Recognition of Fibronectin by the Platelet Integrin αIIbβ3 Involves an Extended Interface with Multiple Electrostatic Interactions†

Adam C. W. Kauf, Scott M. Hough, and Ron D. Bowditch*

Department of Biochemistry and Molecular Biology, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

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ABSTRACT: Normal platelet function is dependent on the ability of integrin αIIbβ3 (glycoprotein IIb/IIIa) to interact with components of the subendothelial matrix, such as fibronectin (Fn), exposed at sites of vascular injury. Studies using synthetic peptides derived from human Fn sequences Asp^{1373}−Thr^{1383} and Arg^{1493}−Asp^{1495} have suggested a role for both the 9th (3fn9) and 10th (3fn10) type III repeats of this ligand in binding to αIIbβ3. In this study, we have taken a charge-to-alanine mutagenesis approach to evaluate the importance of these sites, and other charged residues, within the context of recombinant 3fn9−10 modules for binding to αIIbβ3. To identify residues that are involved in Fn binding to αIIbβ3, recombinantly expressed 3fn9−10 module pairs with alanine substitutions introduced into each of the 38 charged residues were individually assayed for the ability to inhibit Fn binding to purified αIIbβ3. Substitutions at Fn residues Arg^{1493} and Asp^{1495} of the RGD sequence were found to have the greatest effect on αIIbβ3 binding, as expected. However, Fn residues Arg^{1369}, Arg^{1371}, Arg^{1379}, Arg^{1445}, and Arg^{1448} were needed for optimal interaction of the 3fn9−10 module pair with αIIbβ3. All Fn residues found to affect binding of 3fn9−10 to αIIbβ3 are located on the same face and extend from the surface of the molecule. Additionally, the epitopes for two anti-Fn monoclonal antibodies that inhibit binding of this ligand to αIIbβ3 were found to overlap the sites identified. These results demonstrate that αIIbβ3−Fn binding involves multiple electrostatic interactions.

Normal platelet function is dependent on the ability to adhere to components of the subendothelial matrix at sites of vessel wall damage. Fibronectin (Fn),[1] a constituent of plasma, platelet α-granules, and the subendothelial matrix, is a ligand for the platelet integrin αIIbβ3 (glycoprotein IIb/IIIa) (*1*). Although the function of Fn in hemostasis is not completely understood, this protein does play a significant role in platelet adhesion and spreading on Fn-containing surfaces under both static and shear conditions. In addition, the presence of Fn within the fibrin clot suggests a role for αIIbβ3−Fn interaction in clot retraction.

Fn is a dimeric glycoprotein that also functions in other physiological processes involving cell adhesion and cell migration (*1*). From a structural standpoint, the Fn molecule is a mosaic protein made up of three different types of homologous repeating modules that have been designated type I, type II, and type III (3fn) (*2*). Representative tertiary structures for each of these modules have been solved by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography (*3*−*8*). Fragmentation of Fn with proteases has divided the molecule into functional domains that interact with fibrin, gelatin, glycosaminoglycans, prokaryotic receptors, and integrins.

The integrins are a widely distributed family of noncovalent heterodimeric glycoproteins that play a vital role in cellular adhesion, migration, and signaling (*9*, *10*). At least eight integrins recognize the central cell binding domain of Fn which encompasses the 9th (3fn9) and 10th (3fn10) type III modules. All these integrins recognize the sequence Arg-Gly-Asp (RGD) found in the 3fn10 module. Among these receptors are the integrins αIIbβ3 (*11*−*15*) and α5β1 (*16*, *17*). These two integrins also require residues located within the 3fn9 module for optimal binding of Fn (*18*−*23*). A synthetic peptide derived from the human Fn sequence Asp^{1373}−Thr^{1383} located within the 3fn9 module was shown to interact directly with αIIbβ3, inhibit Fn and fibrinogen binding to purified αIIbβ3, and inhibit platelet aggregation (*24*, *25*). The significance of these residues in αIIbβ3 recognition of Fn has not been evaluated within the context of the macromolecule. The central cell binding domain of Fn does not require glycosylation or formation of disulfide bonds for integrin recognition and is therefore particularly amenable to expression in prokaryotic systems and useful for mutagenesis studies examining integrin−ligand recognition mechanisms.

A structure-based mutagenesis study has shown that seven residues in addition to the RGD sequence are involved in α5β1-dependent cell adhesion (*23*). On the basis of our previous studies and those with the integrin α5β1, we

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* To whom correspondence should be addressed. Phone: (405) 271-5992. Fax: (405) 271-3910. E-mail: ron-bowditch@ouhsc.edu.

[1] Abbreviations: Fn, fibronectin; NMR, nuclear magnetic resonance; RGD, Arg-Gly-Asp; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ESMS, electrospray mass spectrometry; ELISA, enzyme-linked immunosorbent assay.

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hypothesize that residues in addition to RGD interact with αIIbβ3 and form a single binding site. In the present work we examined the significance of the Asp[1373]−Thr[1383] sequence within the context of the recombinant 3fn9−10 module pair using a charge-to-alanine mutagenesis approach (*26*). Only Fn residue Arg[1379] within this sequence was found to have any effect on 3fn9−10 binding to αIIbβ3. Our studies were then expanded to the remaining charged residues within the 3fn9−10 module pair to identify other potential electrostatic interactions outside the Asp[1373]−Thr[1383] and Arg[1493]−Asp[1495] (RGD) sites of Fn that may be involved in binding to αIIbβ3. Four other Arg residues, Arg[1369], Arg[1371], Arg[1445], and Asp[1448], were found to be required for optimal 3fn9−10 binding to αIIbβ3. The results presented demonstrate that the integrins αIIbβ3 and α5β1 share a common Fn recognition mechanism that involves an extended interface between the 3fn9−10 modules and receptor.

