Structural Requirements for Biological Activity of the Ninth and Tenth FIII Domains of Human Fibronectin

Richard P. Grant‡, Claus Spitzfaden§, Harri Altroff§, Iain D. Campbell§**, and Helen J. Mardon‡‡‡

From the ‡Nuffield Department of Obstetrics and Gynaecology, University of Oxford, The Women’s Centre, John Radcliffe Hospital, Headington, Oxford OX3 9DU and the §Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom

The ninth and tenth type III domains of fibronectin each contain specific cell binding sequences, RGD in FIII10 and PHSRN in FIII9, that act synergistically in mediating cell adhesion. We investigated the relationship between domain-domain orientation and synergistic adhesive activity of the FIII9 and FIII10 pair of domains. The interdomain interaction of the FIII9–10 pair was perturbed by introduction of short flexible linkers between the FIII9 and FIII10 domains. Incremental extensions of the interdomain link between FIII9 and FIII10 reduced the initial cell attachment, but had a much more pronounced effect on the downstream cell adhesion events of spreading and phosphorylation of focal adhesion kinase. The extent of disruption of cell adhesion depended upon the length of the interdomain linker. Nuclear magnetic resonance spectroscopy of the wild type and mutant FIII9–10 proteins demonstrated that the structure of the RGD-containing loop is unaffected by domain-domain interactions. We conclude that integrin-mediated cell adhesion to the central cell binding domain of fibronectin depends not only upon specific interaction sites, but also on the relative orientation of these sites. These data have implications for the molecular mechanisms by which integrin-ligand interactions are achieved.

The regulated adhesion of cells to the extracellular matrix (ECM) is essential for the development and function of normal tissues, and aberrant regulation of cell adhesion is often associated with disease. Fibronectins (FNs) are adhesive proteins that are abundant in the ECM of many cell types and have a critical role in many biological processes. Twenty isoforms of human FN, the expression of which is developmentally regulated, can be generated as a result of alternative splicing of the primary FN transcript (2–4). The FN molecules are dimers of disulfide-linked 235-kDa monomers. Each monomer is composed of type I, type II, and type III domains (FIII, FII, and FIII), identified as repeating amino acid motifs in the primary structure (5) (Fig. 1). These motifs occur in many diverse cellular and extracellular proteins (6). Separable, functional regions of the FN molecule have been identified that contain binding sequences for other components of the ECM, including collagen, fibrin, and heparin (1). Cells bind to FN via the central cell binding domain (CCBD) spanning the eighth, ninth, and tenth FIII domains (FIII8–10) (7) and via the CS1 and CS5 sites in the alternatively spliced IIICS region (8, 9) (see Fig. 1).

The adhesion of cells to FN in the ECM is mediated by the integrin family of transmembrane receptors (10–12). Synthetic peptides that contain the RGD sequence exhibit cell adhesive activity but do not mimic the full adhesive function of the FN molecule (7, 28, 29). The FN molecule is required for the expression of other components of the ECM, including collagen, fibronectin, and laminin. The interactions between FN and the integrin family of transmembrane receptors (10–12) are of great interest.

An earlier nuclear magnetic resonance (NMR) study of FIII10 demonstrated that the RGD sequence resides in a mobile loop between the F and G β strands of the domain (27). After introduction of flexible glycine residues, we showed previously that the structural integrity of the FIII9–10 pair of domains is required for the synergistic activity of the two domains in supporting cell spreading (36). Here we have investigated the relationship between domain-domain interactions and the biological activity of the FIII9–10 pair in specific cell adhesion events. Our strategy was to modify the strength of the domain-domain interaction and the relative mobility of the two domains by introducing flexible polypeptide interdomain linkers of different lengths. The influence of the linkers on the structure of the FIII9–10 pair was analyzed by NMR and found to affect biological activity of FIII9–10.
assessed by the ability of immobilized wild type and mutant GST-FI11 fusion proteins to support events involved in cell adhesion. We discuss the correlation between our biological data and the underlying structural changes as examined by NMR and thermodynamic methods.

**EXPERIMENTAL PROCEDURES**

**Biophysical Studies—**NMR measurements were performed on fully 13N-labeled samples of FI110 and FI110–10 at 25 °C in 20 mM sodium acetate, 5% D2O, pH 4.8. Protein concentrations were 4 mM (FI110) or 1.5 mM (FI110–10). Three-dimensional 13N-correlated H.H NOESY spectra (37) were recorded on a home-built spectrometer operating at a proton frequency of 600 MHz.

