

TITLE: Regions of functional upstream domain of protein F1 of *Streptococcus pyogenes* necessary for exposure of tenth type III module in fibronectin

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Regions of functional upstream domain of protein F1 of *Streptococcus pyogenes* necessary for exposure of tenth type III module in fibronectin

The 49 amino acid portion of the F1 surface protein of *Streptococcus pyogenes* known as the functional upstream domain (FUD) interacts with Fn in an unknown manner that prevents assembly of a Fn matrix. When FUD binds to Fn, it causes a conformational change that exposes ¹⁰FnIII. This study examined the important regions of FUD that are needed for binding of FUD to Fn by using 18 mutant FUD peptides. A monoclonal antibody (mAb) was used to detect an epitope on ¹⁰FnIII that becomes available upon a conformational change in Fn. Mutant FUD peptides that had residues deleted lost their function to expose ¹⁰FnIII. Other mutant FUD peptides that had block alanine substitutions had varying abilities to expose ¹⁰FnIII, dependent upon the specific residues that were mutated. Heparin, known to bind to Fn in the same region as FUD, can bind to Fn and cause exposure of ¹⁰FnIII, but does not inhibit FUD binding to Fn.

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Abstract

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Introduction

Fibronectin (Fn) is a ubiquitous and abundant protein required for many important physiological functions, such as cell adhesion, growth, and wound healing (1). To accomplish these functions, Fn must be assembled into a fibrillar network in a process known as Fn assembly (1). Fn is a 500-kDa glycoprotein dimer, composed of two ~250-kDa subunits connected by a disulfide-bond (2). Each subunit of Fn is composed of three types of repeating homologous modules, types I, II, and III (2). Sets of these modules create domains that allow binding to various molecules and proteins such as: collagen, fibrin, heparin, and Fn (1). The assembly into fibrils is a cell-mediated process that depends in part on interactions between Fn and integrin receptors (3). The RGD sequence on the tenth fibronectin type III domain (¹⁰FnIII) is responsible for binding to integrins and activating the Fn assembly process (4). The process also requires the N-terminal 70-kDa fragment (70K) of Fn. This was demonstrated by studies that showed Fn and 70K bind to the surface of fibroblasts or platelets

with the same affinity and location (5,6). Also, Fn lacking 70K is incapable of fibril assembly (7), and it has also been shown that deletion of any of the first five Type I modules of 70K reduces the affinity of the N-terminal region for cellular fibronectin assembly sites (7). Thus, interactions or disruptions of the N-terminal Type I modules affects fibronectin assembly.

70K is composed of various binding regions. The 27-kDa N-terminal region (27K) of 70K contains five type I modules, which are essential in matrix assembly (8). Type I modules are about 45 residues long and have a pair of disulfide bonds that contribute to the formation of the tertiary structure. This region is also the site of fibrin binding, which is an important part of blood clot formation (9). Fibrin is crosslinked to the 27K region by covalently linking glutamine residues on Fn to the ϵ -amino group of a lysine residue on fibrin (9). This process is mediated by thrombin-activated factor XIII. Another region of 70K is the 40-kDa N-terminal region (40K) that is the site of collagen binding. The binding of Fn to collagen is important for the formation of the extracellular matrix (ECM) (10). An important function of this interaction occurs during wound repair. Fn increases macrophage phagocytic activity (11) by forming a complex with tissue debris and binding to hepatic macrophages (12). It is also shown that Fn can enhance the ingestion of gelatin-coated particles by macrophages (12).

One class of proteins known to interact with Fn is the microbial surface components recovery adhesive matrix molecule (MSCRAMM) (13). One such protein that has been intensely studied is Fn binding protein A (FnBPA) of *Staphylococcus aureus*. *S. aureus* is a hazardous human pathogen that causes life-threatening infections. Bingham *et al.* investigated the mechanism by which FnBPA binds and adheres to Fn, in order to determine if there are new ways of inhibiting this interaction. By inhibiting the interaction that allows *S. aureus* to invade cells, it may be possible to prevent the infections from ever occurring. Using x-ray crystallography, Bingham *et al.* showed that binding

repeats in FnBPA binds to Type I modules 2, 3, 4, and 5 of the N-terminus of Fn. Each type I module contains a major and minor β -sheet, comprised of 5 β -strands. FnBPA binds by adding an extra β -strand to the major β -sheet (14), and this interaction is referred to as the tandem β -zipper (21).

Similarly, *Streptococcus pyogenes*, a group A streptococcus, is known to cause many human infections and does so by using its own MSCRAMM, the F1 surface protein and its allelic variant Sfb1, to invade host tissue (15). The functional upstream domain (FUD) of F1 is a 49-residue N-terminal non-repetitive sequence (16). It is the smallest segment of the F1 protein that allows for the binding of F1 to the 70K (16). Given that the 70K is essential for Fn assembly, binding of FUD should inhibit the ability of Fn to assemble, and this was observed by Tomasini-Johansson *et al.* (16). The binding of FUD to Fn is also known to cause a conformational change in Fn, exposing 10 FnIII. Also, 10 FnIII contains the RGD sequence responsible for integrin binding, thus FUD binding may render Fn more competent to interact with integrin receptors (17). This response could increase the internalization of *S. pyogenes* into cells (17) and alter Fn assembly. In order for Fn to assemble into fibrils, it must be in its native conformation. Disruption by FUD binding disables assembly of exogenous or endogenous Fn (16). Based on modeling FUD into the crystal structure obtained by Bingham *et al.*, we believe that the backbone of FUD binds in an anti-parallel fashion to the β -sheet conformation of the N-terminus of Fn. Recently, it was shown that FUD binds at least to 2 FnI- 1 FnIII (unpublished)

To study the binding interaction between FUD and Fn we used 18 mutant FUD peptides, with alterations ranging from the N-terminal to the C-terminal. The mutagenesis studies indicate that there are specific regions of FUD required for binding to Fn, and that there is an interesting mutant that has the 29th residue deleted, d29, with loss of function of the peptide. Also, other molecules that bind to Fn, such as monoclonal antibodies or heparin, were tested for similar properties. The binding of some

of these molecules caused $^{10}\text{FnIII}$ to be exposed, suggesting that Fn can have multiple interactions that cause its conformation to change.

Methods

Mutagenesis of Recombinant FUD

PCR was used to mutate each variant of the FUD peptide. The resulting cDNA was digested with KpnI and NheI restriction enzymes and subsequently inserted into the pET-28c+ vector. pET-28c+ was modified to contain a previously modified multiple cloning site from pQE-30 (Ensenberg 2004). The modified pQE-30 was engineered to contain a NcoI restriction site at the N-terminal methionine. pQE-30 was also modified to contain an N-terminal 6x His-tag followed by a thrombin cleavage site. Once sequenced, the construct was transformed into BL21(DE3) cells for protein expression.

