The Eighth FIII Domain of Human Fibronectin Promotes Integrin \( \alpha_5\beta_1 \) Binding via Stabilization of the Ninth FIII Domain*

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Binding of the extracellular matrix molecule fibronectin to the integrin receptor \( \alpha_5\beta_1 \) elicits downstream signaling pathways that modulate cell function. Fibronectin-\( \alpha_5\beta_1 \) interaction occurs via the conserved RGD sequence in the tenth FIII (FIII10) domain of fibronectin. A synergistic site containing the sequence PHSRN in the adjacent FIII9 domain has also been identified. Here we investigate the function of the eighth FIII domain in integrin-mediated cell adhesion using a wide range of methods, including biochemical, biological, and biophysical assays of integrin binding, cell adhesion, and protein denaturation. Mutation of the FIII9 synergistic site (PHSRN to PHAA) in FIII9-10 reduced the binding activity for integrin \( \alpha_5\beta_1 \) to levels observed for FIII10 alone, but the corresponding mutant in FIII9-9-10 showed no loss of binding activity. Cell adhesion assays also demonstrated enhanced functional activity of constructs containing FIII8. Equilibrium chemical denaturation studies indicated that FIII8 confers conformational stability upon FIII9, but only if the exposed loops, PHSRN and VKNEED on FIII9 and FIII8, respectively, are intact. These results demonstrate that the loss of integrin binding activity, observed upon alteration of the PHSRN synergistic site of FIII9-10, results partly from a loss of conformational stability of FIII9. Our data suggest a mechanism for integrin \( \alpha_5\beta_1 \)-fibronectin interaction, which, in addition to the primary RGD binding event, involves a conformation-sensitive scanning by the integrin for accessible sites on the ligand, whereupon full activation of downstream signaling occurs.

The binding of fibronectin (FN) to the integrin family of transmembrane receptors elicits downstream signaling events that can modulate diverse cellular processes including cell adhesion, spreading, proliferation, migration, and invasion. The regulated adhesion of cells to FN thus has a key function in embryonic development and tissue homeostasis, and aberrant regulation of this process is often associated with disease. The understanding of the molecular basis of FN-integrin interactions has wide implications for the manipulation of normal and disease tissue processes.

FNs are large glycoproteins that are abundant in the extracellular matrix (ECM) of most tissues (for a review, see Ref. 1). The mature FN molecules are dimers of two C-terminally disulfide-linked monomers of ~220 kDa. Each monomer consists of homologous repeating units or domains, called type I, II, and III domains. The type III FN domains (FIII) are the most common and the largest, composed of around 90 amino acids arranged in seven anti-parallel \( \beta \)-strands (2). FIII domains occur in many diverse intra- and extracellular proteins (3).

Integrins are heterodimeric cell-cell and cell-ECM adhesion receptors composed of one \( \alpha \)-subunit and one \( \beta \)-subunit and classified into eight different groups according to the identity of their \( \beta \)-subunit (for a review, see Ref. 4). Integrin-mediated signal transduction occurring in response to ligand binding involves phosphorylation of intracellular molecules such as focal adhesion kinase (pp125FAK) and paxillin, and consequent induction of second messenger pathways (5). The central cell binding domain (CCBD) of FN contains binding sites for a number of integrins, including \( \alpha_5\beta_1 \). The minimal cell recognition sequence in the CCBD is the RGD motif in the tenth FIII domain (FIII10). Synthetic peptides containing RGD exhibit some cell adhesive activity and block cell adhesion to FN. The RGD motif is a conserved sequence occurring in several other distinct extracellular matrix proteins (6). An additional site, PHSRN, that is required for activity close to that evoked by intact FN, has been identified in the adjacent FIII9 domain (7). Residues associated with this site have been shown to act synergistically with RGD in \( \alpha_5\beta_1 \) and \( \alpha_6\beta_1 \)-mediated cell adhesion (8). In addition, deletion mutagenesis studies have suggested involvement of a further site, N-terminal to FIII9, in \( \alpha_5\beta_1 \) binding, although no specific amino acids were identified (9). More recently, additional \( \alpha_5\beta_1 \) binding activity has been described in the fragment spanning domains FIII6 to FIII10 (10). However, the specific functions of the individual FIII domains N-terminal to FIII9 in FN-integrin binding have not been reported.