EXPERIMENTAL PROCEDURES

Materials. Human Fn was purified as described (*27*). The anti-Fn monoclonal antibodies (mAbs) fnI-8 and fnI-11 have been previously described (*18*). The anti-αIIb mAb PMI-1 (*28*) was a generous gift of Dr. M. Ginsberg, The Scripps Research Institute, La Jolla, CA. All chemical reagents unless otherwise stipulated were obtained from Sigma (St. Louis, MO).

Construction of Native and Mutant Recombinant 3fn9−10 Expression Plasmids. Manipulation of recombinant DNA was by standard techniques (*29*). Restriction enzymes, T4 DNA ligase, and Taq polymerase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Oligonucleotides were synthesized by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center. DNA encoding the human 3fn9−10 module pair (residues G[1326]−T[1510]) was PCR amplified from the plasmid pIH910 (*18*) with the oligonucleotides BGL2910F (GAAGATCTGATTCCCCAACTGGCATTG) and MalR2 (TCGCTATTACGCCAGCTGGCG), digested with *Bgl*II and *Hin*dIII, subcloned into the hexahistidine (H6) expression vector pQE30 (Qiagen Inc., Valencia, CA), digested with the restriction enzymes *Bam*HI and *Hin*dIII, and transformed into *Escherichia coli* M15[pREP4].

Oligonucleotide-directed mutagenesis was performed using PCR overlap extensions with the pIH910 plasmid as the template (*30*, *31*). DNA encoding the mutant form of the 3fn9−10 module pair was digested with the restriction enzymes *Bgl*II and *Hin*dIII and subcloned into the plasmid pQE30 as described above. The correct coding sequences for the native and mutant forms of recombinant 3fn9−10 modules were verified by nucleotide sequencing of the insert in the plasmid.

Expression of Hexahistidine Fusion Proteins. Cells harboring the various pQE30−3fn9−10 recombinant plasmids were grown in SB media plus 100 μg/mL ampicillin and 50 μg/mL kanamycin at 37 °C to an Abs_{600nm} of 0.5−0.7. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 2 mM, and the cells were further incubated at 37 °C for 2 h. The cell culture was centrifuged at 4000*g* for 10 min, and the pellet was resuspended in sonication buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl) and stored at −80 °C. The thawed cell suspension was sonicated and centrifuged at 11000*g* for 20 min at 4 °C, and the supernatant was removed, diluted 1:3 in sonication buffer, and affinity purified on Ni−NTA agarose (Qiagen). Material bound to the Ni−NTA agarose was washed with 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, and 10% glycerol and then eluted with the same buffer at pH 4.0. The purified fusion protein was dialyzed in phosphate-buffered saline (PBS) and examined on a 12% polyacrylamide gel. The correct amino acid composition was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Purified Integrin Microtiter Well Assay. The purified αIIbβ3 binding assay was performed in modified Tyrode's buffer (2.5 mM HEPES, 150 mM NaCl, 2.5 mM KCl, 12 mM Na_2CO_3, and 1 mg/mL bovine serum albumin, pH 7.4) containing 1 mM $MgCl_2$ as previously described (*18*, *32*). Human αIIbβ3 was purified from normal platelets by mAb PMI-1 affinity chromatography. Twenty milligrams of mAb PMI-1 in 0.1 M Na_2HCO_3, pH 8.0, and 0.5 M NaCl was cross-linked to 1 g of CNBr-activated CL4-B Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). The remaining active sites on the CL4B Sepharose were blocked with 100 mM Tris-HCl, pH 8.0, and 0.5 M NaCl. Washed outdated platelets were lysed in platelet lysis buffer consisting of 10 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM *n*-octyl β-D-glycopyranoside, 1 mM $MgCl_2$, 1 mM Pefobloc (Roche Molecular Biochemicals), 10 μM leupeptin, and 1 mg/mL *N*-ethylmaleimide on ice for 20 min. The lysed platelets were centrifuged for 25 min at 25000*g*, and 25 mL of supernatant was loaded onto a 4 mL PMI-1−Sepharose column. The column was washed five times with 5 mL of platelet lysis buffer and eluted with 20 mM sodium acetate, pH 3.0, 2 mM $MgCl_2$, and 50 mM *n*-octyl β-D-glycopyranoside, and 3 mL fractions were collected. The fractions were immediately neutralized by addition of 100 μL of 1 M HEPES, pH 10.

Protein Folding. Analysis of the recombinant 3fn9−10 module pair (r3fn9−10) folding was performed as described (*33*). Briefly, recombinant protein samples were dialyzed in H_2O, pH 4.5, and lyophilized. The dry protein was then resuspended in D_2O (Isotec, Inc., Miamisburg, OH) adjusted to pD 4.5 with DCl. The resuspended protein was incubated at room temperature, and samples were removed at the specified time points. The samples were immediately subjected to electrospray mass spectrometry (ESMS).