Expression/Purification

For protein expression, 5 mL LB containing 30 $\mu\text{g/mL}$ kanamycin overnights were prepared. The following day, 3 mL of overnight culture were added to 300 mL of LB containing 30 $\mu\text{g/mL}$ kanamycin. Bacteria were grown until the absorbance at 590 nm was 0.6, followed by the addition of 1 M IPTG to a final concentration of 1 mM. Four hours after induction, the bacteria were centrifuged for 20 minutes at 5000 x g in the Sorvall. The supernatant was poured off, and the pellet was weighed and stored at -20°C . The next day, or within at most a week, the pellet was thawed on ice. Lysis buffer (50mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8.0) was added (5 mLs/g cells) and the cells were lysed for two hours, centrifuged for 20 minutes at 7500 x g to remove any insoluble debris, and the supernatant collected. 1 mL of packed Ni-NTA resin (Biorad) (2 mL of 50% slurry

equilibrated in lysis buffer) was added to bind to the His-tagged proteins. This was incubated at room temperature for one hour, then overnight at 4°C on a nutator. The next day, the mixture of resin and lysate was added to a disposable polypropylene column (0.8 x 4 cm). The His-tagged proteins were expected to bind to the Ni-NTA resin, and the remaining flow-through was collected. Next, the column was washed with 10 bed volumes (BV) of wash buffer (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, 15 mM imidazole, pH 8.0). Column volume (CV) is the amount of Ni-NTA resin, and BV is 0.7 x CV. The final step was elution of the protein off of the Ni-NTA resin. Initially, 1 BV of elution buffer (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, 250 mM imidazole, pH 8.0) was added. 0.5 BV was collected and labeled elution fraction 1 (E1), and the remaining was then collected and labeled elution fraction 2 (E2). The mixture was allowed to incubate for 2 hours, after which 1 BV of elution buffer was added and collected in E2, and 1 BV was added and allowed to incubate for 15 min. Finally, elutant was collected in E2 until a final volume of 1.5 x BV, and the remaining collected and labeled elution fraction 3 (E3). 20 µL of the flow-through, wash, E1, and E3 fractions were mixed with 5 µL of 5x sample buffer (9 M urea, 3% sodium-dodecyl-sulfate (SDS), 62.5 mM Tris pH 6.8, 10% beta-mercaptoethanol). The protein was expected to be in E2, thus only 5 µL of E2 were mixed with 2 µL of sample buffer to prevent overloading of the gel. Fractions were run on a 3% stacking/14% resolving SDS-polyacrylamide gel in order to determine which fraction the protein was in, as well as its purity. The protein was then dialyzed 1:100,000 into 20 mM Tris, 150 mM NaCl, 2.5 mM Ca²⁺, pH 8.4, frozen, and stored at -80°C.

Cleavage and Fast Protein Liquid Chromatography purification

1 U of biotinylated-thrombin (Novagen) in 20 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.4 was added to the protein to remove the N-terminal His-tag. The thrombin was allowed to cleave for two hours, after which 20 μL of streptavidin-agarose (Novagen) (50% slurry) per 1 U thrombin was added. The mixture was allowed to incubate for 30 minutes. This step was repeated to ensure removal of all thrombin. Next, 150 μL of Ni-NTA per 1 U of thrombin were added. Ni-NTA was equilibrated in 20 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.4 and added as a 75% slurry. The mixture incubated for 10 minutes and the protein was collected over a 2 mL disposable polypropylene column. The unbound flow-through fraction, which contained the desired protein, was collected. An SDS-PAGE gel was run to ensure the Histidine-tag was cleaved off, and once it was determined that the protein no longer contained the Histidine-tag, it was dialyzed 1:100,000 into 20 mM Tris, 20 mM NaCl, pH 7.4 and run over a Hi Trap Q column on fast performance liquid chromatography (FPLC). This was done to remove any endotoxins that could affect cell-based assays. The protein was eluted off of the column via a NaCl gradient from 20 mM to 300 mM. If the proteins came off the column between 80 mM and 120 mM NaCl, they were not dialyzed. However, if they came off the column outside of the 80-120 mM NaCl range, they were dialyzed into 20 mM Tris, 100 mM NaCl, pH 7.4.

Biotinylation of mAbIII-10

MAbIII-10 was dialyzed into PBS (8 mM Na₂HPO₄, 137 mM NaCl, 1.76 mM KH₂PO₄, 2.7 mM KCl, pH 7.4). NHS-biotin (Pierce) at 20-fold molar excess was added at room temperature for 1 hr, after which excess biotin was removed by dialysis into PBS.

Competitive Inhibition enzyme-linked immunosorbent assay

The 96-well high-binding plate (CoStar 3590) was coated the previous night with 2 µg/ml Fn. Each protein was diluted to 10 µg/ml in 10 mM Tris, 50 mM NaCl, pH 7.4 containing 0.2% BSA and mixed with 100 µg/ml Fn and allowed to incubate for 30 minutes. After 30 min, the plate was washed once with low salt TBS-T (10 mM Tris, 50 mM NaCl, 0.05% tween, pH 7.4), and blocked with 1% BSA for 1 hr. Meanwhile, a 5:0.5 Fn:FUD (µg/mL:µg/mL) dilution was made of each protein. 25 µl of mAbIII-10 ascites at 1:7142 (final concentration 1:50000 in 10 mM Tris, 50 mM NaCl, 0.35% tween, pH 7.4) was added to 150 µl aliquots of each dilution. The dilution and antibody mixture were allowed to incubate for 1 hr. The plate was washed three times with low salt TBS-T and 50 µl of each dilution was added into wells and incubated for 2 hr. After which the plate was washed two times with low salt TBS-T and then two times with high salt TBS-T (10 mM Tris, 150 mM NaCl, 0.05% tween pH 7.4). 50 µl of 1:5000 dilution of alkaline phosphatase donkey anti-mouse secondary (Jackson Immuno Research Laboratories, Inc., West Grove, PA) in high salt TBS-T was added and incubated for 1 hr. The plate was washed four times with high salt TBS-T. 50 µl of 1 mg/ml substrate in 10 mM Tris, 150 mM NaCl, pH 9.0 was added and the plate was read at 405 nm.

For experiments using mAbs, the assay was run according to the procedure above, except mAbs were used instead of FUD. The Fn and mAbs were at equimolar concentrations of 20 nM. Also, biotinylated mAbIII-10 (b-mAbIII-10) was used instead of mAbIII-10 because the secondary Ab used in the previous experiment would bind to any Ab that is bound to Fn. By using b-mAbIII-10 and AP streptavidin detection, we were able to determine if the Abs could expose ¹⁰FnIII. For experiments involving heparin, the experiment again was run according to the previous assay, except heparin was used instead of FUD. The concentration of heparin was increased from 0.001-0.25 mg/mL and the concentration of soluble Fn was 20 nM.