**Construction of pGEXFI9–10 Mutants—**The constructs pGEXFI110 and pGEXFI110–10 have been described previously (36). The FI110–10 linker mutant FI110-PG-10, containing one additional proline and one glycine residue between threonine 1415 at the COOH terminus of FI110, and valine 1416 at the NH2 terminus of FI110 (according to the domain boundaries in Kornblith et al. (38)), was expressed from the construct pGEXFI110–10-PG-10. For construction of pGEXFI110–10-PG-10, FI110, and FI110-PG-10 were amplified separately from FN cDNA (pFHIII (2)) by Pfu polymerase (Stratagene, La Jolla, CA) in the polymerase chain reaction. Oligonucleotide primers were designed that introduced a Bgl II site into the 5′ end of the amplified FI110 cDNA, an Xho I site into the 3′ end of FI110, and a Smal site, CCCGGG, encoding the extra proline and glycine residues, into the FI110–10 linker. The primers used were: FI110 5′, TGAAGATCTGGTCTGATGTTCCGAG, GGGGTTTCTGATGTTCCGAG, FI110 3′, TCATCTGAGCGATGGTCCGGATTAATGGA. The polymerase chain reaction products were cleaved with either Bgl II (FI110) or Xho I (FI110–10), gel-purified using QIAEX II (Qiagen Ltd., Dorking, United Kingdom (UK)) according to the manufacturer’s instructions, and cloned into the BamHI/Xho I sites of pGEX4T (Pharmacia, Uppsala, Sweden). The sequence of the inserts in the recombinant clones was confirmed using the Sequenase 2.0 kit (Amersham International plc, Amersham, UK).

The mutant pGEXFI110–10-PG, encoding the fusion protein GFIII9–10-PG, was generated by the introduction of the sequence GGAGGCGGAGGC encoding three glycine residues, into the FI110–10 linker. The primers used were: FI110 5′, TGAAGATCTGGTCTGATGTTCCGAG, FI110 3′, GGGGTTTCTGATGTTCCGAG, FI110 3′, GGGGTTTCTGATGTTCCGAG. The polymerase chain reaction products were cleaved with either Bgl II (FI110) or Xho I (FI110–10), gel-purified using QIAEX II (Qiagen Ltd., Dorking, United Kingdom (UK)) according to the manufacturer’s instructions, and cloned into the BamHI/Xho I sites of pGEX4T (Pharmacia, Uppsala, Sweden).

**Inhibition of Cell Adhesion by Integrin Antibodies—**The surface of duplicate wells of 96-well flat-bottomed, tissue culture-grade plates (Becton Dickinson, Oxford, UK) was coated with doubling dilutions of 100 μg ml−1 GST-FI110 fusion proteins in PBS for 16 h at 4 °C and washed with PBS. Uncoated plastic was blocked by incubation in 1% bovine serum albumin in PBS for 1 h at 37 °C. Either BHK or hESF cells (104 in 100 μl of GMEM or Dulbecco’s modified Eagle’s medium, respectively) were inoculated into each well and incubated in 5% CO2 for 1 h at 37 °C. Cells were washed gently in PBS, fixed in 4% formaldehyde, 4% glutaraldehyde in PBS, and scored for spreading as described previously (36). Total cell attachment was assessed by staining with 0.1% crystal violet as described elsewhere (40). Bound dye was measured at A570 nm with a Titertek Multiscan plate reader.

For the inhibition assays, the surface of duplicate wells of a 96-well plate was coated with 5 μg ml−1 FN and washed and blocked as described above. Test protein diluted in 25 μl of PBS was incubated with 104 BHK cells in 25 μl of GMEM for 2 min. Cells and protein were then inoculated into the precoated wells equilibrated with 50 μl of GMEM. Cells were incubated, fixed, and analyzed as described above.

**Immunoprecipitation and Western Blotting of FAK—**The sequences GRID5 and PSFRN are displayed in ball-and-stick format.

**Fig. 2. Crystal structure of the FI110–10 pair showing linker insertion point.** Ribbon structure of FI110–10 pair from Leahy et al. (44). Linkers were inserted immediately before valine 1416 (arrow), the first valine of FI110. The sequences GRID5 and PSFRN are displayed in ball-and-stick format.
for each residue, a pair of strips from $^1$H-$^1$H NOESY planes of three-dimensional $^{15}$N-correlated NOESY spectra of uniformly $^{15}$N-labeled FIII10 and FIII9–10 is shown. Strips were taken at the $^{15}$N frequency of the $^{15}$N-$^1$H direct correlation peak. Left- and right-hand strips of each pair correspond to the spectra of FIII10 and FIII9–10, respectively. The positions of NH and C-$^1$H cross-peaks are indicated by rectangles and circles, respectively.