Resolution of the structure of the human FIII7-10 string of four domains by x-ray crystallography (11) and heteronuclear NMR studies of the human FIII10 domain (12) have provided key information for the understanding of integrin-ligand recognition and binding. The NMR and crystal structures reveal that the RGD sequence of FIII10 resides in a loop extending from the domain surface, and NMR-derived data further suggest that this RGD loop is flexible (13, 14). This flexibility may...
contribute to the promiscuity of RGD-integrin interactions and provide an explanation for the blocking of cell adhesion function by small flexible RGD peptides. The PHSRN sequence in FI19 and the RGD loop are on the same side of the FI19-10 pair but PHSRN, which is also located on a loop, is less exposed. Structure-function studies have indicated that the relative spatial configuration of the RGD and PHSRN loops in FI110 and FI19 is critical for function (15).

Analysis of specific cellular events, elicited in response to cell adhesion to wild-type and mutant FI19, FI110, and FI19-10 domains, suggests that cell attachment occurs primarily via FI110, while maximal activation of pp125FAK and downstream signaling is dependent upon FI19 (16). The two-site model of FN-integrin interaction implied in these studies is in agreement with earlier data proposing that the synergy site binds to the $\alpha_5$-subunit and the RGD site binds to the $\beta_3$-subunit (10).

The characterization of binding sites on integrins that interact with the FI1 domains has largely focused on the RGD and PHSRN motifs, although it is not clear whether or not these are the only motifs required for ligand-receptor interaction. Indeed, recent studies have suggested additional sites in FI19 and FI110 that contribute to ligand binding (17). In this study we investigate the specific role of the FI11 domain in integrin $\alpha_5\beta_3$ recognition by characterizing wild-type and mutant FI11-9-10 proteins, and by biological assays, our data suggest that native FI11 and various mutations in FI11 and FI19 can modulate FN binding to integrin $\alpha_5\beta_3$. We show that there is a strong correlation between structural stability of FI11 and its integrin-mediated function.

**EXPERIMENTAL PROCEDURES**

**Construction of pGEX-FII1 Clones**—The construction of pGEX-FI19, pGEX-FI19-10, and pGEX-FI19-10-10 has been described elsewhere (18). The DNA sequences of the FI1 constructs were amplified from the plasmid pFHL1 (19). pGEX-FI10 was constructed using the primers FIII8-5 and TTATTATGTTTTCTGCATAC. pGEX-FI10, and pGEX-FI19-10-10 has been described elsewhere (18).

**DNA Sequences of the FI1 Constructs** were amplified from the pGEX-FI10 constructs containing the PHSRN mutation in the FI19 domain were further mutated in the VKNEED loop of FI110 using the primers described above. Notations used for the various mutants are as follows: PHSRN to PHAAA (mSRN); VKNEED to AAAAAE (mVK); VKNEED to VKNAAA (mEED); PHSRN to PHAAA and VKNEED to AAAAAE (mSRN, mVK); PHSRN to PHAAA and VKNEED to VKNAAA (mSRN, mEED) (see Table I). The DNA sequence of all created constructs was confirmed using Sanger DNA sequencing methodology (Dept. of Biochemistry, University of Oxford).

**Expression and Purification of FI1 Proteins**—GST-FI1 fusion proteins were expressed in Escherichia coli and purified as described previously (18). For cell spreading inhibition assays and equilibrium chemical denaturation studies, cleaved FI1 proteins were obtained by thrombin digest of the respective GST fusion proteins bound to the GST-conjugated Sepharose resin (2.5 units of thrombin per mg of fusion protein). Cleaved FI1 protein was subsequently washed off the resin with PBS, dialysed exhaustively in 40 mM Tris-HCl, pH 8 (buffer A), and subjected to ion exchange chromatography using Q-Sepharose resin developed with a NaCl gradient of 0–1 M in buffer A over 30 min.