Direct enzyme-linked assay

A 96-well high-binding plate (CoStar 3590) was coated with 10 µg/ml Fn overnight. Wells were then washed once with high salt TBS-T, and blocked with 5% BSA in TBS-T for 1 hr. Meanwhile, a 0.3 nM biotinylated-FUD (b-FUD) solution was made in high salt TBS-T containing 0.1% BSA. Next, 175 µL dilutions of 100 nM FUD and 0.3 nM b-FUD were prepared. The plate was washed 3 times with high salt TBS-T, and 50 µL of each dilution were added to the wells and incubated for 2 hr. The wells were then washed 4 times with high salt TBST and 50 µL of 1:20000 dilution of alkaline phosphatase-conjugated streptavidin (Jackson Immuno Research Laboratories, Inc., West Grove, PA) in high salt TBS-T, 0.1% BSA were added and incubated for 1 hr. The plate was washed 4 times with high salt TBS-T and 50 µL of 1 mg/ml alkaline-phosphatase substrate in 10 mM Tris, 150 mM NaCl, pH 9.0 was added and the plate was read at 405 nm.

For experiments using heparin, plates coated with 10 µg/mL Fn were first incubated with heparin for 2 hr. The plate was washed and then incubated with b-FUD for 2 hr. The plate was then washed and incubated with AP streptavidin secondary and run according to the above experiment.

Statistical Analysis

The raw data for each replicate, relative to control, was used for statistical analysis. For example, on the experiment involving FUD binding to Fn and exposing ¹⁰FnIII, the raw data for replicate 1 was compared to the Fn control, and the raw data for replicate 2 was compared to the Fn control for that replicate. The raw data of relative values ($\frac{FUD}{Fn}$) was then analyzed to determine the mean and standard deviation. These values were also used with the Fn values ($\frac{Fn}{Fn}$) in a two-tailed t-test with $\alpha=0.05$ to determine if there was a significant difference between the two averages.

Results

Effect of FUD mutants on b-FUD binding to Fn

A study by Ensenberger *et. al.* showed that dimeric FUD peptide bound to both 27K and 40K (13), and monomeric FUD bound to Fn, 70K, 27K, but not 40K (13). For the purpose of this study, monomeric FUD was used to study its interaction with Fn. There has yet to be a crystal structure of FUD binding to Fn, thus to better understand this interaction, we generated 18 mutants to learn the residues of FUD that are important for Fn binding.

We tested the ability of mutant FUD peptides to compete with b-FUD for the binding to Fn. In this experiment, the WT or mutant FUD peptides were at a concentration of 100 nM compared to the 0.3 nM concentration of b-FUD. The values were compared to the control of b-FUD without the addition of a FUD peptide. d29 is incapable of blocking Fn assembly (5), thus other mutants involving the 29th residue were generated: d27-31, d28-30, and the insertion of one alanine between I29 and D30 (i29/30). As can be seen in Figure 2A, each lost its ability to compete with b-FUD, as well as the other mutants with deletions: d21-25, d32-36, d37-41, and d43-47. We also tested other important binding sites by using block alanine substitutions. At a concentration of 100 nM, A7-11, A12-16, A17-20, A27-31, A37-41, and A43-47 lost their ability to compete with 0.3 nM b-FUD (Figure 2B). However, A2-6, A21-25, and A29 still had some blocking capabilities, with A2-6 having the greatest blocking ability at 18.6% of WT FUD.

Ability of FUD to bind to Fn and expose ¹⁰FnIII

Previous studies showed that FUD binding to Fn is capable of causing a conformational change that exposed ¹⁰FnIII (17). Even though some FUD mutants were incapable of competing with b-FUD for binding to Fn, we tested their ability to expose ¹⁰FnIII in the absence of b-FUD. To study this we

used a competitive inhibition ELISA and mAbIII-10. The use of mAbIII-10 was pioneered by Ugarova *et. al.* who demonstrated that binds to an epitope in the RGD containing $^{10}\text{FnIII}$ (18). In low salt solutions containing soluble Fn, the mAbIII-10 epitope is hiding. However, when FUD binds to Fn and exposes $^{10}\text{FnIII}$, the RGD sequence is made available for binding of mAbIII-10. In this experiment, WT and mutant FUD peptides were pre-incubated with Fn to allow any exposure of $^{10}\text{FnIII}$. MAbIII-10 was then added to the solution and allowed to bind to epitopes in the RGD sequence. The solution was then added to the plate coated with 2 $\mu\text{g/ml}$ Fn, in which $^{10}\text{FnIII}$ site is known to be exposed, and the amount of mAbIII-10 bound to Fn in solution was measured by use of AP donkey anti-mouse secondary, followed by addition of substrate. The data obtained was compared to the amount of mAbIII-10 bound to the plate in the control of soluble Fn without an FUD peptide. Using this, we were able to determine relative amounts of the ability of WT and mutant FUD to cause exposure of $^{10}\text{FnIII}$.

FUD was able to bind to Fn and expose $^{10}\text{FnIII}$ with equimolar concentrations; however, many mutant peptides cannot compete with FUD for binding to Fn, thus a 20:1 FUD:Fn ratio was used to determine if FUD mutants could expose mAbIII-10 binding site. Pre-incubation of Fn and FUD allowed for the binding of FUD to Fn to expose the $^{10}\text{FnIII}$ site (Figure 3A,B). This allowed mAbIII-10 to bind to its epitope on soluble Fn, confirming the results seen by Ensenberger that FUD alters the conformation of Fn (17). However, d29, d28-30, and d27-31 were not sufficient to expose the $^{10}\text{FnIII}$ site on Fn in solution to compete for mAbIII-10. d29 and d28-30 were not statistically different than Fn alone, and this was confirmed by a two-tailed t-test ($p>0.1$). Thus, deletion of the 29th residue region of FUD greatly decreases its ability to cause a conformational change in Fn. Other deletions, d21-25, d32-36, d37-41, and d43-47 lost some or most of their ability to expose the mAbIII-10 binding

site in Fn (Figure 3A). However, i29/30 still retained some ability to change the conformation of Fn, and its mean value was statistically different from Fn alone ($p < 0.05$).

Conversely, the mutations involving alanine substitutions were not as detrimental to the function of FUD. A2-6, A21-25, and A29 were observed to function like WT FUD on binding to Fn. A29 is interesting because deletion of the 29th residue removes the function of FUD, but changing the residue does not significantly affect its function. All but one of the WT and mutant alanine FUD peptides were statistically different from the control of Fn alone ($p < 0.05$), with only A37-41 showing no significant difference ($p > 0.1$).