Biochemical Procedures. Protein concentrations were determined by the BCA assay (Pierce Chemical Co., Rockford, IL). Proteins were biotinylated as described (*34*), except the protein was in 100 mM Na_2HCO_3, pH 8.0, and dialyzed in 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl after biotinylation. Competitive enzyme-linked immunosorbent assays (ELISA) were performed by standard procedures (*35*) in Immulon 2 (Dynatech Laboratories, Inc., Chantilly, VA) 96-well plates, and bound biotinylated immunoglobulin was detected by incubation with avidin−biotin−horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA) and developed using *o*-phenylenediamine.

RESULTS

Residue Arg[1379] of the Fn Asp[1373]−Thr[1383] Site Is Required for Optimal Binding to αIIbβ3. Our previous studies

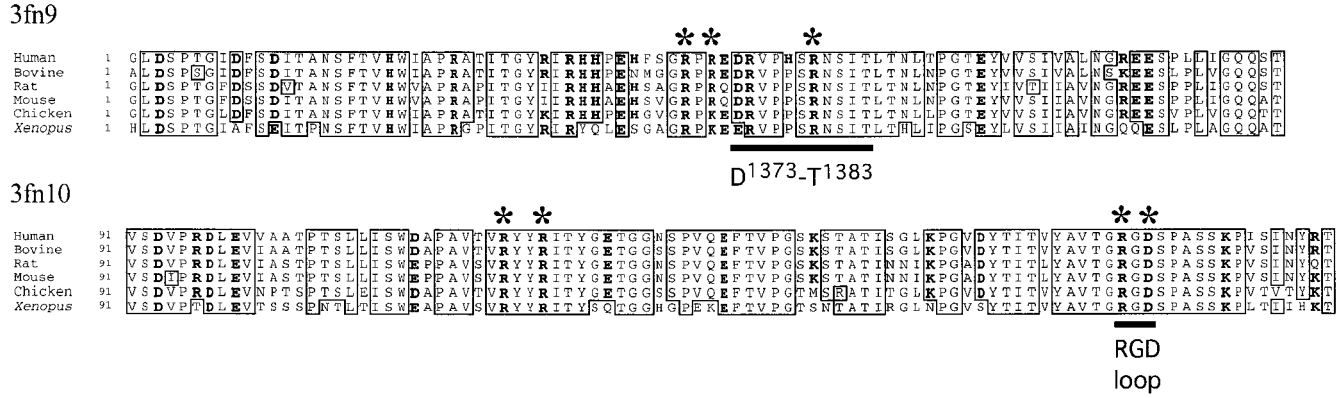


FIGURE 1: Species comparison of the Fn 9th and 10th type III domains. Shown are the complete residue sequences of the 9th type III domain (3fn9) and 10th type III domain (3fn10) of human, bovine, rat, mouse, chicken, and *Xenopus*. Residues with charged side groups are in bold type. Regions of identity are boxed. The regions from which two peptides that inhibit Fn binding to the integrin αIIbβ3 were derived (underlined) are shown as the Asp[1373]−Thr[1383] and the RGD loop. In addition, residues at which an alanine substitution perturbed binding of r3fn9−10 to purified αIIbβ3 are labeled (asterisks).

demonstrated that synthetic peptides derived from Fn residues Asp[1373]−Thr[1383] contained a minimal Fn sequence, Asp[1373]−Arg[1379] (DRVPHSR), that inhibited ligand binding to the platelet integrin αIIbβ3 (*24*). To determine whether these residues in the Fn macromolecule are required for the optimal interaction of this region with αIIbβ3, alanine substitutions were individually introduced, expressed as recombinant (r) 3fn9−10 module pairs, and the ability of each of these to inhibit whole human Fn binding to purified αIIbβ3 was assessed. The residues with charged side groups, Asp[1373], Arg[1374], His[1377], and Arg[1379], were mutated to alanine (Figure 1). The inhibitory potency of each r3fn9−10 module pair was compared to native r3fn9−10 (Figure 2a). Results from a single representative experiment are shown; however, IC_{50}s and standard deviations were obtained from at least three independent experiments using different preparations of purified r3fn9−10 modules. The Arg[1379]Ala substitution inhibited Fn binding to purified αIIbβ3 with an IC_{50} of 360 ± 42 nM, which was twice that observed for native r3fn9−10 (IC_{50} = 180 ± 7 nM). Alanine substitutions at other residues within the Asp[1373]−Arg[1379] site, Asp[1373], Arg[1374], and His[1377], did not significantly alter the IC_{50} from that of the native sequence. In addition, a Ser[1378]Ala substitution was examined (IC_{50} = 187 ± 16 nM) and found not to significantly affect r3fn9−10 inhibition of Fn binding to αIIbβ3. These experiments provide evidence within the context of the macromolecule that Arg[1379] contributes in the interaction between the Fn Asp[1373]−Thr[1383] sequence and αIIbβ3.