FIG. 3. NMR spectroscopy of the RGD site of FIII10 and FIII9–10. For each residue, a pair of strips from $^1$H-$^1$H NOESY planes of three-dimensional $^{15}$N-correlated NOESY spectra of uniformly $^{15}$N-labeled FIII10 and FIII9–10 is shown. Strips were taken at the $^{15}$N frequency of the $^{15}$N-$^1$H direct correlation peak. Left- and right-hand strips of each pair correspond to the spectra of FIII10 and FIII9–10, respectively. The positions of NH and C-$^1$H cross-peaks are indicated by rectangles and circles, respectively.

Extension of the FIII9–10 Linker Leads to Reduced Interdomain Interaction—Detailed NMR and thermodynamic studies of the five cleaved constructs (FIII9, FIII10, FIII9–10, FIII9-$^{10}$, and FIII9-$^{10}$) are presented in a separate publication. The requirement for structural integrity of FIII9–10 for cell attachment via integrins in addition to $\alpha_5\beta_3$ was also investigated in cell attachment assays using hESF cells, attachment of which is mediated by multiple integrins including $\alpha_5\beta_3$. The data for these experiments are shown in Fig. 5B. At high coating concentrations (100 $\mu$g ml$^{-1}$) the level of attachment to wild type GFI19-10 was similar to that on FN. In addition, hESF cells attached efficiently to both GFI19-10, GFI19-$^{10}$, and GFI19-10 coated at 100 $\mu$g ml$^{-1}$, at a similar level to FN and GFI19-10. At lower coating concentrations (e.g., 6 $\mu$g ml$^{-1}$, Fig. 5B), while GFI19-10 supported maximal attach-

2 H. J. Mardon, unpublished data.
ment of stromal cells there was a stepwise decrease with the mutant FIII9–10 proteins with increase in the length of the interdomain linker. Immobilized GFIII10 supported approximately 10% the attachment on FN or GFIII9–10 at this concentration.

**Cell Spreading Activity of FIII9–10 Is Progressively Reduced as the FIII9–10 Interdomain Linker Region Is Increased—**The morphology of BHK cells plated on FN, GFIII9–10, GFIII9-PG-10, GFIII9-P[G]5-10, GFIII10, or GST is shown in Fig. 6. Cells plated on FN (Fig. 6A) exhibited a spread morphology. Cells plated onto GFIII9–10 were also spread, but not to the same extent as on FN (Fig. 6, compare B with A). The cells spread less on GFIII9-PG-10 (Fig. 6C), and very few cells exhibited a spread morphology on GFIII9-P[G]5-10 (Fig. 6D) or GFIII10 (Fig. 6E). Very few cells attached to GST (Fig. 6F) and those that did attach remained rounded.

Cells attached to plates coated with the GST-FIII fusion proteins and FN were scored for spreading, expressed as the percentage of attached cells that exhibited a spread morphology. Both BHK (Fig. 7A) and hESF (Fig. 7B) cells plated on GFIII9–10 at a coating concentration of 100 µg ml⁻¹ exhibited similar spreading to cells on FN. However, 50% spreading activity was obtained at a much lower coating concentration of FN (~0.2 µg ml⁻¹) compared with 50% spreading activity on GFIII9–10 (12.5 µg ml⁻¹). Both BHK and hESF cells spread poorly on GFIII10, which for both cell types supported less than 10% the spreading activity of GFIII9–10 at a coating concentration of 100 µg ml⁻¹.

Spreading of BHK cells on GFIII9-PG-10 coated at 100 µg ml⁻¹ (Fig. 7C) was reduced to 70% compared with GFIII9–10, similar to the relative levels of cell attachment observed for these proteins. Spreading on the mutant GFIII9-P[G]5-10, containing the longer linker, was substantially reduced at 100 µg ml⁻¹, exhibiting levels of spreading activity less than 25% that of GFIII9–10. These differences were more pronounced at lower coating concentrations. At a coating concentration of 6.25 µg ml⁻¹ (Fig. 7C) spreading activity of BHK cells on GFIII9–10 was approximately 30% that of FN, spreading on GFIII9-PG-10 was ~25% that of GFIII9–10, and there was no spreading on GFIII9-P[G]5-10.