Effect of mAbIII-10 on ability of Fn to bind FUD

Further, we wanted to know if other molecules and proteins that are known to bind to Fn could also cause a conformational change. These experiments involved the use of Fn binding monoclonal antibodies. The FUD binding site on Fn is located on 70K (16), and we were curious if FUD had the same binding affinities for 70K and full-length Fn. Looking at Figure 4, it is clear that FUD has a higher binding affinity for 70K than full-length Fn. The next experiment investigated the hypothesis that just as binding of FUD increases the ability of Fn to bind mAbIII-10, the binding of mAbIII-10 would increase the ability of Fn to bind FUD. 70K, Fn alone, or Fn with mAbIII-10 were incubated with b-FUD for 30 min to test whether mAbIII-10 could increase the binding of FUD to full-length Fn. Figure 4 supports the idea that mAbIII-10 increases the binding of b-FUD to soluble Fn; however, 70K is still better at competing for b-FUD.

Ability of mAbs to expose ¹⁰F_nIII

Other antibodies that bind to Fn were used to assay for the conformational change of Fn that is seen when FUD binds. The monoclonal antibodies 9D2, 4D1, 5C3, 7D5, and L8 all bind to ²F_nI-¹F_nIII, the same region that FUD binds, thus we were curious if they could expose ¹⁰F_nIII. Analogous to the competitive inhibition ELISA using WT and mutant FUD followed by addition of mAbIII-10, this experiment used mAb instead of FUD and biotinylated-mAbIII-10 (b-mAbIII-10) instead of mAbIII-10. b-mAbIII-10 was used because the secondary Ab used in the competitive inhibition ELISA would bind to any Ab that is bound to Fn. By using b-mAbIII-10 and AP streptavidin secondary, we were able to determine if the Abs could expose ¹⁰F_nIII. 4D1 and L8 do not cause a conformational change in Fn (Figure 5). A t-test was performed to test for statistically significant differences, and with $\alpha=0.05$, each had a p-value greater than 0.1. However, FUD, 9D2, 7D5, and 5C3 cause a conformational change in Fn, and were statistically different from the Fn control ($\alpha=0.05$, $p<0.05$). Compared to Fn alone, 9D2, 7D5, and 5C3 caused an increase of about 40% of mAbIII-10 binding to Fn in solution. This is less than FUD, which caused an 80% increase of mAbIII-10 binding to Fn in solution.

Effect of heparin on FUD binding to Fn

Heparin enhances the rate of binding of Fn to collagen and the process of Fn-mediated phagocytosis of gelatin-coated particles (19). Heparin binds in the 27K region, and was shown by Ugarova *et al.* to change the conformation of Fn (18). Concentrations of 0.001 to 0.25 mg/mL heparin were incubated with soluble, and mAbIII-10 was used to determine if heparin could expose ¹⁰F_nIII. As seen in Figure 6A, heparin was able to bind to Fn and expose ¹⁰F_nIII, confirming the results seen by Ugarova *et al.* (18) We were then curious if heparin binding affected b-FUD binding to Fn. To do so,

wells coated with 10 $\mu\text{g}/\text{mL}$ Fn were incubated with 1, 10, 100, and 1000 $\mu\text{g}/\text{mL}$ heparin for 2 hr. After this, 0.3 nM b-FUD was incubated for 2 hr. Heparin binding to Fn does not affect the binding of b-FUD to Fn (Figure 6B).

Discussion

The interaction between FUD and Fn is important to understand due to the biological importance of Fn. Thus, a better understanding of this interaction is necessary to provide more information on processes such as Fn assembly and its role in angiogenesis (20). The interaction between bacterial proteins and Fn often involves the binding of Fn binding repeats (FnBRs) to Fn. This interaction often involves the FnBR adding an extra β -strand to the major β -sheet in Type I modules in Fn, and the interaction is known as a tandem β -zipper (21). Previous studies demonstrated that FnBPA of *Staphylococcus aureus* binds to ^2FnI - ^5FnI (14), and recent studies suggest that FUD binds at least to ^2FnI - $^1\text{FnIII}$ (unpublished). This study investigated the binding of FUD to Fn and the importance of the 29th residue of FUD. Fn can also interact with many other proteins and molecules, and subsequent experiments studied whether the binding of these molecules could also change the conformation of Fn.

The enzyme-linked assay that studied the competing power of FUD mutants with b-FUD showed interesting results. While mutant FUD concentrations were more than 100-fold greater than b-FUD, the highest blocking any mutant had was A2-6 that blocked 19% of the control FUD. It should be noted that the concentration of the mutant peptides was 100 nM, and when this is increased to 10,000 nM, more mutants are able to block b-FUD binding (unpublished). Although most FUD mutants minimally blocked b-FUD binding to Fn, they still may bind to Fn and expose $^{10}\text{FnIII}$.

The competitive ELISA tested the mutant's ability to bind to Fn and expose $^{10}\text{FnIII}$. The 20:1 ratio of FUD:Fn used concentrations of 400 nM:20 nM, respectively, thus at 400 nM, A2-6, A21-25,

A29, and i29/30 were all able to bind to 20 nM Fn and expose ¹⁰FnIII. It is interesting that deletion of the 29th residue removes the ability of FUD to bind to Fn and cause a conformational change, yet changing the 29th residue to an alanine residue does not have an effect. However, if there is a block alanine substitution for residues 27-31, again the binding ability is lost. This suggests that the 29th residue is necessary for FUD binding, yet its surrounding region greatly determines if FUD will be able to bind to Fn. There were 4 tandem pairs of mutants that involved block alanine substitution or deletion of the same region, and all but one completely lost their ability to bind to Fn. A block alanine substitution for residues 21-25 did not greatly affect the binding ability of FUD; however, if the same residues are deleted, the binding ability is lost. This suggests that it is not the specific residues that are needed for FUD binding; it is the 21-25 residue region that is needed for binding. This supports that idea of a tandem β -zipper interaction that FUD adds an extra β -strand to the major β -sheet in Fn modules. A deletion would disrupt the hydrogen-bonding between each strand, and the interaction would not be sufficient to expose ¹⁰FnIII.

FUD is known to bind to the 70K region of Fn, yet it is somewhat surprising that FUD has a higher binding affinity for 70K than full-length Fn. One reason for this observation could be that the FUD binding site is hidden in the tertiary structure of Fn and exposed in 70K. When FUD binds to Fn, it causes the exposure of ¹⁰FnIII and the RGD sequence is made available for binding to mAbIII-10 (17). It is interesting, however, that the addition of mAbIII-10 to soluble Fn increases FUD binding. This interaction is not completely understood, mainly because mAbIII-10 only binds to Fn after FUD binds and exposes ¹⁰FnIII. A future experiment will explore this interaction by mutating the epitope on Fn that binds to mAbIII-10. When this is done and the experiment repeated, FUD should have the same binding affinities for full-length Fn and full-length Fn that can no longer bind to mAbIII-10.