Optimal Fn Binding to αIIbβ3 Requires Fn Residues Arg[1369], Arg[1371], Arg[1445], and Arg[1448]. To determine whether residues outside the Fn Asp[1373]−Arg[1379]site and the Arg[1493]−Asp[1495] (RGD) sequence are required for optimal recognition of Fn by αIIbβ3, alanine substitution mutagenesis was applied to all residues within the 3fn9−10 module pair that possess a charged side group (Figure 1). Each of these alanine substitutions were tested for the ability to inhibit the binding of Fn to purified αIIbβ3 and compared to the native r3fn9−10 module pair as described above (Figure 2b). In addition to the effect seen with the Arg[1379]Ala, alanine substitutions at Arg[1369] and Arg[1371] located in 3fn9 demonstrated a decreased inhibitory potency (IC_{50} = 510 ± 42 and 980 ± 53 nM, respectively) as compared to the native r3fn9−10 module pair. Residues Arg[1445] and Arg[1448] located within the 3fn10 module were also required for maximal Fn−αIIbβ3 interaction (Figure 2b). The IC_{50}s for the Arg[1445]Ala and Arg[1448]Ala substitutions were 850 ± 69 and 1920 ± 83 nM, respectively. As would be predicted, alanine substitutions at Fn residues Arg[1493] and Asp[1495] of the RGD sequence abolished the ability of these mutant r3fn9−10 modules to inhibit Fn binding to αIIbβ3 (Figure 2b). A conservative Asp[1495]Glu substitution also did not inhibit Fn binding to αIIbβ3 at concentrations as high as 10 μM (data not shown). To rule out any contribution of altered intermolecular association between r3fn9−10 molecules, each of the mutant r3fn9−10 module pairs shown to affect binding to αIIbβ3 were tested under the same conditions as the purified receptor assay except that αIIbβ3 was replaced by one of various anti-Fn mAbs. None of the constructs differed from the native r3fn9−10 module pair with regard to interaction with the mAbs (data not shown). These results suggest that several positively charged residues outside the Asp[1373]−Thr[1383] site and the RGD loop of Fn were involved in binding the integrin αIIbβ3.

Each of the alanine substitution mutations observed to have an effect on binding to purified αIIbβ3 were examined with regard to their spatial localization on the 3fn9−10 module pair (Figure 3a). On the basis of the tertiary structure of Fn (*4*) all of the mutations that negatively affected integrin binding were located on the same face of the 3fn9−10 module pair and project from the surface of the molecule. Therefore, these residues would be accessible for binding a single integrin. We also noted that alanine substitutions at Fn residues Glu[1372], Glu[1392], and Glu[1462] consistently exhibited enhanced binding to αIIbβ3 (Figure 2). Fn residues Glu[1372] and Glu[1392] are structurally proximal to Arg[1369] and Arg[1371]. Similarly, Glu[1462] is adjacent to Arg[1448] in the 3fn10 module. This suggests that an increase in the net positive charge in the areas identified may enhance the electrostatic interactions between Fn residues Arg[1369], Arg[1371], and Arg[1448] and αIIbβ3.

Evaluation of Mutant r3fn Folding. The Fn type III module adopts an immunoglobulin-like fold made up of seven antiparallel β-strands that form two β-sheets of three and four strands (*5*, *36*). Since substitution mutations may potentially disrupt folding of individual 3fn modules, we utilized a combination of proton−deuteron exchange and ESMS to investigate whether the r3fn9−10 mutations that altered integrin binding were a result of gross changes in