**Purity and $M_r$ of all proteins was assessed by SDS-PAGE, visualized with Coomassie Blue. Protein concentration was calculated using absorption of the solution at 280 nm, with the extinction coefficient estimated using the program peptidesort (Wisconsin package ver. 10.0, Genetics Computer Group, Madison, WI), exploiting the procedure of Gill and von Hippel (20).**

**Purification of Integrin $\alpha_5\beta_3$**—Integrin $\alpha_5\beta_3$ was purified from human placenta as described previously (21), with some modifications. Briefly, a placenta was homogenized in 200 ml ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 1 mM MnCl2, 1 mM CaCl2, 150 mM NaCl, 1% Triton X-100) containing sodium vanadate (1 mM), leupeptin (10 µg ml$^{-1}$), aprotonin (10 µg ml$^{-1}$), and phenylmethylsulfonyl fluoride (1 mM). The homogenate was centrifuged at 20,000 $\times$ g for 1 h at 4°C, and the supernatant was loaded onto protein A-conjugated Sepharose resin (Amersham Pharmacia Biotech) and subsequently onto Sepharose resin conjugated to antibody clone BIIG-2 raised against the $\alpha_5$ subunit, both resins having been pre-equilibrated with lysis buffer. The integrin was eluted in 2 bed volumes of 20 mM sodium acetate, pH 3.1, containing 30 mM octyl-$\beta$-glucoside (Sigma-Aldrich). 1-ml fractions were collected and simultaneously neutralized in 10× lysis buffer without Triton X-100 and containing 300 mM octyl-$\beta$-glucoside. The integrin was stored in a buffer containing the divalent cations Mn$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$.

**ELISA**—96-well flat-bottomed plates (Nunc) were coated with a 10-fold dilution of the integrin stock solution (0.1 mg ml$^{-1}$) in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MnCl2, 0.1 mM MgCl2, 0.1 mM CaCl2 (EB) and left overnight at 4°C. The plates were then washed three times with EB and blocked with 5% bovine serum albumin (BSA) in EB for 1 h at 37°C. The wells were washed as above, and the plates were incubated for 2 h at 37°C with the relevant GST-FI1 fusion proteins diluted in EB containing 1% BSA. The wells were processed as above, and mouse anti-GST antibody (1:2000 dilution in EB containing 1% BSA) (Sigma) was added. The plates were incubated for 1 h at room temperature, the wells washed with EB, and sheep anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Sigma) was added (1:2500 dilution in EB containing 1% BSA). The plates were incubated for 1 h at room temperature, the wells washed as above, and incubated with the Sigma Fast HRP tablet set according to the manufacturer's instructions. The absorbance of the solution was measured at 405 nm. Assays were performed in triplicate, and background antibody binding in the absence of ligand was subtracted from the data. Nonspecific binding of GST fusion proteins to uncoated wells containing BSA only was measured separately for each ligand concentration point and subsequently subtracted from the corresponding values for total binding. Dose-response data from the assays were analyzed by non-linear regression using a sigmoidal curve fit (Prism, GraphPad Software).

**Cell Attachment and Spreading Assays**—Baby hamster kidney
The Role of Eighth FIII Domain in Integrin Binding

RESULTS

FII18 Compensates for a Defective FII19 Synergy Site in Integrin α5β1 Binding.—The influence of FII18 upon the interaction of FN with integrin α5β1 was analyzed in solid-phase ligand binding assays. An initial assessment of the integrity of the integrin preparation was made by comparing the binding affinities of the individual GST-FII10, GST-FII9, and GST-FII18 domains, the GST-FII19-10, and GST-FII18-9 domain pairs, the GST-FII18-9-10 domain triplet, and GST alone (Fig. 1A). The observed binding affinities of the recombinant proteins were considered not to be perturbed by the GST carrier protein or its dimerization, since the isolated GST dimer had negligible binding activity to α5β1 integrin, and the same carrier GST was present in all the proteins tested. Of the individual FII1 domains, only FII10 exhibited α5β1 binding activity (Fig. 1A), as is consistent with previous data (18). Comparison of the dose-response curves for FII10 and FII110 (Fig. 1A) shows that addition of FII19 increases the affinity for α5β1 (the apparent Kₐ was ~50-fold lower than for FII10 alone), confirming the synergistic action of FII19. No binding was observed for the FII18-9 domain pair. The addition of FII18 N-terminal to FII19-10 did not significantly alter the integrin binding affinity (Fig. 1A).