FUD is not the only compound that can bind to Fn and cause a conformation change. 4D1 binds to ¹FnI-³FnI and L8 binds to ⁹FnI-¹FnIII (unpublished), and both do not cause a conformational change. However, 7D5 binds to ⁴FnI-⁵FnI and 5C3 binds to ⁷FnI-⁹FnI (unpublished), and both of these mAbs do cause a conformational change. These results indicate that FUD binding to regions ⁴FnI-⁹FnI cause the exposure of ¹⁰FnIII, whereas the interaction between FUD and ²FnI-³FnI and ¹FnIII may not cause this effect. This assay has limitations, though, because the binding affinities of each mAb for soluble Fn are not known. If 4D1 and L8 have lower binding affinities than 7D5 and 5C3, this may be the cause for the difference in their respective abilities to expose ¹⁰FnIII. To determine the binding affinities, an experiment could be run that increases the concentration of soluble Fn while keeping the concentration of the mAb constant. As more soluble Fn is added to the mixture, the amount of mAbIII-10 bound to coated Fn should decrease. By observing the amount of soluble Fn needed to decrease mAbIII-10 binding to coated Fn by 50%, we could obtain relative affinities of the mAb for soluble Fn. Also, heparin binds to Fn in the 27K region and causes the exposure of ¹⁰FnIII (18). However, this interaction does not affect the binding of FUD to Fn. This result, though, could be questioned because it was not determined whether heparin was bound to the Fn on the coated wells when FUD was added. Another experiment should be run, using a primary mAb specific for heparin, to determine if heparin does indeed bind to coated Fn. This will provide a more conclusive result for the effect of heparin on FUD binding to Fn.

The sum of the FUD, mAb, and heparin experimental data suggests that there are multiple interactions that can cause a conformation change in Fn. Future experiments that include the mAbs effect on FUD binding to Fn will provide more information on the interaction between FUD and Fn. For example, an experiment could be run that is analogous to the heparin experiment, but instead of

heparin, different mAb could be used. By observing which mAb, if any, affect FUD binding to Fn, we may be able to determine the modules that are essential for FUD binding.

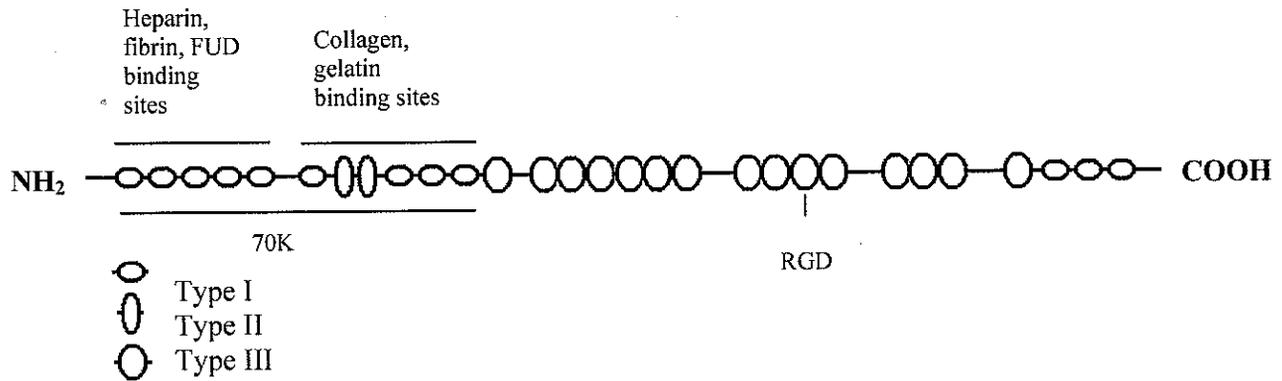
Previous studies have shown that FUD binding to Fn causes a conformation change in Fn, thereby exposing ¹⁰FnIII containing the RGD sequence responsible for integrin binding (17). The findings presented in this paper provide more information on the interaction between FUD and Fn. The 29th residue of FUD is essential; however, there are many other regions necessary for proper FUD binding to Fn. Even when the mutations allow for binding of FUD to Fn, they may effect the competing power of the peptide. Also, the binding of other molecules to Fn such as mAb or heparin can also cause a conformational change, yet do not alter the binding of FUD, thus Fn can have multiple interactions that cause its conformation to change.

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Fig 1

A



B

FUD	KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFSGNMVETEDT
D29	KDQSPLAGESGETEYITEVYGNQQNPVD-DKKLPNETGFSGNMVETEDT
A29	KDQSPLAGESGETEYITEVYGNQQNPVD AD KKLPNETGFSGNMVETEDT
I29/30A	KDQSPLAGESGETEYITEVYGNQQNPVD AD KKLPNETGFSGNMVETEDT
A2-6	KAAAAA GESGETEYITEVYGNQQNPVDIDKKLPNETGFSGNMVETEDT
A7-11	KDQSP LAAAAA ETEYITEVYGNQQNPVDIDKKLPNETGFSGNMVETEDT
A12-16	KDQSPLAGESG AAAAA TEVYGNQQNPVDIDKKLPNETGFSGNMVETEDT
A17-20	KDQSPLAGESGETEYI AAAA GNQQNPVDIDKKLPNETGFSGNMVETEDT
A21-25	KDQSPLAGESGETEYITEVY AAAAA PVDIDKKLPNETGFSGNMVETEDT
D21-25	KDQSPLAGESGETEYITEVY-----PVDIDKKLPNETGFSGNMVETEDT
A27-31	KDQSPLAGESGETEYITEVYGNQQNP AAAAA KLPNETGFSGNMVETEDT
D27-31	KDQSPLAGESGETEYITEVYGNQQNP-----KLPNETGFSGNMVETEDT
A37-41	KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNE AAAAA NMVETEDT
D37-41	KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNE-----NMVETEDT
A43-47	KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFSGN AAAAA DT
D43-47	KDQSPLAGESGETEYITEVYGNQQNPVDIDKKLPNETGFSGN-----DT
D28-30	KDQSPLAGESGETEYITEVYGNQQNPV---KLPNETGFSGNMVETEDT
D32-36	KDQSPLAGESGETEYITEVYGNQQNPVDIDK-----TGFSGNMVETEDT

