1 M medium bicarbonate, pH 8. FN and recombinant proteins were immobilized at 50 ng/well, and mAb 32E6 was used at 25 ng/well in all proliferation assays. Coinmobilization of OKT3 and costimulatory proteins were performed by first incubating 50 μl/well anti-CD3 mAb OKT3 (1 μg/ml) for 60 min at room temperature, followed by the addition of 50 μl of costimulatory mAbs or proteins at indicated concentrations. This was incubated at 4°C overnight or at room temperature for 2 h. Plates were blocked with 5% (v/v) BSA (Intergen)/PBS. The wells were washed four times with PBS, once with RPMI 1640, and used immediately. Falcon 35-mm petri dishes (Becton Dickinson, Franklin Hills, NJ) were used in the shear flow cell detachment assays, recombinant proteins rFnbpA(37-605), rFnbpA(37-881), control GST protein, and Fn were immobilized at 4 μg/ml and 4 mg/ml, respectively. The FN concentration of 20 μg/ml. Plates were incubated with the proteins for 2 h at room temperature, then blocked with 5% (v/v) BSA for 2 h. The plates were washed and used immediately.

Preloading of immobilized recombinant proteins with FN, vitronectin, collagen I, BSA, and Fg

Proteins or mAbs were immobilized on 96-well plates or 35-mm petri dishes as previously described. The plates were blocked with excess 5% (v/v) BSA/PBS. The wells or 35-mm petri dishes were washed four times with PBS and once with RPMI 1640. Soluble FN, vitronectin, collagen I, BSA, and Fg (20 μg/ml) were added to wells or the 35-mm petri dishes, which were precoated with immobilized proteins and incubated for 4 h at 37°C. The wells or petri dishes were then rinsed three times with RPMI 1640 to remove any unbound FN, and were used immediately.

T lymphocyte costimulation and addition of soluble proteins and FN

Purified human peripheral T lymphocytes were plated at a density of 2.5 × 105 cells/ml (5 × 106 cells/well) onto coated Conring plates (Costar) in 200 μl of complete medium. Approximately 2–3 days after plating, cells were pulsed with 50 μl/well of 0.5 μCi [3H]thymidine (Amersham, Arlington Heights, IL) in complete medium for 18–24 h. The cells were harvested onto glass fiber filter mats (Whatman, Madison, U.S.) using a PHD cell harvester (Cambridge Technology, Cambridge, MA). [3H]Thymidine incorporation was measured by standard liquid scintillation counting (Beckman LS2800; Beckman Instruments, Fullerton, CA). Soluble mAbs, recombinant proteins, or FN were added at the onset of culture at a concentration of 10 μg/ml; Beckman Instruments, Fullerton, CA).

Staphylococcus aureus PRESENTATION OF FN TO T CELLS

![Staphylococcus aureus](http://www.jimmunol.org/) by guest on January 13, 2018 http://www.jimmunol.org/ Downloaded from
Shear flow cell detachment assay

Immobilized substrate plates were prepared by placing 1.5 ml of 0.1 M NaHCO₃, pH 8, containing rFnbpA(37-605), rFnbpA(37-881), control GST protein, or rFnbpA(620-881) at 4 μg/ml, or FN at 20 μg/ml into a 35-mm easy-grip petri dish (Falcon) and incubated at 4°C overnight. The non-specific binding sites were then blocked using 0.5 ml of 5% (w/v) BSA/PBS for 2 h at room temperature. Cells were washed once in RPMI 1640 then washed once with Modified Tyrode’s Running Buffer (MTRB) (10 mM Tris pH 7.4, 103 mM NaCl, 24 mM NaHCO₃, 5.5 mM glucose, and 5.4 mM KCl) and resuspended in 0.5 ml MTRB. The flow chamber apparatus consists of a thin, rubber spacer with two parallel grooves cut, a 35-mm petri dish making the bottom of the chamber; a hard, plastic cylinder is placed into the 35-mm petri dish and clamped down. The aperture with four openings forms the top of the chamber. The flow chamber apparatus is placed into the 35-mm petri dish and clamped down. The apparatus is assembled and MTRB runs through one opening, over the 35-mm petri dish along one of the grooves in the spacer, and exits through the other opening. The flow chamber apparatus uses a 37°C reservoir of MTRB, the chambered gasket mounted to the microscope, and a flow chamber pump (Harvard Apparatus, Holliston, MA) controlled by a Macintosh SE computer (MacBasic programming). MTRB is pulled through the chamber to remove air bubbles and to warm the plate to 37°C. The cells are injected into the system with a 1-ml syringe and pulled into the flow chamber by manipulation of an efferent syringe outside of the chamber. The cells are allowed to settle for 10 min before starting the pump while maintaining the 37°C incubation. The pump is programmed to run in reverse in a linear mode, to pull the MTRB through the system at a specified rate of 0.04–0.08 ml/min over 300 s. After the cells settle within the chamber, the flow chamber pump is initiated, the flow rate is increased in a linear fashion over time, and the events are recorded using time lapse VCR and later analyzed using the technique described below.