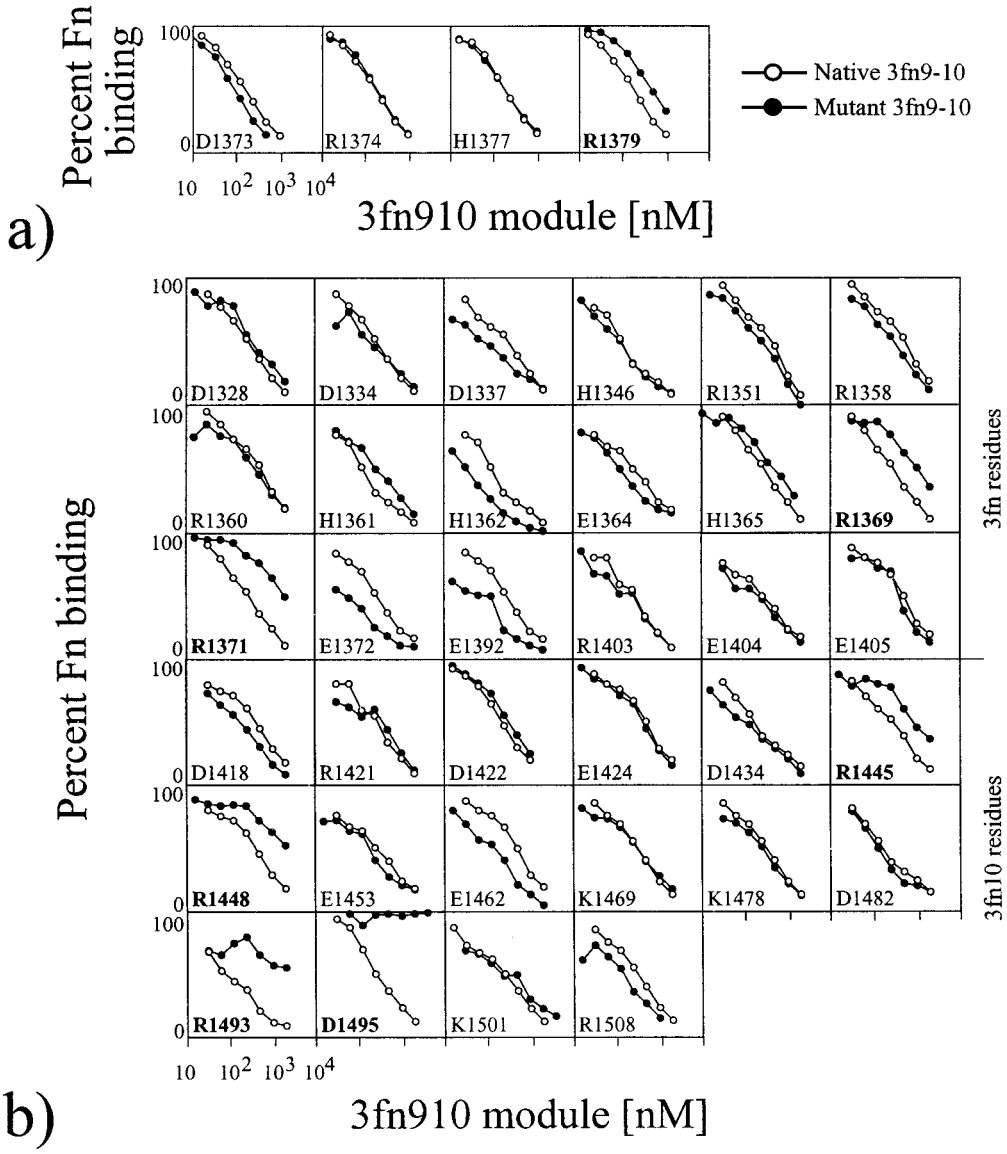


FIGURE 2: Inhibition of Fn binding to αIIbβ3 by alanine-substituted r3fn9−10. Panel a: Asp[1373]−Thr[1383] residues with charged side groups. Panel b: Each of the remaining 3fn9−10 residues with charged side groups. Recombinant proteins with alanine substitutions were assessed for the ability to inhibit Fn binding to purified αIIbβ3. Biotinylated Fn (10 nM) was added to αIIbβ3-coated microtiter wells with varying concentrations of native r3fn9−10 (○) or with an alanine substitution at the specified residue (●). The amount of bound Fn was determined by incubation with avidin-conjugated peroxidase and developed with *o*-phenylenediamine. Percent Fn binding = $[(B_{\text{r3fn9-10}} - B_{\text{EDTA}})/(B_{\text{no inhibitor}} - B_{\text{EDTA}})] \times 100$. Results shown are the average of triplicate determinations from a single experiment.

the global folding. Previous investigators have found a high level of correlation between the slowing of proton exchange and intramolecular hydrogen bonding (*33*, *37*, *38*). Lyophilized r3fn modules were suspended in D_2O (pD 4.5), and the change in mass was monitored at regular time intervals. As shown for r3fn9−10 (Figure 4), there is an initial rapid increase in mass of 213 Da. Heat-denatured r3fn9−10 in D_2O (pD 4.5) was found to exhibit a mass increase of 284 Da. Subtracting the change in mass of the r3fn9−10 module pair at 1 h from that of the denatured r3fn9−10, a difference in mass of 71 ± 4 Da was obtained. This value was in good agreement with a value of 70 slowly exchanging backbone amide protons expected for two 3fn modules (*39*).

These experiments were extended to r3fn9−10 mutant modules that exhibited a negative effect on binding to αIIbβ3 (Figure 5). Each of these recombinant proteins had the expected number of slowly exchanging protons and did not differ significantly from the profile observed for the native form. The Asp[1495]Ala substitution exhibited an aberrant migration in SDS−PAGE. However, the Asp[1495]Ala-containing r3fn9−10 module pair exhibited a mass of 21 185 Da as expected and appeared to fold correctly (Figure 5). Other proteins with neutral residue substitutions of aspartic acid or glutamic acid residues have also exhibited an aberrant migration in SDS−PAGE (*40*). These results suggest that the recombinant 3fn modules used in these studies are correctly folded as β-barrel structures and that the effects observed for individual alanine substitutions are not a result of global misfolding.

The Epitopes for Two Inhibitory Anti-Fn mAbs Overlap Binding Sites Identified for αIIbβ3. Two mAbs, fnI-11 and fnI-8, recognize epitopes located in the 3fn9 and 3fn10 modules, respectively. Both mAbs inhibit the binding of Fn to the platelet integrin αIIbβ3 (*18*). Each of the 38 r3fn9−10 alanine substitution mutations was examined for the ability to bind fnI-11 and fnI-8 in a competitive ELISA. As shown