Fig 2

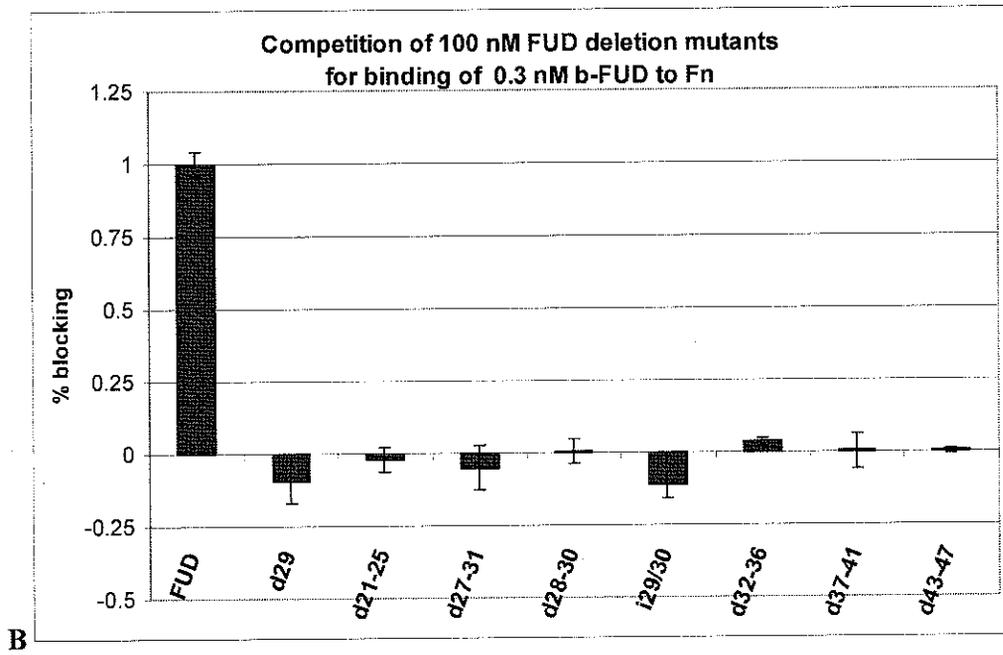
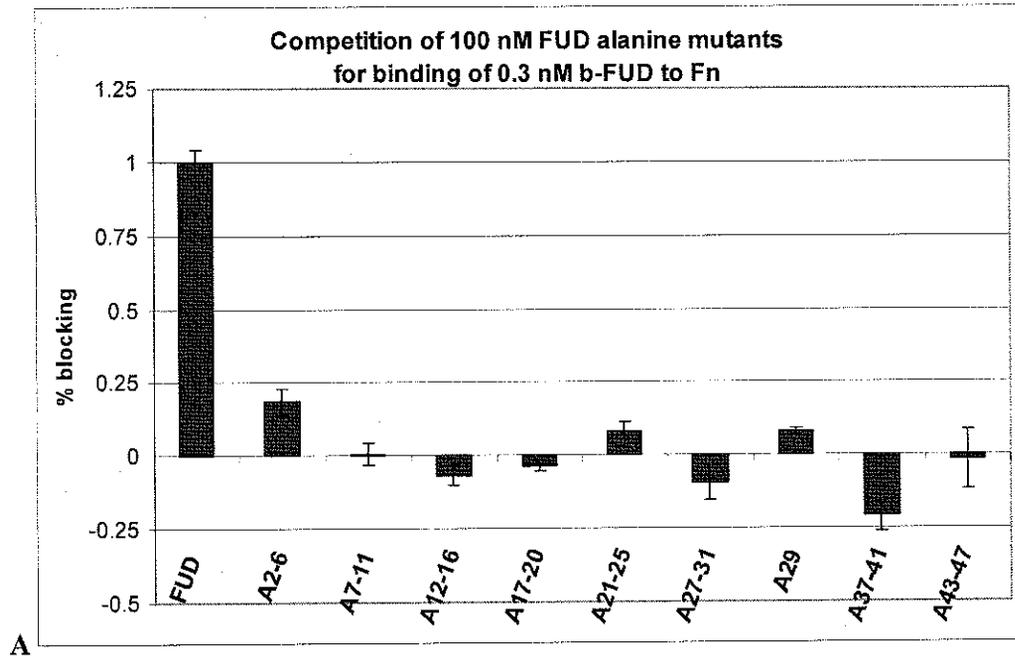


Fig 3

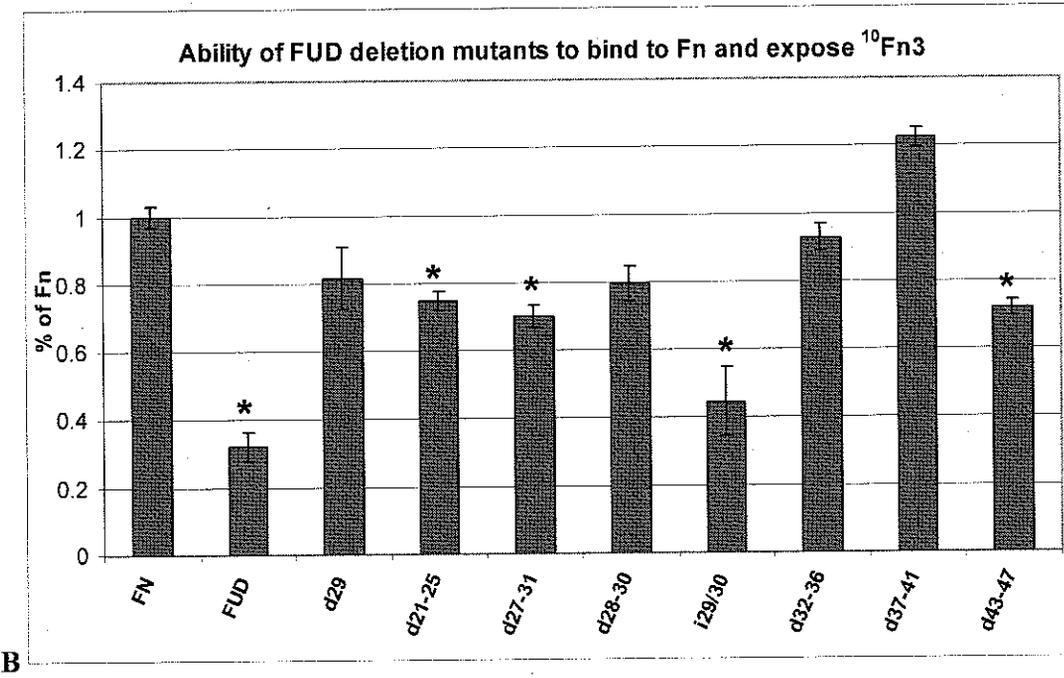
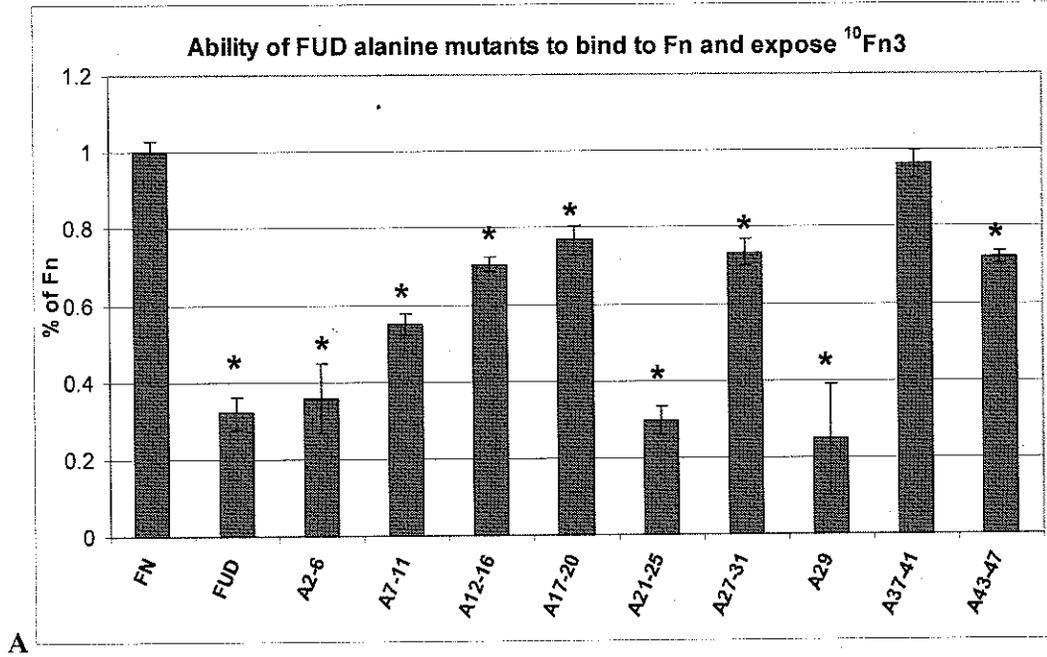