Data analysis for the shear flow cell detachment assay

A Nikon inverted microscope was used with a ×20 objective to observe cell adhesion during shear flow cell detachment runs. A charge-coupled device camera was mounted to the microscope and was connected to a time lapse VCR (Panasonic, Secaucus, NJ) to collect real time images for later analysis. Scion imaging software (NIH Image) was used to capture and enhance images from the VCR tapes to produce clear black and white images. Once the images were printed, the cells at each time point were counted and converted to percentages of the initial cell number. The number of cells at a given time point was divided by the number of cells at time 0 and multiplied by 100 (percent cells bound). Shear stress values were calculated using the formula: \( \frac{3Q}{wh^2} \), where \( Q \) is the flow rate in cm³/s, \( w \) is the width of the chamber, and \( h \) is the height of the chamber. The resulting value of the shear stress calculation is in dynes/cm². Each time point correlates with a specific shear stress, in dynes/cm², and the results were shown as a percentage of the cells in a given field before the start of a run: [(number of cells bound at a given flow rate)/(number of cells before the start of the run)] × 100.

Results

rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) mediated T lymphocyte coactivation in the presence of anti-TCR mAb

The 6xHis-tagged protein rFnbpA(37-881) encompasses the entire FnbpA protein of S. aureus strain 8325-4 but lacks the N-terminal signal sequence and the cell wall anchoring regions (19). The 6xHis-tagged protein rFnbpA(37-605) encompasses the Fg binding A region and B repeat region of FnbpA and lacks the FN-binding D repeat regions (19). The GST-fusion protein rFnbpA(620-881) encompasses the D repeat units (Du through to D4) of FnbpA(620-881), and the amount of proliferation was measured by [³H]thymidine incorporation. The results of the proliferative signals generated by rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881), are shown in Fig. 2. The amount of proliferation induced by OKT3 (25 ng/well) alone, FN (50 ng/well) alone, control GST protein (50 ng/well) alone, rFnbpA(37-881) (50 ng/well) alone, rFnbpA(37-605) (50 ng/well) alone, and rFnbpA(620-881) (50 ng/well) alone were all <2000 cpm.

However, when rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) were coimmobilized with mAb OKT3, the amount of [³H]thymidine incorporation was 38,387, 44,704, and 39,371 cpm, respectively. These counts were comparable to the 50,218 cpm generated by the positive control OKT3 coimmobilized with FN. Control GST protein coimmobilization with OKT3 did not coactivate the T cells and resulted in 7427 cpm.

Effect of soluble rFnbpA on T cell proliferation mediated by OKT3 coimmobilized with rFnbpA

T cell proliferation assays were set up to determine whether soluble rFnbpA proteins could alter T cell coactivation mediated by each of the immobilized rFnbpA proteins (Fig. 3). Soluble rFnbpA(37-881) (stippled column) inhibited T cell proliferation generated by OKT3 + rFnbpA(37-881), OKT3 + rFnbpA(37-605), OKT3 + rFnbpA(620-881) by 87.9, 93.6, and 94.3%, respectively. Similar to soluble rFnbpA(37-881), soluble rFnbpA(620-881) (filled column) inhibited proliferation mediated by OKT3 + rFnbpA(37-605) by 93.4%, OKT3 + rFnbpA(37-881) by 95.4%, and OKT3 + rFnbpA(620-881) by 94.6%. However, soluble rFnbpA(37-605) (hatched column) inhibited costimulation mediated by OKT3 + rFnbpA(37-605) by 87.1%, and only slightly inhibited OKT3 + rFnbpA(37-881) and OKT3 + rFnbpA(620-881) proliferation by 13.1 and 21%, respectively. This result may be due to the fact that rFnbpA(37-605) lacks the D repeat region, which is the previously identified FN-binding region of FnbpA. It is possible that the A region and B repeat region, contained within rFnbpA(37-605), are not sufficient to competitively block both the FN-binding and coactivation activity of rFnbpA(37-881) and rFnbpA(620-881).