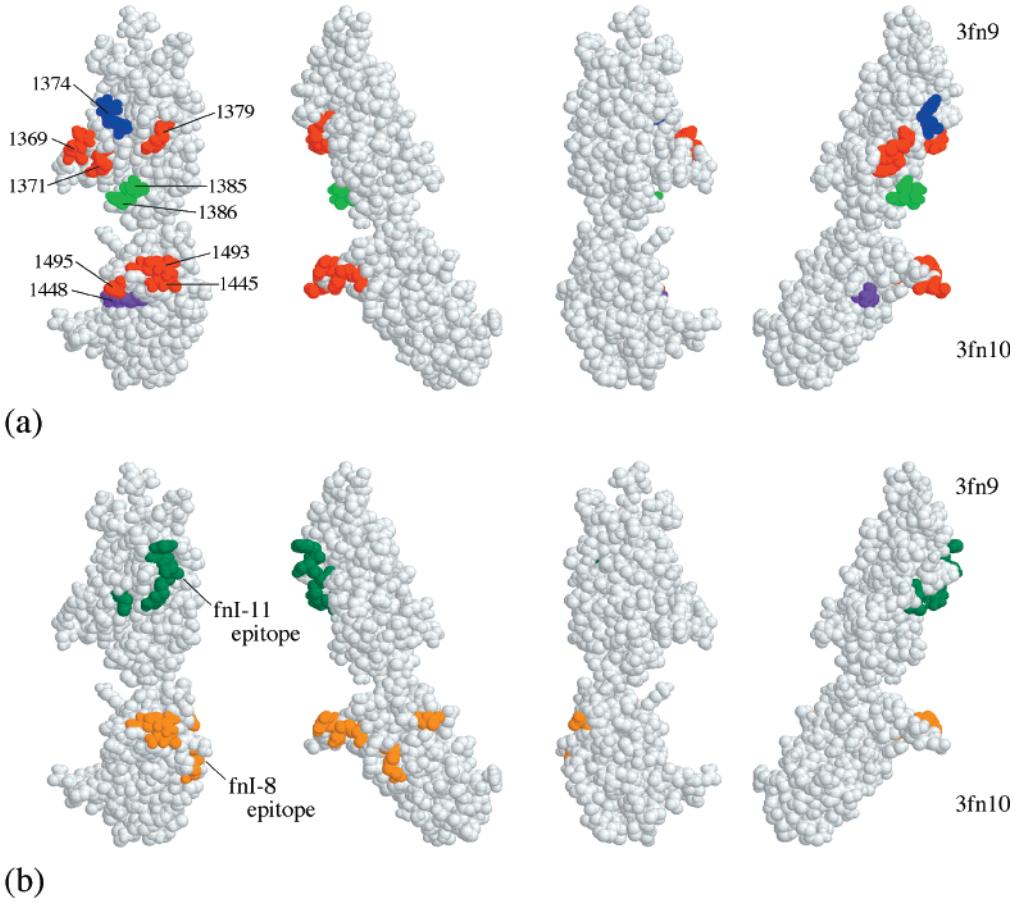


FIGURE 3: Spatial localization of 3fn9−10 regions involved in binding to the integrins αIIbβ3 and α5β1. Shown are space-filling representations (rotated 90°) of the 3fn9−10 domains. (a) Residues 1369, 1371, 1379, 1445, 1493, and 1495 (red) were required for optimal interaction with both αIIbβ3 and α5β1. Residue 1374 (blue) was required for α5β1 and not for αIIbβ3. Residue 1448 (purple) was required for αIIbβ3 and was not examined with α5β1. Residues 1385 and 1386 (green) were required for α5β1 and were not examined with αIIbβ3. Results shown were obtained from this study, Obera et al. (*19*), and Redick et al. (*23*). Residues are numbered on the basis of their position in Fn according to Kornblihtt et al. (*46*). (b) Residues identified by alanine scanning mutagenesis that participate in the formation of the epitopes for the inhibitory mAbs fnI-11 (dark green) and fnI-8 (orange) were determined by competitive ELISA (Table 1).

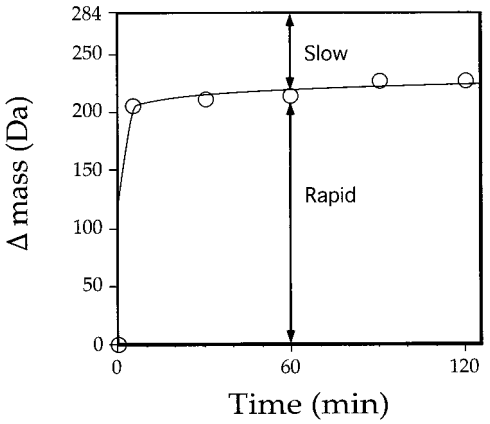
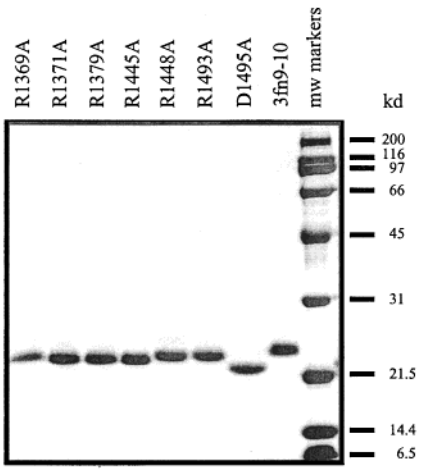


FIGURE 4: H−D exchange of the recombinant 3fn9−10 module in D_2O. Lyophilized r3fn9−10 was resuspended in D_2O at pD 4.5 and the increase in mass (Δmass) monitored over time by electrospray mass spectrometry. The maximal value for the change in mass was that observed for heat-denatured r3fn9−10 in D_2O at pD 4.5. Each mass unit increase of 1 Da corresponds to a proton exchanged for a deuteron. This method allows the number of slowly exchanging backbone amides to be determined.

in Table 1, alanine substitutions at residues Asp^{1373}, His^{1377}, Ser^{1378}, and Arg^{1379} affected the epitope for mAb fnI-11. This indicates that the epitope for fnI-11 overlaps the region within the 3fn9 module that was required for optimal binding of Fn to αIIbβ3 but does not require the exact same residues (Figure 3). Alanine substitutions at residues Asp^{1438}, Arg^{1445}, Lys^{1469}, and Arg^{1493} affected the epitope for mAb fnI-8, demonstrating that the epitope for this mAb overlaps the RGD loop and Arg^{1445} in the 3fn10 module (Table 1, Figure 3b). Thus, the epitopes for two anti-Fn mAbs that inhibit this ligand binding to αIIbβ3 overlap the regions identified for optimal binding to this integrin.