Fig 4

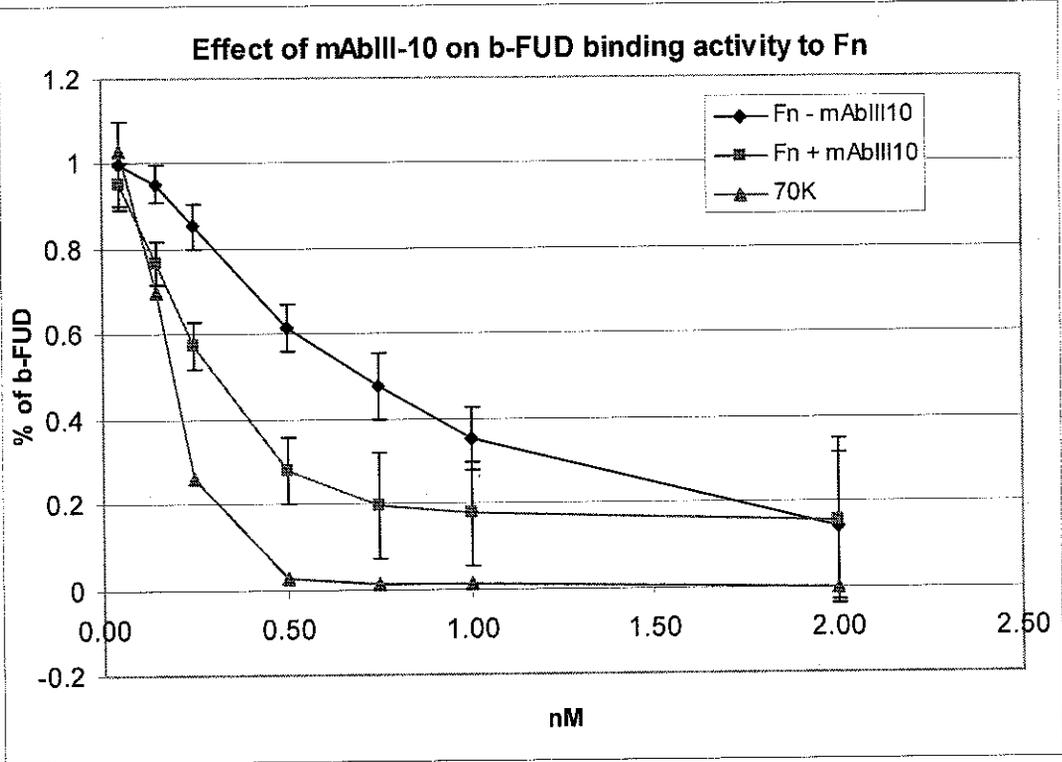


Fig 5

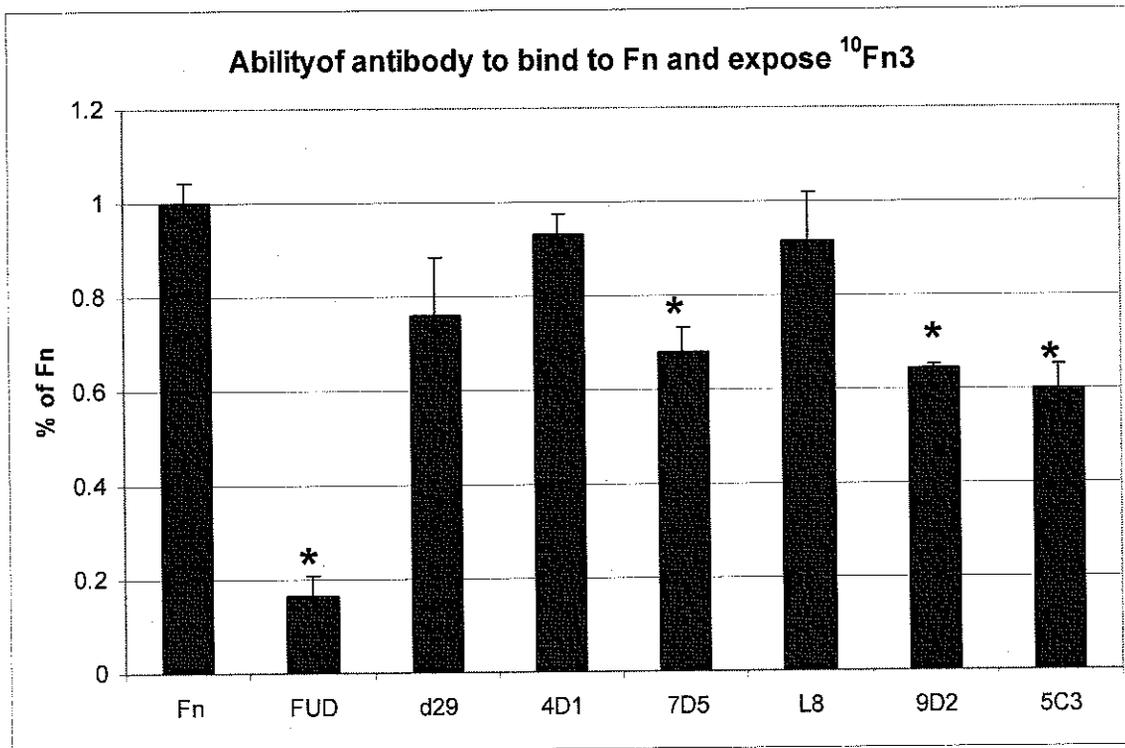


Fig 6

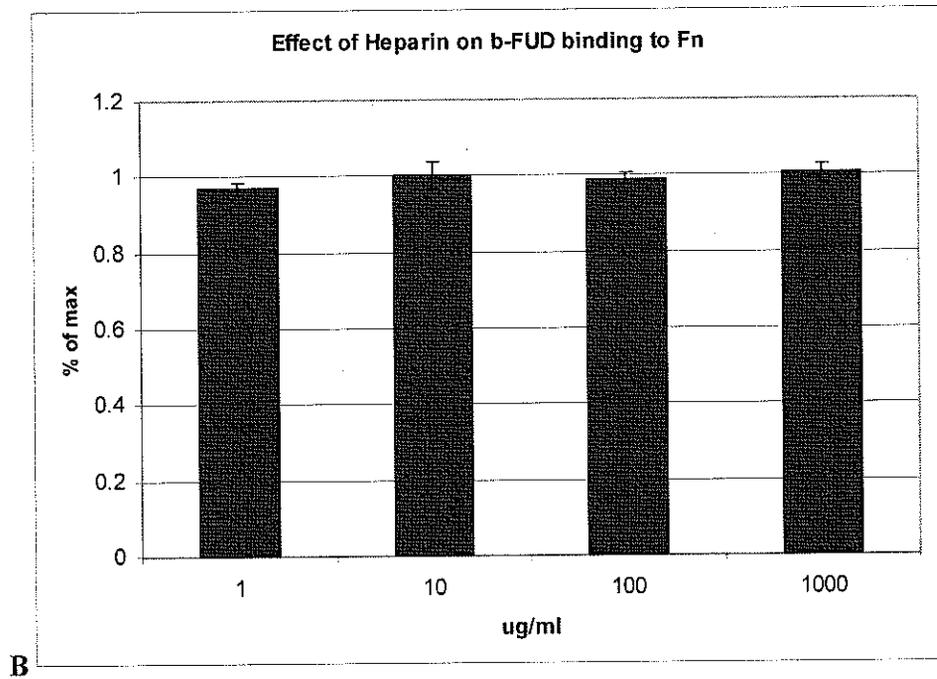
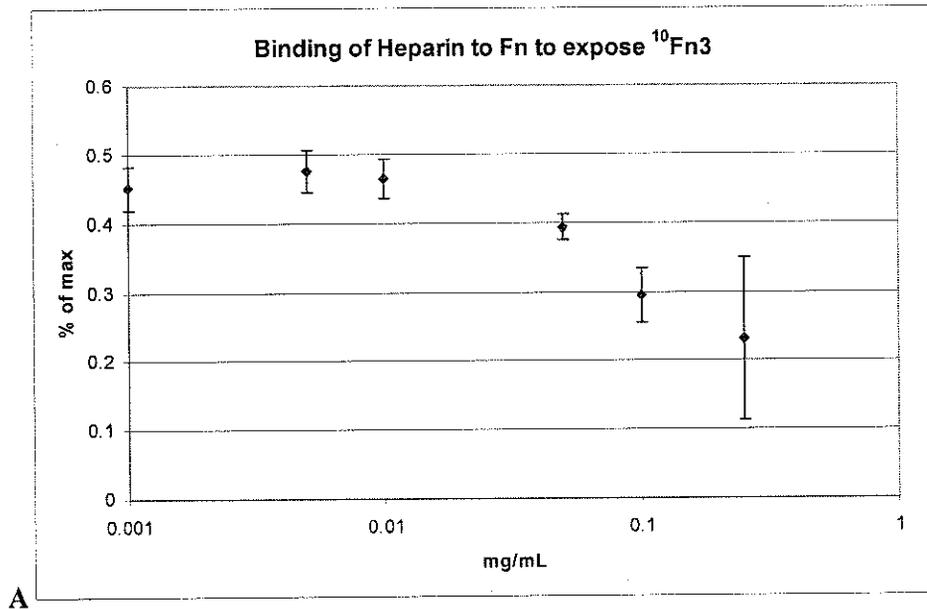


Figure 1: **Diagram of Fn fragments and FUD alignment.** (A) Diagram of Fn showing important regions such as 70K and the RGD sequence in ¹⁰FnIII. Also, binding sites for heparin, fibrin, collagen, gelatin, and FUD are shown. (B) Alignment of WT FUD with mutant FUD peptides.

Figure 2: **FUD mutants competing with b-FUD for binding to Fn.** (A) The effect of 100 nM FUD deletion mutants on competing with 0.3 nM b-FUD and blocking binding to coated Fn. (B) The effect of 100 nM FUD alanine substitution mutants on competing with 0.3 nM b-FUD and blocking binding to coated Fn. The amount of b-FUD bound was compared to the signal from wells with 0.3 nM b-FUD alone. Values are relative to FUD blocking, and are mean values with error bars representing standard deviation (n=3).

Figure 3: **FUD sequence requirements for exposure by FUD of the mAbIII-10 epitope in Fn.** (A) Mutant FUD deletion peptides pre-incubated with Fn in a 20:1 molar ratio, respectively. (B) Mutant FUD alanine substitution peptides pre-incubated with Fn in a 20:1 molar ratio, respectively. The Fn in solution competes poorly with the coated Fn for mAbIII-10, and values were compared to Fn alone. Thus, exposure of the epitope leads to greater competition. Values are mean with error bars representing standard deviation (n=1, 2, or 3). Asterisk denotes that these values were statistically different from the Fn control using a two-tailed t-test (p<0.05).