The integrin binding capacities of the synergy site mutants GST-[mSRN]FII18-9-10 and GST-[mSRN]FII19-10 (see Table I) were subsequently compared by ELISA. Alanine substitution of the amino acids SRN in the FII19 synergy site reduced the α5β1 binding affinity of FII19-10 to the level observed for the single FII110 domain (Fig. 1B). Comparison of the synergy site mutants GST-[mSRN]FII18-9-10 and GST-[mSRN]FII19-10, however, showed that the FII18 domain can recover α5β1 binding activity that was lost as a result of mutation of part of the FII19 synergy site (Fig. 1B). These data thus indicate a role for FII18 in integrin α5β1 binding in the absence of a functional PHSRN site in FII19.

FII18 Confers Additional Cell Adhesion Activity on FII19-10.—We further explored the possible contribution of FII18 to FN-integrin interaction in biological assays of cell attachment and spreading (Fig. 2). Attachment assays with BHK fibroblasts (Fig. 2A) showed that neither FII18 alone nor the FII18-9 pair exhibited any cell attachment activity. The activity of FII18-9-10 was greater than that of FII19-10, although this difference was less pronounced at higher concentrations of coating protein. In comparison with cell attachment, cell spreading was more sensitive to the presence of FII18 (Fig. 2B); the ability of FII18-9-10 to induce cell spreading was ~50 and ~30% higher than that of FII19-10 at coating concentrations of ~0.2 and ~2 μM, respectively.

An alternative approach to the assays described above was adopted in which the binding of FII19 domains in solution to the cell surface integrins was assessed, thus negating the effect of possible conformational constraints imposed by the immobilization of the protein ligand to the plastic substrate. Assays of
inhibition of cell adhesion on FN-coated surfaces, testing the ability of FIII8 to enhance the inhibitory activity of FIII9-10, were performed using independent FIII domains cleaved from the GST carrier protein (Fig. 2C). The FIII8 and FIII10 domains in isolation had no effect upon cell adhesion at the concentrations tested, confirming our previous data (18). Wild-type FIII8-9-10 had a more marked effect on cell spreading than FIII9-10, showing ~20% more inhibitory activity at the concentration of ~2 μM. These results thus differ from the ELISA data in which addition of FIII8 to the wild-type FIII9-10 ligand produces no enhancement of solid-phase integrin binding. This probably reflects the different nature of the two assays. ELISA only provides information about the requirements for primary ligand binding event and is not influenced by integrin responses secondary to the initial recognition, such as receptor clustering (23) and inside-out signaling, which affects ligand affinity (24).

Biological assays were further employed to assess the contribution of FIII8 to the induction of downstream cellular responses in the absence of a functional synergy site in FIII9-10 (Fig. 3). The FIII9-10 mutant carrying alanine substitutions in the synergy site (SRN to AAA: [mSRN]FIII9-10) exhibited reduced levels of cell attachment, approaching those observed for FIII10 (Fig. 3A). Addition of FIII8 to [mSRN]FIII9-10 (in the mutant [mSRN]FIII8-9-10) recovered the cell attachment activity lost upon mutation of the synergy site in FIII9 (Fig. 3B). These observations are therefore in accordance with ELISA data for integrin binding activity of these mutants (see Fig. 1B).