DISCUSSION

We have employed a charge-to-alanine mutagenesis strategy to identify residues in Fn that are required for full binding activity to the integrin αIIbβ3. Five arginine residues in addition to Arg^{1493} and Asp^{1495} of the RGD site were required for optimal binding of Fn to purified αIIbβ3. Each of these residues extended from the surface and was located on one face of the molecule (Figure 3). Our findings are remarkably consistent with those found for α5β1-dependent cell adhesion (Table 2), indicating that there is overlap in Fn residues required for binding these integrins. Thus, a common Fn recognition mechanism for αIIbβ3 and α5β1 involving an extended interface with multiple electrostatic contacts emerges. Although the RGD site in Fn is clearly critical for binding these integrins, more complex interactions at the binding interface may be required for sufficient binding



a)

3fn module	Number of slow exchanging protons: expected	observed[a]
3fn9	35	36±3
3fn10	35	35±3
3fn9-10	70	71±4
R1369A	70	68±2
R1371A	70	69±3
R1379A	70	73±3
R1445A	70	68±3
R1448A	70	70±3
R1493A	70	71±2
D1495A	70	69±3

[a]Change in mass after 60 min exposure to D_2O, pD 4.5 was used for determination

b)

FIGURE 5: Structural evaluation of r3fn modules. Each of the r3fn9−10 module pairs with alanine substitutions that perturbed binding to purified αIIbβ3 were examined by SDS−PAGE (15%) (panel a) and by H−D exchange (panel b). Panel a: 5 μg per lane of Ni−NTA-purified r3fn9−10 was examined on a 15% SDS−PAGE and stained with Coomassie for total protein. Each of the recombinant proteins demonstrated similar purity and migration, except Asp[1495]Ala. Panel b: Experiments similar to that described in Figure 4 were carried out for each of the designated r3fn modules. The change in mass after 1 h is shown as the observed value. The value expected was that predicted from the NMR structure of individual 3fn modules.

affinity to function in adhesion under physiological conditions or induce conformational changes in these integrins that lead to downstream signaling events.

The 3fn9 module of Fn is known to be required for optimal binding of Fn to αIIbβ3 (*18*). Initially our analysis of αIIbβ3−Fn binding focused on residues 1373−1379, because a synthetic peptide based on the 3fn9 sequence Asp[1373]−Thr[1383] was shown to directly interact with αIIbβ3 and inhibit its ability to bind ligand (*24*). In this study we demonstrate that within the context of the r3fn9−10 module pair the residue Arg[1379] is required for the region Asp[1373]−Thr[1383] to interact with αIIbβ3. The αIIbβ3-related integrin, αvβ3, does not require the 3fn9 domain for optimal binding of Fn (*24*). Thus, we found the Arg[1379]Ala substitution had no effect on r3fn9−10 binding to purified αvβ3 (data not shown). Mutagenesis studies demonstrate that Arg[1379] is also important in the recognition of Fn by the integrin α5β1 (*23*, *41*, *42*). Interestingly, neither the Asp[1373]−Thr[1383] peptide nor

Table 1: Localization of Anti-Fn Monoclonal Antibody Epitopes

position of alanine substitution	IC_{50} of r3fn9−10 mutant modules (μg/mL)[a]: mAb fnI-8	mAb fnI-11
native	0.40 (±0.15)[b]	0.35 (±0.15)[b]
D1328	0.65	0.50
D1334	0.30	0.35
D1337	0.30	0.50
H1346	0.40	0.30
R1351	0.30	0.25
R1358	0.30	0.25
R1360	0.60	0.30
H1361	0.40	0.30
H1362	0.40	0.30
E1364	0.25	0.30
H1365	0.50	0.35
R1369	0.50	0.35
R1371	0.50	0.25
E1372	0.25	0.40
D1373	0.45	**>50**
R1374	0.50	0.35
H1377	0.35	**>50**
S1378	0.50	**50**
R1379	0.50	**>50**
E1392	0.30	0.25
R1403	0.40	0.25
E1404	0.25	0.35
E1405	0.25	0.35
D1418	0.40	0.30
R1421	0.45	0.35
D1422	0.35	0.35
E1424	0.40	0.30
D1438	**>50**	0.45
R1445	**>50**	0.30
R1448	0.35	0.30
E1453	0.30	0.30
E1462	0.30	0.30
K1469	**2.0**	0.30
K1478	0.40	0.30
D1482	0.30	0.25
R1493	**>50**	0.30
D1495	0.35	0.35
K1501	0.30	0.35
R1508	0.40	0.25

[a] The concentration of r3fn9−10 modules required to inhibit 50% of 1 μg/mL biotinylated mAb binding to wells coated with 10 μg/mL native r3fn9−10 (see Experimental Procedures). The average of triplicate determinations is reported. [b] SD of 10 independent experiments. Demarcation of 3fn9 and 3fn10 is represented by a line of space.

derivatives of this peptide inhibited Fn binding to purified α5β1 or α5β1-dependent K562 cell adhesion to Fn (R. D. Bowditch, unpublished observations). This suggests that these two integrins may use a distinct mechanism for Fn recognition or α5β1 may be more sensitive to the conformation of this sequence than αIIbβ3. Our mutagenesis studies demonstrate that the integrins αIIbβ3 and α5β1 both require Arg[1379] located in 3fn9 for optimal recognition of this ligand. Thus, αIIbβ3 and α5β1 share a common Fn residue for recognition of the Asp[1373]−Thr[1383] or "synergy" site located within the 3fn9 module.