The number of hESF cells that spread on GFIII9–10 coated at 100 µg ml⁻¹ (Fig. 7C) was reduced to 70% compared with GFIII9–10, similar to the relative levels of cell attachment observed for these proteins. Spreading on the mutant GFIII9-P[G]5-10, containing the longer linker, was substantially reduced at 100 µg ml⁻¹, exhibiting levels of spreading activity less than 25% that of GFIII9–10. These differences were more pronounced at lower coating concentrations. At a coating concentration of 6.25 µg ml⁻¹ (Fig. 7C) spreading activity of BHK cells on GFIII9–10 was approximately 30% that of FN, spreading on GFIII9-PG-10 was ~25% that of GFIII9–10, and there was no spreading on GFIII9-P[G]5-10.

The number of hESF cells that spread on GFIII9–10 coated at 100 µg ml⁻¹ was similar to BHK cells (Fig. 7, compare D with C). The number of hESF cells spread on GFIII9-PG-10 or GFIII9-P[G]5-10 was approximately 90 and 30%, respectively, of the number of cells spread on GFIII9–10. At a coating concentration of 6.25 µg ml⁻¹, spreading of hESF cells on GFIII9–10 was 10% that of cells on FN. Cells plated onto GFIII9-PG-10 and GFIII9-P[G]5-10 exhibited 35 and 10%, respectively, levels of spreading on GFIII9–10. Thus spreading activity of hESF cells on GFIII9-PG-10 was elevated compared...
with BHK cells. Spreading of hESF cells on GFIII10 at 100 μg ml\(^{-1}\) and at 6.25 μg ml\(^{-1}\) was 19 and 5\%, respectively, relative to spreading on GFIII9–10, similar to levels found for BHK cells.

Activity of Wild Type and Mutant FIII9–10 Domains in Soluble Phase Assays—The capacity of purified wild type and mutant FIII domains to inhibit adhesion of BHK cells to fibronectin was also assessed in inhibition assays. The FIII domains were purified from GST adsorbed to glutathione-Sepharose beads by cleavage with thrombin and were tested for their capacity to inhibit attachment of BHK cells to immobilized FN coated on plastic at 5 μg ml\(^{-1}\). These data are shown in Fig. 8. Attachment of BHK cells to FN was completely abolished by the presence of 125 μM FIII9–10 in the culture. At a concentration of 125 μM, FIII9-PG-10 inhibited attachment of BHK cells to FN by 20%, and no significant inhibition was observed with FIII9-P[G]5-10 within the molar range used in these experiments. Relative to the controls, in which no protein was added to the cells prior to plating out, 50% inhibition of spreading of BHK cells on FN was achieved in the presence of 125 μM FIII9–10 and 125 μM FIII9-PG-10. No inhibition of cell spreading was observed with FIII9-P[G]5-10. At a concentration of 125 μM, FIII10 and GRGDS peptide both inhibited attachment of BHK cells to FN by 20 and 30%, respectively, and had a small effect on cell spreading. Thus insertion of two amino acids in the interdomain linker of FIII9–10 both immobilized and in solution had different effects on cell attachment and cell spreading.

Phosphorylation of FAK Is Strictly Dependent upon Structural Integrity of FIII9–10—Given that perturbation of the FIII9–10 had a profound effect on cell spreading, and given that integrin-mediated cell spreading appears to be preceded by FAK phosphorylation (11), we examined the relationship between cell spreading and FAK phosphorylation in response to adhesion to the wild type and mutant FIII9–10 proteins. The relative amounts of phosphorylated FAK in BHK cells plated

---

**Fig. 7. Cell spreading on wild type and mutant linker proteins.** BHK cells (A, C) and hESF cells (B, D) were assayed for spreading on FN (●), GFIII9–10 (○), GFIII10 (×), GFIII9-PG-10 (■), GFIII9-P[G]5-10 (□), or GST (▲) on doubling dilutions of each test protein (A and B). The percentage of cells spread on proteins coated at 100 μg ml\(^{-1}\) (shaded columns), and 6.25 μg ml\(^{-1}\) (white columns) is shown in C and D. The data shown represent the mean ± S.E. of at least four experiments.