The ability of FIII8 to participate in integrin-induced cellular signaling was further determined by examining focal adhesion complex formation in response to [mSRN]FIII9-10 carrying the FIII9 synergy site mutation (Fig. 4). Vinculin-positive focal adhesion complexes observed in cells plated onto FIII9-10 and FIII8-9-10 (Fig. 4, A and C) did not form in response to the mutant domain pair [mSRN]FIII8-9-10 (compare Fig. 4, A and B). However, the ability to promote focal adhesion complex formation was restored by the presence of FIII8 in [mSRN] FIII8-9-10 (Fig. 4D), providing further evidence that FIII8 contributes specifically to the induction of integrin-dependent downstream signaling responses.
The VKNEED Sequence in the Exposed C-C’ Loop of FIII8 Has a Function in Cell Adhesion—The crystal structure of FIII7-10 (11) reveals a protruding loop between the β-strands C and C’ of FIII8, on the same face of the molecule as the exposed loops containing the RGD and PHSRN sites (Fig. 5). Given the location and configuration of this loop in FIII8, we tested the possibility that it contributes specifically to the integrin-binding activity of FIII8 observed in the GST-[mSRN]FIII8-9-10 mutant. The residues VKNEED within the loop were substituted with alanine both in the wild-type GST-FIII8-9-10 construct and in the GST-[mSRN]FIII8-9-10 synergy site mutant (Table I). The resultant mutants GST-[mVKN]FIII8-9-10 and GST-[mEED]FIII8-9-10 did not show any difference in $\alpha_5\beta_1$ binding affinity when compared with the native GST-FIII8-9-10 protein (Fig. 6A). The binding affinities of double mutants GST-[mSRN,mVKN]FIII8-9-10 and GST-[mSRN,mEED]FIII8-9-10 were also similar to that of GST-FIII8-9-10 (data not shown). These results therefore suggest that the VKNEED sequence in FIII8 is not a primary recognition motif for $\alpha_5\beta_1$ integrin.

The possible function of the VKNEED sequence of FIII8 in promoting cell adhesion was determined using the above described mutants in cell spreading assays (Fig. 6B). In experiments comparing the activity of the VKN and EED mutants (Table I), lower numbers of cells spread on surfaces coated with [mVKN]FIII8-9-10 and [mEED]FIII8-9-10 than on those coated with wild-type FIII8-9-10 (−65 and −80% of FIII8-9-10 activity, respectively, at the concentration of −0.1 μM) (Fig. 6B).

Cell spreading in response to GST-[mSRN,mVKN]FIII8-9-10 and GST-[mSRN,mEED]FIII8-9-10 at low coating concentration (∼0.1 μM) was reduced to ∼5 and ∼35% of wild-type FIII8-9-10 activity, respectively, but remained higher than that supported by [mSRN]FIII9-10 (Fig. 6B). The mutants [mVKN] FIII8-9-10 and [mSRN,mVKN]FIII8-9-10 were biologically less active than [mEED]FIII8-9-10 and [mSRN,mEED]FIII8-9-10, respectively, suggesting that the residues EED were more tolerant to substitution by AAA than the residues VKN. These data, while contrasting those from solid-phase integrin binding assays (see Fig. 6A), reveal that the VKNEED loop contributes
to cell adhesion, accounting for some, but not all, of the additional adhesive activity contained within FIII8.

**FIII8 Confers Conformational Stability on FIII9**—The FIII9 domain in the FIII9-10 pair is relatively unstable in comparison with FIII10 (14), while the configuration of the RGD and PHSRN sites with respect to each other is critical for functional activity (15). The potential effect of FIII8 on the stabilization of FIII9-10, which would have consequent effects on functional activity revealed in the ELISAs and cell spreading assays, was thus assessed. Equilibrium unfolding experiments using GdnHCl were carried out as described previously (22). These experiments exploit the presence of a buried tryptophan in a similar position in each of the three FIII domains, whose fluorescence properties are sensitive to conformation. A $\Delta G$ value can be determined from the ratio of folded to unfolded protein observed at each GdnHCl concentration (designated as [GdnHCl]). Three related parameters, $\Delta G_{(H2O)}$ (the free energy of unfolding in the absence of denaturant), $m$ (the dependence of $\Delta G$ on [GdnHCl]) and $[\text{GdnHCl}]_{1/2}$ (the denaturant concentration giving 50% unfolding) are used to characterize the curves for a two-state unfolding mechanism ($\Delta G_{(H2O)} = m[\text{GdnHCl}]_{1/2}$). This analysis is most robust when there are well-defined pre- and post-transition baselines (25); the problem of ill-defined baselines mainly arises with free FIII9. It has been shown that FII10 undergoes a three-state folding process (26). However, the third step is only readily detected using guanidinium isothiocyanate as a denaturant. For the analysis here, where the goal is to understand relative effects on domain stability rather than folding mechanisms, a two-state mechanism of unfolding for FIII domains is assumed (14).