Recent studies have shown that α5β1-dependent adhesion of K562 cells to plastic-bound r3fn7−8−9−10 modules is affected by mutations at Fn residues Arg[1369], Arg[1371], Arg[1374], Arg[1379], Thr[1385], Asn[1386], and Arg[1445] (*23*). These results are remarkably similar to those found for purified αIIbβ3 (Table 2). Adhesion of K562 cells via α5β1 to recombinant 3fn7−8−9−10 was affected by individual alanine substitutions at Arg[1374], Arg[1379], and Arg[1445] when plastic surfaces were coated at 20 μg/mL. Alanine substitutions at residues Arg[1369],

Table 2: Comparison of Fn Residues Required for αIIbβ3 and α5β1 Binding

position of alanine substitution	αIIbβ3[a]	α5β1[b]
R1369	+[c]	+
R1371	+	+
R1374	−[d]	+
R1379	+	+
T1385	ND[e]	+
N1386	ND	+
R1445	+	+
R1448	+	ND
R1493	+	+[f]
D1495	+	+[f]

[a] Studies presented in this paper. [b] Results from Redick et al. (*23*). [c] Negatively affected binding to purified αIIbβ3 or α5β1 cell adhesion. [d] No significant effect. [e] Not determined for this integrin. [f] Found to be required for α5β1-dependent adhesion of BHK cells (*19*). Demarcation of 3fn9 and 3fn10 is represented by a line of space.

Arg[1371], Thr[1385], and Asn[1386] exhibited an effect when plated at lower concentrations or when combined. Our results demonstrate that single alanine substitutions at Arg[1369], Arg[1371], Arg[1379], Arg[1445], Arg[1448], Arg[1493], and Asp[1495] decrease the apparent affinity of the r3fn9−10 module pair for αIIbβ3 in a purified receptor system and that these effects are not a result of r3fn9−10 self-association, module unfolding, or postadhesion events. Differences in relative effects seen by individual substitutions in the two integrins may be a result of differences in the assays.

The mutations analyzed by Redick et al. (*23*) were chosen using a structure-based approach, and those reported here explored the entire 3fn9−10 module pair surface. As shown in Table 2, at least six Fn residues are required for recognition by both αIIbβ3 and α5β1. Fn residues Thr[1385] and Asn[1386] were not tested with purified αIIbβ3, nor was Arg[1448] examined with α5β1; therefore, no conclusions can be drawn regarding these residues. Interestingly, alanine substitution of Arg[1374] affects α5β1-dependent cell adhesion; however, this substitution had the same apparent affinity for αIIbβ3 as the native r3fn9−10. Overall, recognition of Fn by both αIIbβ3 and α5β1 is similar in the mechanism of Fn binding; however, there are subtle differences in the residues required.

All of the residues identified in human Fn in this study are conserved in bovine, rat, mouse, chicken, and *Xenopus* Fn sequences, indicating functional or structural importance (Figure 1). Examination of the molecular structure of the 3fn9−10 modules revealed that the residues identified in this study are located on the same face of the molecule as the previously characterized binding sites (*4*). Furthermore, the side groups of each residue found to be involved in the interaction of Fn with αIIbβ3 are located on the surface of the ligand and are accessible for integrin binding. In support of this conclusion, the epitopes for two anti-Fn mAbs that inhibit this ligand binding to αIIbβ3 were found to overlap the residues identified for the 3fn9−10 module pair binding to this integrin. It is noteworthy that, with the exception of Asp[1495], the only charged Fn residues that were required for optimal Fn−αIIbβ3 interaction were positively charged. Alanine substitutions of negatively charged residues near the arginine residues identified to be required tended to enhance 3fn9−10 binding to αIIbβ3. This suggests that an increase in the net positive charge in the areas identified may enhance the electrostatic interactions between Fn and αIIbβ3. Thus, the positively charged Fn residues identified most likely interact with anionic charge residues or potentially with anionic carbohydrate moieties of the integrin in a spatially specific manner to stabilize binding between receptor and ligand.

What are the potential ramifications of an extended Fn−integrin binding interface? Incremental increases in the spacing of 3fn9 and 3fn10 by insertion of glycine residues within the linker region disrupted downstream cell adhesion events (*43*), suggesting that the spacing of the 3fn9 and 3fn10 modules is important to integrin binding. In addition to effects on cell adhesion, the Asp[1373]−Thr[1383] sequence or synergy site has been shown to be involved in α5β1-mediated matrix assembly (*44*) and downstream cell adhesion events, such as cell spreading and protein phosphorylation (*43*, *45*). Disruption of any of the multiple electrostatic interactions identified in this study would be expected to lessen the binding affinity and may also inhibit subsequent conformational changes in the integrin that lead to specific downstream signaling.

In summary, multiple positively charged contact residues within the 3fn9 and 3fn10 modules are required for optimal binding to αIIbβ3. These residues were located on the same face of the molecule and extend from the surface. It appears that a common recognition mechanism involving multiple electrostatic contacts between Fn−αIIbβ3 and Fn−α5β1 exists. Thus, αIIbβ3 and α5β1, and potentially other integrins, interact with the cell binding domain of Fn via an extended interface that is required for the native binding affinity and may be responsible for post-ligand-binding conformational changes in the integrin.

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