rFnbpA(37-881) and rFnbpA(37-605) require the presence of OKT3 and medium supplemented with FBS containing FN for costimulation of T cells

To determine whether rFnbpA(37-881) and rFnbpA(37-605) required FN to mediate coactivation, purified T cells were first washed twice in 2 mM EDTA/PBS to remove any bound FN, then

![FIGURE 1. Structural organization of FnbpA of S. aureus strain 8325-4. The recombinant 6xHis-tagged and GST fusion proteins used in this study are also shown. The amino acid residues contained in each construct are indicated in parentheses. S, Signal sequence; A, Fg-binding region; B1 and B2, homologous repeats of unknown function; Du-D4, FN-binding repeat units; W, wall-spanning region; M, membrane-spanning region; +, positively charged tail. The position of the LPXTG motif, involved in anchoring the protein to the cell wall peptidoglycan, is also indicated.](http://www.jimmunol.org/ji620679.html)
the cells were plated in wells containing rFnbpA(37-881) or rFnbpA(37-605) coimmobilized with OKT3 and in RPMI 1640 containing whole FBS or FN-depleted serum (Fig. 4). Washing cells with 2 mM EDTA/PBS did not alter the cells’ capacity to proliferate (data not shown). T cell proliferation was determined by the amount of $^{[3]H}$thymidine incorporated. Wells with BSA or polyethylene, in all combinations with or without OKT3 and with medium containing whole FBS or FN-depleted FBS, did not result in any T cell proliferation. As a positive control, FN coimmobilized with OKT3 mediated T cell proliferation whether the medium contained whole FBS or FN-depleted FBS (hatched and solid gray bars). rFnbpA(37-881) and rFnbpA(37-605) only mediated T cell proliferation when coimmobilized with OKT3 and plated in medium containing whole FBS (hatched columns). The ability of rFnbpA(37-605) to mediate T cell activation in the presence of OKT3 in medium containing whole FBS was surprising because rFnbpA(37-605) does not contain the previously identified FN-binding D repeat region. The recombinant rFnbpA(620-881) protein, which represents the previously identified FN-binding D repeats of the MSCRAMM, also only mediated proliferation when coimmobilized with OKT3 and in the presence of medium with whole FBS (data not shown). These results suggest that the rFnbpA proteins coimmobilized with OKT3 alone is not sufficient for costimulation, and the presence of FBS containing FN is required for the costimulatory effects of recombinant proteins, rFnbpA(37-605), rFnbpA(620-881), and rFnbpA(620-881).

The titration effect of soluble FN with rFnbpA(37-881) and rFnbpA(37-605) is shown in Fig. 5. Cells were washed twice in 2 mM EDTA/PBS then plated into wells with OKT3 coimmobilized with rFnbpA(37-881) (closed circle), rFnbpA(37-605) (open triangle), and rFnbpA(620-881) (open square).
FN (open diamond), or mAb to \( \alpha_2 \beta_1 \) (32E6) (closed square), in RPMI 1640 with FN-depleted FBS (Fig. 5). Different concentrations of soluble FN were added to wells at the onset of the assay. As expected, T cell proliferation mediated by OKT3 with FN (open diamond) and OKT3 with mAb 32E6 (closed square) remained relatively constant at all concentrations of soluble FN. With the addition of soluble FN, coimmobilized rFnbpA(37-881) and OKT3 (closed circle) caused an increase in proliferation until 3.125 \( \mu g/ml \) FN followed by a decrease in proliferation as the amount of soluble FN increased. rFnbpA(37-605) coimmobilized with OKT3 (open triangle) increased proliferation as the amount of soluble FN increased.

Preloading rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) with soluble FN can restore costimulation of T cells in medium containing FN-depleted FBS

As rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) seemed to require FN to mediate a functional costimulatory signal for T cell activation, this specific FN dependency was investigated in more detail. Plates previously immobilized with mAb OKT3 and FN, mAb 32E6, rFnbpA(37-881), rFnbpA(37-605), or rFnbpA(620-881) were preloaded with (filled columns) or without soluble FN (open columns) for 4 h at 37°C, then the wells were washed three times with RPMI 1640 to remove the unbound FN as described in Materials and Methods. Purified T lymphocytes were plated into the wells with RPMI 1640 containing FN-depleted serum, and the amount of proliferation was determined (Fig. 6). OKT3 coimmobilized with FN or mAb 32E6 resulted in strong responses regardless of whether the wells were preloaded with soluble FN or not. In contrast, rFnbpA(37-881), rFnbpA(37-605), or rFnbpA(620-881) coimmobilized with OKT3 required preloading with soluble FN to restore proliferation, and a signal of 41,785 cpm was measured for OKT3 + rFnbpA(37-881), 41,439 cpm was measured for OKT3 + rFnbpA(37-605), and 43,348 cpm was measured for OKT3 + rFnbpA(620-881). These results suggest that rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) bind soluble FN, and this complex can generate a functional costimulatory response.