Synergistic Activity of Fibronectin FIII9 and FIII10 Domains in Soluble Phase Assays—The capacity of purified wild type and mutant FIII domains to inhibit adhesion of BHK cells to fibronectin was also assessed in inhibition assays. The FIII domains were purified from GST adsorbed to glutathione-Sepharose beads by cleavage with thrombin and were tested for their capacity to inhibit attachment of BHK cells to immobilized FN coated on plastic at 5 μg ml\(^{-1}\). These data are shown in Fig. 8. Attachment of BHK cells to FN was completely abolished by the presence of 125 μM FIII9–10 in the culture. At a concentration of 125 μM, FIII9-PG-10 inhibited attachment of BHK cells to FN by 20%, and no significant inhibition was observed with FIII9-P[G]5-10 within the molar range used in these experiments. Relative to the controls, in which no protein was added to the cells prior to plating out, 50% inhibition of spreading of BHK cells on FN was achieved in the presence of 125 μM FIII9–10 and 125 μM FIII9-PG-10. No inhibition of cell spreading was observed with FIII9-P[G]5-10. At a concentration of 125 μM, FIII10 and GRGDS peptide both inhibited attachment of BHK cells to FN by 20 and 30%, respectively, and had a small effect on cell spreading. Thus insertion of two amino acids in the interdomain linker of FIII9–10 both immobilized and in solution had different effects on cell attachment and cell spreading.

Phosphorylation of FAK Is Strictly Dependent upon Structural Integrity of FIII9–10—Given that perturbation of the FIII9–10 had a profound effect on cell spreading, and given that integrin-mediated cell spreading appears to be preceded by FAK phosphorylation (11), we examined the relationship between cell spreading and FAK phosphorylation in response to adhesion to the wild type and mutant FIII9–10 proteins. The relative amounts of phosphorylated FAK in BHK cells plated
onto immobilized wild type or mutant GFIII9–10 domains were determined by immunoprecipitation of FAK and subsequent Western blotting with anti-phosphotyrosine antibodies as shown in Fig. 9A. The intensity of bands identified with anti-phosphotyrosine antibodies and representing phosphorylated FAK was expressed relative to total FAK as shown in Fig. 9B.

Introduction of the short interdomain linker in GFIII9-PG-10 reduced levels of FAK phosphorylation by one-third. The longer linker in GFIII9-P[G]5-10 led to a further reduction in FAK phosphorylation, giving levels approximately 30% of that obtained with cells plated onto GFIII9–10 and similar to that of cells plated on GFIII10.

For comparison of cell attachment, cell spreading and FAK phosphorylation in response to GFIII10 and the mutant FI11g9–10 proteins, the levels of activity obtained in each assay were expressed relative to those obtained for FI11g9–10 (Table I). Phosphorylation of FAK and cell spreading activities were tightly correlated and affected to the same degree by insertion of additional linker sequence, whereas levels of cell attachment were consistently higher.

**FIG. 8.** Inhibition of BHK cell adhesion by wild type and mutant FI11g9–10 domains. Attachment (A) and spreading (B) of BHK cells to FN (coated at 10 μg ml⁻¹) was assessed in the presence of doubling dilutions of FI11g10 (×), FI11g9–10 (○), FI11g9-PG-10 (■), FI11g9-P[G]5-10 (□), or GRGDS peptide (△).

**FIG. 9.** Phosphorylation of FAK in BHK cells on wild type and mutant FI11g9–10 fusion proteins. Total FAK and phosphorylated FAK (coprecipitated with anti-FAK antibodies) from BHK cells plated on plastic coated with 100 μg ml⁻¹ fusion proteins were detected by Western blotting (A). The percentage of phosphorylation of FAK on the mutant proteins relative to phosphorylation on GFIII9–10 (100%) was determined by densitometric analyses of the Western blots in A (B).

**DISCUSSION**

Previous studies have identified the FN peptide sequences RGD in FI11g10 and PHSRN in FI11g9 as integrin binding sites (32, 34) and have demonstrated the importance of structural integrity and contiguity of the FI11g9–10 pair for synergistic cell adhesive activity of the two domains (36). In this study we have further dissected the intra- and intermolecular interactions involved in the cell adhesion activity of the FI11g9–10 pair. We show that 1) precise spatial positioning of the two binding sites in FI11g9 and FI11g10 is critical for synergistic activity, and 2) downstream cell adhesion events of FAK phosphorylation and cell spreading are more sensitive than cell attachment to disruption of the spatial relationship of FI11g9 and FI11g10.

A number of experimental approaches have been used to dissect the cell adhesive activity of FN. The synergistic site containing PHSRN was recently identified in two important experiments (32, 34). In the case of αβ₃-mediated cell adhesion, Aota et al. (32) used hybrid constructs consisting of the eighth and tenth FI11g domains. In this experiment putative synergy regions from FI11g9 were swapped into the corresponding position in FI11g8 in the FI11g8–10 hybrid. The synergistic site was mapped in this way to the loop between the C' and E β strands in FI11g9. In a second study by Bowditch et al. (34) the
sequence DRVPHSRSNIT was identified as the candidate synergistic site for α1β1-mediated cell adhesion, based upon the ability of the peptide to inhibit binding of a 20-kDa fragment of FN containing FIII9 and FIII10 to the platelet-specific integrin αIIbβ3.

