Characterization of Regions of Fibronectin Besides the Arginine-Glycine-Aspartic Acid Sequence Required for Adhesive Function of the Cell-binding Domain Using Site-directed Mutagenesis

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Previous studies of adhesion mediated by the central cell-binding domain of fibronectin suggest that additional polypeptide information besides the Arg-Gly-Asp sequence is required for full activity. We analyzed this putative second, synergistic region of fibronectin more extensively by deletion analysis and oligonucleotide-based site-directed mutagenesis. Resulting mutated fusion proteins expressed using Agt1ll were assayed for baby hamster kidney fibroblast cell spreading activity. Deletion mutants truncating from the amino terminus showed a decrease of activity in two apparently discrete steps. Complementary studies using a series of overlapped internal deletions designed to retain the repetitive fibronectin structure also indicated that two distinct peptide regions besides the RGDS sequence were necessary for full activity. Removal of the carboxyl-terminal region resulted in the greatest loss of activity (≥20-versus 3-5-fold). Very similar results were obtained with HT-1080 cells dependent on the αβ3 integrin receptor for adhesion to fibronectin. An anti-fibronectin monoclonal antibody that inhibits cell adhesion was found to bind to the carboxyl-terminal functional region, and a point mutation caused specific loss of its epitope. These studies reveal unexpected complexity in the organization of these functional regions, which contrasts with adhesion models based only on simple, short peptide recognition sequences.

The interaction of cells with extracellular matrix proteins plays important roles in a variety of biological processes, such as embryonic development, wound healing, and metastasis. Among the many extracellular proteins identified as embryonic development, wound healing, and metastasis. The costs of publication of the article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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protein solutions were dialyzed against PBS at 4 °C.

Measurement of Adhesive Activity—The cell adhesive activity of each mutated protein was determined quantitatively by a cell-spread- ing assay. Wells of a 96-well plastic tissue culture plate were coated with the proteins to be assayed using a series of 2-fold dilutions. BHK cells were washed twice with Hanks’ balanced salt solution, detached by brief treatment with 0.05% trypsin, 0.53 mM EDTA, then allowed to recover from proteolysis for 20 min in regular culture medium at 37 °C. The cells were washed twice with PBS and placed in the coated wells at 5 × 10³ cells/well. After 45 min of incubation at 37 °C, the cells were fixed with 3% formaldehyde, 3% glutaraldehyde, and the percentage of BHK fibroblasts that attached firmly and spread was determined at each dilution by phase contrast microscopy. The protein concentration required to produce 50% BHK fibroblast cell spreading could be determined reproducibly from dose-response curves based on multiple concentration points for each fusion protein. Human HT-1080 fibrosarcoma cells were assayed similarly, except that the cells were detached using 0.25% trypsin and allowed to spread in serum-free Dulbecco’s modified Eagle’s medium for 45 min at 37 °C.

Monoclonal Antibody Binding Studies—Rat monoclonal antibodies 3BS, 11E5, 12B4, 13G12, and 16G3 directed against the 37-kDa FN cell-binding domain2 were purified from serum-free hybridoma media as described (7). Binding to fusion proteins was determined by en- zyme-linked immunosorbent assay in 96-well microtiter plates. Pu- rified fusion proteins in PBS (75 µl at 10 µg/ml) were incubated in microwells for 1 h. After blocking with 2 mg/ml bovine serum albumin in PBS and rinsing, wells were incubated with 75 µl of purified anti- FN monoclonal antibody in 0.5% Tween-20 in PBS for 2 h at room temperature, washed, then incubated with horseradish peroxidase-conjugated affinity purified anti-rat IgG (ICN) at a dilution of 1:500 in Tween/PBS for 1 h. After washing, wells were incubated with peroxidase substrate consisting of 2 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 2.5 mM H₂O₂ in 0.1 M sodium acetate, 0.05 M NaH₂PO₄, 0.01% thimerosal, and absorbances of the reaction product were measured at 405 nm.

RESULTS

Detailed Mapping by 5’ Deletion—In our previous study, several lengthy deletions from the 5’ end of FN cDNA caused losses of activity of the corresponding expressed fusion pro- teins (13). This result was consistent with those obtained from the study of proteolytic fragments, and we postulated the existence of a “synergistic” second site required for the full cell binding and cell spreading activity of fibronectin. We utilized two complementary approaches to obtain a more definitive functional map in this paper. First, a series of six additional deletions from the 5’ end of FN cDNA was con- structed by the use of restriction enzyme recognition sites as described under “Materials and Methods.” Fig. 1 summarizes these truncation mutants.