The isolated FIII8 domain denatured at slightly higher GdnHCl concentrations than the isolated FIII9 domain ([$\text{GdnHCl}]_{1/2}$ being equal to 1.13 M, as opposed to 0.78 M for...
Table II
Equilibrium denaturation parameters for the first denaturation step of the recombinant FIII protein

| FIII species | (GdnHCl)$_{1/2}$ | $\Delta G_{(H2O)}$ | m |
|--------------|-----------------|-------------------|---|
|              | kcal mol$^{-1}$ | kcal mol$^{-1}$   | μM |
| FIII8        | 1.13            | 8.40              | 7.44 |
| FIII9        | 0.78            | ~2.25$^a$         | 2.89 |
| FIII10       | 4.91            | 9.81              | 2.00 |
| FIII8-9      | 2.03            | 6.31              | 3.11 |
| FIII9-10     | 2.18            | 8.14              | 3.74 |
| FIII9-9-10   | 2.79            | 16.42             | 5.88 |
| [mSRN]FIII8-9-10 | 2.34 | 14.83             | 6.34 |
| [mVKN]FIII8-9-10 | 2.48 | 16.47             | 6.65 |
| [mEED]FIII8-9-10 | 2.51 | 16.12             | 6.43 |
| [mSRN,mVKN]FIII8-9-10 | 2.23 | 12.74             | 5.71 |
| [mSNR,mEED]FIII8-9-10 | 2.31 | 12.47             | 5.41 |

$^a$ The experimental error on $\Delta G_{(H2O)}$ was a result of the lack of baseline fluorescence at low concentrations of denaturant.

We have applied a combination of biological, biochemical, and biophysical approaches to elucidate the nature of the α$_3$β$_3$-FN interaction beyond the known requirements for the binding sites in FIII9 and FIII10. The main findings from this study are as follows: (i) the presence of the FIII9 domain maintains ligand binding potency in the absence of an intact FIII9 synergy site; (ii) FIII8 has a function in the stabilization of the FIII9 domain, and (iii) short amino acid sequences in FIII8 and FIII9 have short and long range stabilization effects that are necessary for the biological activity of the CCBD.

In this study, we have attempted to dissect further the functional sites in the CCBD of human FN that are required for integrin α$_3$β$_3$-mediated cell adhesion. Previous data have shown that substitution of Asp for Arg$^{1379}$ in FIII9 results in a marked decrease in α$_3$β$_3$-mediated adhesion activity, and substitution of other residues in the PHSRN motif in chimeric FIII9-10 pairs has a lesser effect on cell adhesion (7). Our data demonstrate that alanine substitution of only three residues in the motif (mSRN) abolishes the synergistic effect of FIII9 on α$_3$β$_3$ binding.

One important observation in our study is that FIII8 increases the biological activity of FIII9-10 and is able to recover the biological activity lost on mutation of the synergy site (PHSRN) to PHAAA (see Figs. 1B and 3). This suggests that the requirement for PHSRN may only be critical for integrin binding in the case of the isolated FIII9-10 pair and that in the FN molecule the criteria for integrin α$_3$β$_3$ recognition are likely to include effects from additional residues in the CCBD and from domains N-terminal to FIII9. Our results are in general agreement with a recent study (17) exploring the effect of alanine substitution of residues both in the PHSRN motif and elsewhere in the FIII7-8-9-10 domain tandem. While confirming that Arg$^{1379}$ is the single most important synergistically acting residue within FIII9, the authors see only a minimal reduction in cell adhesion when this site alone is mutated. However, in combination with additional substitutions (notably, for Arg$^{1369}$, Arg$^{1371}$, Arg$^{1374}$, or Thr$^{1385}$. Asn$^{1386}$) the drop in adhesive activity is much more pronounced; an indication that the synergistic effect in FIII7-10 is not limited to the PHSRN sequence but actually involves a larger region of FIII9. This finding may explain why the mutant [mSRN]FIII8-9-10 does not show reduced adhesive potential in our assays, especially when considering that this mutant and a single Arg$^{1379}$ mutant of FIII8-9-10, equivalent to the one used by Redick et al. (17), exhibit very similar activities.²