Akiyama et al. (35) recently reported that cell adhesion capacity of FN fragments derived from the CCBD is enhanced when the fragments are presented in solution by nonfunction blocking antibodies adsorbed onto plastic. In consideration of the possible steric and conformational effects of the presenting antibody molecule (the mass of which is up to one order of magnitude larger than the FN fragments we tested) on the adhesive properties of the FN fragments, we chose an alternative method in which the GST-FIII fusion proteins were adsorbed directly onto the plastic. A further consideration was that in situ, FN exists as one of many closely associated components of the ECM, and dimeric FN is presumably immobilized within the matrix, by virtue of its interaction with other ECM components, rather than being mobile above the surface. Ugarova et al. (43) reported that in solution only one of the two FIII10 cell binding sites in dimeric FN is accessible to a monoclonal antibody specific for that site, whereas adsorption of dimeric FN to polystyrene results in a conformational change in the molecule resulting in accessibility of both sites to the antibody, thus having possible implications for our interpretation of solid and soluble phase assays.

The structure of the proteins tested was also taken into account. Recombinant FIII9–10 fragments, in which the domain boundaries were strictly maintained as defined previously (27, 38), were used in this study. The strategy we adopted to investigate the molecular mechanisms involved in the adhesion of cells to FIII9–10 was thus chosen so that the nature of the synergistic interaction between FIII9 and FIII10 could be examined under conditions that mimic as closely as possible, the conditions as they exist within the native FN molecule in the ECM.

We dissected the molecular mechanisms involved in the adhesive activity of FIII9 and FIII10 by assaying cell attachment as an indicator of primary, integrin-ligand interaction, and FAK phosphorylation and cell spreading as indicators of downstream cell adhesion events, in response to wild type and mutant linker FIII9–10 proteins. The introduction of just two amino acid residues in the interdomain linker had little additional effect on cell attachment. Levels of FAK phosphorylation and cell spreading were, however, reduced even further. The data presented here show that in contrast to cell attachment, signaling via FAK phosphorylation and cell spreading are highly dependent upon the physical interaction between FIII9 and FIII10. Furthermore, the close correlation between FAK phosphorylation and cell spreading activities on the wild type and mutant proteins suggests that these events are tightly coupled. While FIII10 is sufficient for integrin binding, our data further demonstrate that the precise structural relationship between FIII9 and FIII10 must be maintained for facilitation of downstream cell adhesion events. The foregoing arguments suggest that there is a temporally resolvable sequence of FN-mediated adhesion events that can be tested experimentally.

There are a number of possible physical effects of the interdomain linkage that might account for reduced biological activity of the mutant linker FIII9–10 proteins. One reason could be that the mutant FIII protein structure may be incorrectly folded. Thermodynamic data (described in further detail in Spitzfaden et al. (45)) show that FIII10 has a significant stabilizing effect on FIII9. This effect is greatest in FIII9–10 and is progressively reduced as the interdomain linker is extended, but the change in ∆G° from FIII9–10 to FIII9-PG[II]10 is relatively small compared with the change from FIII9-PG[II]10 to free FIII9. Additionally, comparison of 1H, 15N NMR spectra shows that the polypeptide backbones of FIII9 and FIII10 in the wild type and mutant domain pairs are almost identical (45). These data indicate that the mutant FIII9–10 proteins have a three-dimensional, folded structure comparable with the wild type FIII9–10.

Another possible explanation for reduced biological activity of the mutants could be possible steric interference between the RGD-containing loop in FIII10 and another part of the molecule. Whereas the overall mobility of FIII10 increases in the mutant proteins compared with the wild type (T3 data (45)), there are no significant changes in the chemical shifts and proton-proton NOE effects of backbone amides and side chain α-carbons in the RGDs peptide (Fig. 3). Therefore, the RGD loop appears to form no new atomic contacts as FIII9 and FIII10 are separated, neither are existing contacts broken, which rules out the possibility of steric interference.