Fig. 2 shows examples of dose-response curves for several of these constructs. The protein concentrations required for 50% BHK cell spreading as determined from these types of curves are shown in Fig. 3. Clones Apal, C6, SacI, and PvuII showed high activities, which were comparable to those of intact fibronectin on a molar basis. The three shortest clones displayed approximately 100-fold losses of activity, consistent with previous results. This newer fine structure mapping revealed, however, three clones of intermediate length that showed the same intermediate levels of activity 8–10-fold lower than the fully active clones; as a result, cell spreading activity decreased in at least two discrete steps (Fig. 3). This result was confirmed in multiple assays, and it suggests that this second, synergistic region actually consists of two distinct subregions.

Mapping by Internal Deletion—In the previous section, the deleted cDNA fragments were fused to the lacZ gene. In order to avoid potential steric effects accompanying the progressive narrowing of the distance between the lacZ protein portion and the GRGDS site, a series of internally deleted mutants designed to retain the type III repeated motif of FN were constructed (Fig. 4). The corresponding fusion proteins expressed in E. coli were assayed for BHK cell spreading activity, and the estimated protein concentrations required for 50% cell spreading are also shown in Fig. 4.

This experiment indicated the presence of two distinct polypeptide subregions besides the GRGDS sequence that were required for full biological activity; the amino-terminal boundaries of the sites where activity dropped as determined by assays of a series of internal deletions are indicated by the gray boxes labeled A and B in Fig. 4. Deletions of the A region resulted in a 3–5-fold loss of activity. Deletions between the


FIG. 1. Construction of 5’-terminal deleted mutants. A schematic diagram of the domain structure of FN is shown at the top, along with previously characterized proteolytic fragments of 75, 37, and 11.5 kDa. A cDNA fragment representing the cell-binding domain of FN was deleted progressively from the 5’ terminus and cloned into Agt11. The type III repeating homology units of FN are indicated as numbered boxes. M57 is the original undeleted cDNA clone, and other clones are designated by the restriction enzyme used to generate deletions. Clones starting with C were constructed previously (13), and the remaining six clones were constructed and expressed as described under “Materials and Methods.”

FIG. 2. Dose-dependent activity curves for the deletion clonesSac, C67, C18, and C1. Dose-dependent activity curves for some of the 5’-terminal deletion clones are shown. Sac (V) showed activities comparable to that of intact human plasma FN (O). Although C67 (□) and C18 (●) were much less active than Sac, they had higher activities than C1 (■), which had activity comparable to that of the 11.5-kDa fragment fusion protein.
A and B regions showed a partial recovery of activity (Figs. 4 and 5). Representative dose dependence curves for fusion proteins with deletion mutations in the B region are shown in Fig. 5. The three clones with internal deletions more toward the amino terminus of the B zone (98, 97, and 107) showed partial losses of activity of 5-8-fold (Figs. 4 and 5). Clones with internal deletions more toward the carboxyl terminus showed ≥20-fold losses of activity (Figs. 4 and 5).

It was not possible to identify which receptor(s) were involved in the recognition of these subregions using the standard BHK cell assays due to the absence of cross-reacting inhibitory anti-receptor antibodies. Instead, human HT-1080 cells were examined. Adhesion of these cells to FN was inhibited by both anti-α5 and anti-β1 monoclonal antibodies (Fig. 6B), indicating that the classical α5β1 FN receptor was crucial for cell adhesion and spreading. As shown in Fig. 6A, the pattern of cell spreading activity on the fusion proteins described above was quite similar to that seen for BHK cells, although the effect of removing the B region was even larger.

HT-1080 cell spreading was blocked by anti-α5P1 antibodies, both on substrates of FN and of deletion mutants; in contrast, anti-vitronectin receptor antibodies were not inhibitory (Fig. 6B).

### Precise Localization of the Binding Site of a Key Monoclonal Antibody against the Cell-binding Domain of FN

Some recently generated monoclonal antibodies against the cell-binding domain of FN have been roughly mapped near the synergistic region, and one of them, termed mAb 3B8, was found to be inhibitory to cell spreading on FN. Five amino acid residue substitutions (Fig. 7) were introduced into this region (subregion B in Fig. 4) for epitope localization of these monoclonal antibodies. None of these point substitutions resulted in any detectable loss of cell spreading activity, suggesting the absence of any major conformational changes due to the substitutions (data not shown). The amino acid substitution S2, in which an Arg residue was replaced by an Asp, abolished the binding of mAb 3B8 (Table I). Another amino acid substitution, S3, caused a partial loss of binding of mAb 3B8, and a third substitution, S5, reduced it slightly (Table I). In contrast, two other point mutations, S1 and S4, had no effects on the binding of mAb 3B8. All five amino acid substitutions had no effect on the binding of other monoclonals against the central cell-binding domain of FN (Table I). These results

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**Fig. 3.** Stepwise loss of adhesive activity after 5'-terminal deletion of the FN cell-binding domain. The protein concentrations required for 50% BHK fibroblast cell spreading were determined for the series of 5'-terminal deletion mutants described in Fig. 1. The values are plotted as a function of the 5'-terminal deletion points. Note that the Arg-Gly-Asp site is significantly beyond the right end of the graph, situated at 4490-4498 bp in this numbering system.