We suggest that the synergistic effect of FIII8 is in part mediated via its effect on the stabilization of the FIII9 domain. The equilibrium denaturation studies designed to test this hypothesis show that although the FIII9-9 pair unfolds cooperatively, the mutual increase in the stability of both domains is equivalent to the increase in stability conferred upon FIII9 by FIII10 in the FIII9-10 domain pair. Thus FIII8 clearly produces an increase in the stability of FIII9 and vice versa.

The C-C' loop of FIII8 contains the motif VKNEED, which resembles the sequence REDV, a known FN adhesion recognition motif (27). In comparison with wild-type FIII8-9-10, the FIII8-9-10 mutants [mVKN] and [mEED] support lower functional activity in cell spreading assays, but not in ELISA, with the residues VKN appearing more sensitive to substitution than EED in functional assays. These results imply that the residues VKNEED are not recognized by the ligand binding site of α$_3$β$_3$ per se but influence downstream, ligand-induced cellular responses. Our data further suggest a requirement for

² H. Mardon, unpublished observations.
the integrity of the VKNEED sequence for the stability of the syner

The confomational stability of the wild-type and mutan

This is highlighted by the comparison of the dependence of ΔG on GdnHCl concentration (m) for the FIII8-9-10 mutants (see Table II). Furthermore, loss of cell spreading activity of the mutants matches their respective loss of the stability of the FIII8-9 pair (Fig. 7C). Such long range effects of the VKNEED sequence, and possibly additional residues, on the stabilization of FIII9 thus provide a mechanism by which cell spreading activity may be modulated by FIII8.

The unfolding assays employed in this study do not necess-

ity reveal local structural deviance but give an indication of the overall foldedness of the proteins. As alanine-scanning mutagenesis is a commonly used approach to identify structural motifs that are important for function, the data we presen-

ent here highlight the exigency for structural evaluation of mutant proteins in these types of studies and reinforce the possibility that amino acid substitutions could critically alter the global structural integrity of proteins. However, despite the correlation we observe between the stability, integrin binding capacity, and biological activity of the FIII8-9-10 mutants, our findings do not allow us to clearly uncouple the effects of domain destabilization from loss of direct contact sites with integrin αβ1. We consider it likely that the PHSRN loop in FIII9 is involved both in direct integrin recognition and in conferring overall conformational stability to FIII9, and that the latter function is an important prerequisite for efficient αβ1 binding and subsequent cell adhesion.

In conclusion, we have revealed a specific function for the FIII8 domain of FN in integrin αβ1 recognition, high-affinity receptor binding and induction of downstream signaling, by elucidation of an indirect effect of FIII8 on the interaction of the CCBD with the αβ1 integrin. The data presented provide a molecular basis for the previously reported enhancement of biological activity by FIII domains N-terminal to FIII9 (9, 10, 28). Our results demonstrate that amino acid sequences in addition to those described previously in FIII9 and FIII10 can contribute to integrin binding and induction of downstream signaling. Our observations support a model for αβ1-FN interaction according to which the primary integrin recognition occurs via the RGD site in FIII10. We propose that secondary binding occurs via multiple, specific amino acid residues that can compensate for each other depending upon the availability and functionality of these sites within the FN molecule. Fi-

nally, our data reinforce the value of using multiple experimen-
tal approaches to determine accurately the specific functions of amino acid motifs in ligand-receptor interactions. The results of this study may have more widespread implications for multidiom ligand-receptor interactions and the identification of specific receptor recognition sites.

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