Figure 4: **Fn incubated with mAbIII-10 competes better than does Fn alone for b-FUD.** Competitive ELISA of 70K, Fn, and Fn incubated with mAbIII-10 competing with 10 µg/mL absorbed Fn for 0.3 nM b-FUD. Values are mean with error bars representing standard deviation of triplicate wells.

Figure 5: **4D1 and L8 do not expose ¹⁰Fn3 in Fn, while 7D5, 9D2, and 5C3 cause a slight conformational change in Fn.** Monoclonal antibodies were pre-incubated with Fn, and the Fn in solution competes with the coated Fn for mAbIII-10. Values are mean with error bars representing standard deviation (n=2). Asterisk denotes that these values were statistically different from the Fn control using a two-tailed t-test (p<0.05).

Figure 6: **Heparin binds to Fn and exposes ¹⁰Fn3, but does not affect FUD binding to Fn.** (A) Concentrations of heparin ranged from 0.001 to 0.25 µg/mL, and heparin was allowed to bind to coated Fn. The conformational change was detected by use of mAbIII-10, and values were compared to amount of mAbII-10 binding alone. Values are mean with error bars representing standard deviation (n=1). (B) Heparin at concentrations of 1, 10, 100, and 1000 µg/mL was incubated with coated Fn. The plate was washed and 0.3 nM b-FUD was then incubated with the coated Fn bound to heparin. AP conjugated-streptavidin was used to detect amount of b-FUD, and compared to the signal from wells with 0.3 nM b-FUD alone.

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