T lymphocyte proliferation is specifically mediated by preloading rFnbpA proteins with FN

To determine whether the effects of preloading rFnbpA proteins were specific to FN, other extracellular proteins were tested as indicated in Fig. 7. FN-depleted soluble Fg, BSA, vitronectin, collagen I, and FN were preloaded on plates previously immobilized with BSA alone, OKT3 alone, or OKT3 coimmobilized with control 6xHis-tagged protein ClfA, rFnbpA(37-881), rFnbpA(37-605), rFnbpA(37-881), rFnbpA(620-881), FN, or anti-\( \alpha_2 \beta_1 \) mAb 32E6. Only preloading with soluble FN mediated proliferation of OKT3 + rFnbpA(37-881) (11,657 cpm), OKT3 + rFnbpA(37-605) (10,812 cpm), OKT3 + rFnbpA(620-881) (10,812 cpm). OKT3 coimmobilized with FN or mAb 32E6 resulted in strong responses (counts higher than 15,000 cpm) regardless of whether the wells
were treated with extracellular proteins or not. OKT3 immobilized with control 6xHis-tagged protein, ClfA did not mediate any proliferation above that measured for OKT3 alone in any condition. These results confirm that preloading of rFnbpA proteins with soluble FN is specifically responsible for mediating T cell proliferation.

Integrin $\alpha_5\beta_1$ is the major receptor involved in rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) costimulation

To identify the T cell surface proteins that were responsible for the signals mediated by rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881), soluble mAbs to various cell surface proteins were used to inhibit the costimulatory signals mediated by the recombinant rFnbpA proteins (Fig. 8). Soluble mAb to the costimulatory molecule CD28 only slightly affected the costimulation mediated by rFnbpA(37-605) (10.4% inhibition). Soluble anti-$\alpha_5\beta_2$ mAb 32E6 did not inhibit costimulation by OKT3 coimmobilized with rFnbpA(37-881), rFnbpA(37-605), or rFnbpA(620-881). When a $\beta_1$ integrin specific mAb 33B6 was added, it inhibited costimulation by OKT3 + rFnbpA(37-605) (71%), OKT3 + rFnbpA(37-881) (70%), and rFnbpA(620-881) (71%). Soluble mAb to $\alpha_5$ inhibited costimulation mediated by OKT3 + rFnbpA(37-605) (45%), OKT3 + rFnbpA(37-881) (46.2%), and rFnbpA(620-881) (38.7%). In other experiments, $\alpha_5$ inhibition of all three recombinant proteins was as high as 80% (data not shown). A soluble mAb to integrin $\alpha_5\beta_1$ (30) was also used in the proliferation assay, but the inhibition was minimal and inconsistent (data not shown). The results suggest that the integrin $\alpha_5\beta_1$ is a major surface protein involved in mediating rFnbpA(37-605), rFnbpA(37-881), and rFnbpA(620-881) coactivation.

rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) can support FN-mediated adhesion of lymphocytes

To confirm that the rFnbpA proteins are mediating cell binding, a shear flow cell detachment assay was performed using various human cell lines expressing different levels of the integrins $\alpha_5\beta_1$ or $\alpha_4\beta_1$. Jurkat T cells that express both $\alpha_5\beta_1$ and $\alpha_4\beta_1$. Jurkat T cells that express both $\alpha_5\beta_1$ and $\alpha_4\beta_1$ were used to