The most likely explanation for the strong influence of the linkers upon biological activity is that synergy of the integrin-biding sites within the FIII9–10 pair is absolutely dependent upon specific interdomain interactions and therefore maintenance of the relative orientation of the RGD and PHSRN peptides. The data we present here are consistent with previous reports (34, 44) that the RGD and PHSRN sites in FIII9 and FIII10 are not in physical contact. The importance of the FIII9–10 interdomain interface is surprising considering that in the crystal structure the interdomain area is only 333 Å2 (44) and relatively small in comparison with interface areas between other FN FIII domains. Furthermore, solution NMR measurements revealed that specific contacts between the two modules are scarce. Our data nevertheless show that synergistic activity not only requires a specific distance between RGD and PHSRN, but also precise spatial positioning of the two sites.

In the wild type FIII9–10 domain pair, the orientation of the integrin binding sites with respect to each other is dependent upon the twist and tilt angles between FIII9 and FIII10. These angles are maintained by specific interdomain interactions at the interface between the two domains and by the torsional restraints of the interdomain amino acid residues. Partial disruption of this interface results in random flexing and rotating of the two domains between active and inactive conformations, and this effect is amplified in the longer linker mutant. It is therefore possible that the incremental nature of the changes observed in cell adhesion on immobilized FIII9–10 wild type and mutant proteins is a result of stabilization of a subset of active conformations. However, our data showing lower activity

| Protein       | FAK phosphorylation | Cell spreading | Cell attachment |
|---------------|---------------------|---------------|-----------------|
| FIII9–10      | 1.00                | 1.00          | 1.00            |
| FIII9-PG[II]10| 0.67                | 0.65          | 0.73            |
| FIII9-PG[II]10| 0.29                | 0.29          | 0.65            |
| FIII910       | 0.14                | 0.10          | 0.55            |
Synergistic Activity of Fibronectin FIII9 and FIII10 Domains

of the mutant in inhibition assays is in agreement with Aota et al. (32) who found that substitution mutants of PHSRN also showed lower activity in inhibition assays than in direct adhesion assays. Our biophysical data clearly show that there is a significant breakaway of the specific interdomain contacts after the addition of two extra linker residues, resulting in increased mobility of the two domains relative to each other. The differences in biological activity of the mutant proteins in the direct adhesion and inhibition assays can be explained if, in the direct adhesion assays, the two integrin-binding peptides are fixed in the optimal relative orientation for binding the integrin, whereas in solution the mutants oscillate between optimal and suboptimal orientation. Thus it would be expected that immobilized fibronectin competes more effectively for the integrin receptor than does soluble protein. This is not surprising in view of the proposed effect on FN of adsorption to surfaces (43).

In conclusion, our findings demonstrate a requirement for the precise positioning of the RGD loop and the synergy site in FIII9–10 for cell adhesion and strongly suggest that the different synergistic regions of the CCBD have separable, and possibly sequential, functions in cell adhesion. These data have implications for our understanding of the inter- and intramolecular mechanisms involved in integrin-mediated adhesion and signaling.