**FIGURE 7.** Effect of preloading rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) with soluble extracellular proteins. The indicated proteins were immobilized and were preloaded with or without 20 $\mu$g/ml soluble proteins (as identified in figure) in RPMI 1640 without FBS. The preloading of soluble proteins was performed as described in Materials and Methods. Purified T cells ($5 \times 10^5$ cells/well) were washed twice in 2 mM EDTA/PBS before plating in RPMI 1640 containing FN-depleted FBS, and the amount of proliferation was measured by the amount of $[^{3}H]$thymidine incorporated. Data are expressed as mean cpm ± SEM of triplicate samples. Where error bars are not indicated, SEM was smaller than the data point symbol. This experiment was repeated four times with similar results.
determine whether rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) can maintain adhesion under increasing shear force and whether this adhesion was also dependent on soluble FN (Fig. 9). Immobilized FN mediated Jurkat cell adhesion throughout the duration of the assay with increasing number of cells detaching as the shear flow increased (X symbol). Control GST protein with (filled squares) or without incubation with soluble FN (open squares) did not mediate Jurkat cell adhesion. rFnbpA(37-881) (open diamonds) and rFnbpA(37-605) (open triangles) did not mediate adhesion. By preloading with FN, rFnbpA(37-881) (filled diamonds) and rFnbpA(37-605) (filled triangles) both gave increased cell adhesion. rFnbpA(620-881) protein did not mediate adhesion without FN (open circles) but when preloaded with soluble FN (filled circles), it mediated adhesion similar to that of rFnbpA(37-881) + FN. The adhesive differences between rFnbpA(37-881) and rFnbpA(37-605) may be due to differences in FN-binding affinities and/or available FN-binding sites.

Fig. 10 shows the results from the shear flow cell detachment assay using \( \alpha_5 \beta_1 \) expressing HPB-ALL. All the recombinant proteins in this assay were preloaded with soluble FN as described in Materials and Methods. The results show that rFnbpA(620-881) + FN (filled circles) and rFnbpA(37-605) + FN (filled triangles) did not mediate cell adhesion even when preloaded with soluble FN. rFnbpA(37-881) + FN (filled diamonds) mediated cell adhesion and as expected immobilized FN (\( \times \)) mediated adhesion of HPB-ALL cells. When K562 cells, which express \( \alpha_5 \beta_1 \) exclusively, were used in the shear flow cell detachment assay (Fig. 11) all three recombinant proteins mediated cell adhesion when preloaded with FN. Altogether these results indicate that all three rFnbpA proteins can support FN-dependent cell adhesion and suggests that it is predominantly mediated by the integrin \( \alpha_5 \beta_1 \).

![Graph](attachment://image.png)
Discussion

Integrins play a major role in host defense mechanisms against infections by recruiting circulating cells to the site of infection, coordinating cell transmigration through the endothelial barrier, basement membrane, and ECM, and functioning as costimulatory molecules for T cell activation (3). During the early stages of infection, bacteria can adhere to host tissue and the immune system can become activated and eliminate the bacteria; however, in many cases the bacteria can evade or suppress the immune system, thus avoiding elimination (8). Microorganisms, such as the pathogenic bacterium *S. aureus*, have surface proteins that can modulate the host immune system. *S. aureus* expresses superantigens (SAgs), which bind to human MHC class II molecules, primarily HLA-DR, and TCR (31–35). SAg binding to human T lymphocytes alone can inappropriately activate the cells, and this can result in T cell anergy or the elimination of the activated cells by apoptosis (36–38). The primary target cells of SAgs are the CD4$^+$ T cells and their activation can result in T helper-type 1 cytokine release, which can lead to reduced Ab production and possibly decreased clearance of the bacteria (39, 40). However, when a second signal is provided in conjunction with a signal through the TCR by costimulatory molecules such as integrins, normal T lymphocyte activation can occur (41, 42). In this study, we have shown that the FN-binding MSCRAMM FnbpA of *S. aureus* can capture FN and properly present it for T cell coactivation, suggesting that a potentially powerful immunomodulatory system can be initiated and supported by *S. aureus*.

In this report, to investigate the effect of FN-binding MSCRAMMs of *S. aureus* on human T cells, the recombinant proteins rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) were used in T lymphocyte proliferation assays and shear flow cell detachment assays. The results from these studies indicate that all three recombinant proteins were able to provide a functional costimulatory signal when coimmobilized with the mAb to CD3, and this coactivation required the presence of soluble FN (Figs. 4–7). The ability of rFnbpA(37-605) to mediate coactivation in an FN-dependent manner was a surprising result. The result was unexpected because, although both rFnbpA(37-881) and rFnbpA(620-881) were used in T lymphocyte proliferation assays and shear flow cell detachment assays. The results from these studies indicate that all three recombinant proteins were able to provide a functional costimulatory signal when coimmobilized with the mAb to CD3, and this coactivation required the presence of soluble FN (Figs. 4–7). The ability of rFnbpA(37-605) to mediate coactivation in an FN-dependent manner was a surprising result. The result was unexpected because, although both rFnbpA(37-881) and rFnbpA(620-881) contain the previously defined FN-binding D repeat region, rFnbpA(37-605) lacks this region and, therefore, was not anticipated to interact with FN. Despite this fact, rFnbpA(37-605) was still able to provide a functional costimulatory signal suggesting that it may contain an unidentified region that can bind FN, possibly with a lower affinity than the proteins containing the D repeat region. This may explain the results in Fig. 3 in which soluble rFnbpA(37-605) can inhibit itself from FN-mediated coactivation, but cannot inhibit coactivation of rFnbpA(37-881) or rFnbpA(620-881), which both contain the FN-binding D repeat region. This is the first evidence suggesting an additional FN-binding site for *S. aureus* FnbpA. Experiments are being performed to localize this site. It is clear from Figs. 9 and 11 that although rFnbpA(37-605)
does not support the adhesion of Jurkat and K562 cells as well as rFnbpA(37-881) and rFnbpA(620-881), nonetheless, measurable adhesion occurred. This adhesion appeared to be sufficient to co-activate T cells in a situation where static interactions occurred between rFnbpA(37-605), FN, and T cells.

In the proliferation assays the predominant integrin involved was determined to be α5β1. In the adhesion assays, rFnbpA(37-881) and rFnbpA(620-881) proteins supported strong adhesion to α5β1- and α4β1-expressing Jurkat cells and α5β1-expressing K562 cells, whereas rFnbpA(37-605) supported weaker adhesion to both Jurkat and K562 cells. No adhesion was detected for rFnbpA(37-605) and rFnbpA(620-881) with the α5β1 expressing HPB-ALL cells, and weak adhesion was found with FnbpA(37-881). Thus, it appears that when FN is presented by the immobilized rFnbpA proteins, α5β1 is not able to support adhesion as well as α5β1. These results, coupled with the proliferation studies, suggest that rFnbpA(37-881), rFnbpA(37-605), and rFnbpA(620-881) can mediate adhesion and coactivation signaling to T lymphocytes through an FN bridge that is predominantly mediated by integrin α5β1.

In this study, we have shown that recombinant forms of FnbpA are able to mediate T lymphocyte costimulation and cell adhesion in the presence of FN through the integrin α5β1. We and others have previously shown that FN-binding MSCRAMMs and host cell integrins are involved in bacterial adhesion to and invasion of a variety of nonphagocytic mammalian cells (20–24, 45–47). Furthermore, in the case of S. aureus, host cell invasion was shown to involve an FN bridge between the bacterial FN-binding MSCRAMMs (FnbpA and FnbpB) and β1 integrin (23, 24). Consistent with this, the method by which the rFnbpA proteins in this study use FN to interact with human T cells is one that requires FN to form a bridge between the recombiant proteins and the integrin α5β1. This complex is sufficient to mediate cell adhesion as well as to generate a functional T cell costimulatory signal through integrin α5β1. Adhesion and activation of T cells by the bacteria FnbpAs may be a useful mechanism to alter the immune response of the host against the invading bacteria. This scenario may occur after the T cells are recruited to the site of infection. At this time, activated integrins on the T cells can bind to the FN and can attach to the FnbpA on S. aureus. These adherent T cells or newly recruited T cells may be in sufficient proximity to allow for coactivation of the T cells by bacteria-derived SAgS or activation by host-derived cytokines.

 Independently, the attachment of bacteria to ECM components through MSCRAMMs or SAg binding to TCR and MHC class II complex are mechanisms that favor bacterial colonization and survival in the host. However, these events occur simultaneously, the resulting circumstances can be detrimental to the bacteria. If proper activation signals to the TCR and costimulatory molecules are generated, a T lymphocyte can be activated and the mechanisms to clear the bacteria can be initiated by the immune system. We have demonstrated that recombiant forms of the FN-binding MSCRAMM FnbpA can provide functional costimulatory signals to the integrin α5β1 through an FN bridge. If the bacteria generate a signal to integrins by binding FN via MSCRAMMs and sends another signal generated by SAg binding to the TCR complex, this creates a scenario that can lead to the activation of the immune system and results in either the elimination of the invading microorganism or, in some cases, the pathological conditions conducive to inflammation or autoimmune diseases. Whether bacteria have evolved under selective pressure to express a repertoire of receptors that can effectively bind to ECM components or T cell/MHC class II complex or whether the host has developed a defense mechanism specifically to counter microbial invasion is an unresolved issue.

References


32. Fraser, J. D. 1989. High-affinity binding of staphylococcal enterotoxins A and B to IL-4-DR. Nature 339:221.