REFERENCES
1. Hynes, R. O. (1990) *Fibronectins*, Springer-Verlag, New York
2. Kornblihtt, A. R., Vibe-Pedersen, K., and Baralle, F. E. (1984) *EMBO J.* 3, 221–226
3. Hynes, R. O., Schwarzbauer, J. E., and Tamkun, J. W. (1984) *CIBA Found. Symp.* 108, 75–92
4. Hynes, R. O. (1985) *Annu. Rev. Cell Biol.* 1, 67–90
5. Petersen, T. E., Thogersen, H. C., Skorstengaard, K., Vibe-Pedersen, K., Sottrup-Jensen, L., and Magnusson, S. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 137–141
6. Campbell, I. D., and Spitzfaden, C. (1994) *Structure* 2, 333–337
7. Obara, M., Kang, M. S., and Yamada, K. M. (1988) *Cell* 53, 649–657
8. Humphries, M. J., Akiyama, S. K., Komoriya, A., Olden, K., and Yamada, K. M. (1986) *J. Cell Biol.* 103, 2637–2647
9. Mould, A. P., Komoriya, A., Yamada, K. M., and Humphries, M. J. (1991) *J. Biol. Chem.* 266, 3579–3585
10. Hynes, R. O. (1992) *Cell* 69, 11–25
11. Loftus, J. C., Smith, J. W., and Ginsberg, M. H. (1994) *J. Biol. Chem.* 269, 25235–25238
12. Ruoslahti, E., and Pierschbacher, M. D. (1987) *Science* 238, 491–497
13. Pytelka, R., Pierschbacher, M. D., and Ruoslahti, E. (1985) *Cell* 40, 191–196
14. Takada, Y., Huang, C., and Hemler, M. E. (1987) *Nature* 326, 667–669
15. Vogel, B. E., Tarone, G., Giancotti, F. G., Gailit, J., and Ruoslahti, E. (1990) *J. Biol. Chem.* 265, 5934–5937
16. DeDhar, S., and Gray, V. (1990) *J. Cell Biol.* 110, 2145–2193
17. Smith, J. W., Vestal, D. J., Irwin, S. V., Burke, T. A., and Cheresh, D. A. (1990) *J. Biol. Chem.* 265, 11008–11013
18. Okk, M., Pytelka, R., and Sheppard, D. (1992) *J. Biol. Chem.* 267, 5789–5796
19. Weinacker, A., Chen, A., Agrez, M., Cone, R. I., Nishimura, S., Wayner, E., Pytelka, R., and Sheppard, D. (1994) *J. Biol. Chem.* 269, 6940–6948
20. Gardner, J. M., and Hynes, R. O. (1985) *Cell* 42, 439–448
21. Plow, E. F., McEver, R. P., Collier, B. S., Woods, V. L., Jr., Marguerie, G. A., and Ginsberg, M. H. (1985) *Blood* 66, 724–727
22. Burridge, K., Petic, L. A., and Romer, L. H. (1992) *Curr. Biol.* 2, 537–539
23. Burridge, K., Turner, C. E., and Romer, L. H. (1992) *J. Cell Biol.* 119, 893–903
24. Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1991) *Cell Regul.* 2, 951–964
25. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) *J. Biol. Chem.* 267, 23439–23442
26. Schaller, M. D., and Parsons, J. T. (1994) *Curr. Opin. Cell Biol.* 6, 705–710
27. Main, A. L., Harvey, T. S., Baron, M., Boyd, J., and Campbell, I. D. (1992) *Cell* 71, 671–678
28. Pierschbacher, M. D., and Ruoslahti, E. (1984) *Nature* 309, 30–33
29. Dufour, S., Duband, J. L., Humphries, M. J., Obara, M., Yamada, K. M., and Thiery, J. P. (1988) *EMBO J.* 7, 2661–2671
30. Nagai, T., Yamakawa, N., Aota, S., Yamada, S. S., Akiyama, S. K., Olden, K., and Yamada, K. M. (1991) *J. Cell Biol.* 114, 1285–1305
31. Aota, S., Nagai, T., and Yamada, K. M. (1991) *J. Biol. Chem.* 266, 15938–15943
32. Aota, S., Nomizu, M., and Yamada, K. M. (1994) *J. Biol. Chem.* 269, 24756–24761
33. Bowditch, R. D., Halloran, C. E., Aota, S., Obara, M., Plow, E. F., Yamada, K. M., and Ginsberg, M. H. (1991) *J. Biol. Chem.* 266, 23323–23328
34. Bowditch, R. D., Hariharman, M., Teminna, E. F., Smith, J. W., Yamada, K. M., Getzoff, E. D., and Ginsberg, M. H. (1994) *J. Biol. Chem.* 269, 10856–10863
35. Akiyama, S. K., Aota, S., and Yamada, K. M. (1995) *Cell Adhes. Commun.* 3, 13–25
36. Mardon, H. J., and Grant, K. E. (1994) *FEBS Lett.* 340, 197–201
37. Fenik, S. W., and Zaidelweg, E. R. P. (1988) *Magn. Reson.* 78, 588–593
38. Kornblihtt, A. R., Umezawa, K., Vibe-Pedersen, K., and Baralle, F. E. (1985) *EMBO J.* 4, 1755–1759
39. Fernandez-Shaw, S., Shorter, S. C., Naish, C. E., Barlow, D. H., and Starkey, P. M. (1992) *Hum. Reprod.* 7, 156–161
40. Kueng, W., Silber, E., and Eppenberger, U. (1989) *Anal. Biochem.* 182, 16–19
41. Laemmli, U. K. (1970) *Nature* 227, 680–685
42. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350–4354
43. Ugarova, T. P., Zamarin, C., Veklich, Y., Bowditch, R. D., Ginsberg, M. H., Weisel, J. W., and Plow, E. F. (1995) *Biochemistry* 34, 4457–4466
44. Leahy, D. J., Ikramuddin, A., and Erickson, H. P. (1996) *Cell* 84, 155–164
45. Spitzfaden, C., Grant, R. P., Mardon, H. J., and Campbell, I. D. (1997) *J. Mol. Biol.* 265, 565–579