**Fig. 4.** Internal deletion mutants and their biological activities. A series of overlapped internal deletions designed to retain the type III repeating motif of FN were introduced into the cell-binding domain of FN by oligonucleotide-based site-directed mutagenesis. The protein concentrations required to produce 50% BHK cell spreading were determined from dose-response curves for each fusion protein and are presented in the right column. Two subregions were found to be important for the full cell-binding activity of FN as shown by the gray boxes labeled A and B. Removal of the A region caused about 3-5-fold loss of activity (three to five times increased protein concentration required for 50% cell spreading). Note that removal of the B subregion resulted in a considerably greater loss of activity (20-fold or more). It should be noted that the carboxyl-terminal boundary of B region has not been determined yet, although the total region is probably small (see “Discussion”). Similar results were obtained with independent preparations of fusion proteins.
yet been determined precisely, this subregion appears to be mutagenesis.

The majority of the activity of this region, since its deletion due in the type I11 repetitive motif of the protein. Although the carboxyl-terminal boundary of the B subregion has not activity was assayed at multiple concentrations of fusion proteins as described under “Materials and Methods.” Dose dependence curves for BHK cell spreading on human plasma FN (O), 58 (●), 83 (▼), 98 (□), and 106 (▲) are shown.

Further support the importance of this second, synergistic site, and they map it to the identical region identified by deletion mutagenesis.

**DISCUSSION**

We have used oligonucleotide-mediated mutagenesis to characterize the organization of a putative second, synergistic region of FN distinct from the RGD site that is required for full adhesive activity. Although previous work implied the presence of a single region more than 20,000 daltons of protein away from the RGD site (13), our present results indicate that this region is complex and actually consists of at least two distinct, separated subregions needed for activity.

One of these subregions maps a similar 28,000 dalton distance away from the RGD site, and its deletion results in approximately 3–5-fold losses of activity (site A in Fig. 4). In comparison, 5’ truncation mutants in this region reproducibly showed slightly greater losses of activity (8–10-fold), suggesting that the additional 5’ sequences present in the internal deletion mutants may contribute additional weak activity.

A second subregion (site B in Fig. 4), however, accounts for the majority of the activity of this region, since its deletion results in greater than 20-fold losses in activity. The latter B subregion starts approximately 14,000 daltons of protein away from the RGD site; the apparent step-like loss of B region activity suggests additional complexity that needs future elucidation. The amino-terminal boundary of this subregion according to deletion analysis is consistent with the truncation analysis except for truncation clone ApaLI, which shows low rather than intermediate activity. This mapping discrepancy (the size of which is small in comparison with the distances between A, B, and Arg-Gly-Asp regions) might be the result of steric interference with receptor binding and subsequent adhesion due to the adjacent β-galactosidase portion of the fusion protein, especially since the junction occurs immediately adjacent to a highly conserved tryptophan residue in the type III repetitive motif of the protein. Although the carboxyl-terminal boundary of the B subregion has not yet been determined precisely, this subregion appears to be relatively small, since two noninhibitory monoclonal antibodies were found to bind between this site and the RGD sequence.3

**FIG. 5.** Dose-dependence curves for BHK cell spreading mediated by FN and clones 58, 83, 98, and 106. Cell spreading activity was assayed at multiple concentrations of fusion proteins as described under “Materials and Methods.” Dose dependence curves for BHK cell spreading on human plasma FN (O), 58 (●), 83 (▼), 98 (□), and 106 (▲) are shown.

**FIG. 6.** Dose dependence curves for cell spreading of human HT-1080 cells mediated by FN and fusion proteins and inhibitory effects of anti-integrin antibodies. Cell spreading assays were performed using human HT-1080 fibrosarcoma cells as described under “Materials and Methods.” Panel A, dose dependence curves on human plasma FN (O), amino acid residue substitution clone S3 (▼), and deletion clones 83 (●), 58 (⊙), 97 (●), 98 (□), 107 (●), 106 (▲). Panel B, inhibitory effect of anti-integrin antibodies. Cell spreading assays were performed in untreated controls (a) or in the presence of anti-vitronectin receptor polyclonal antibody (b), mAb 13 anti-β1 monoclonal antibody (c), or mAb 16 anti-α5 monoclonal antibody (d); the anti-vitronectin receptor antibody was from Telios Pharmaceuticals (San Diego, CA), and the monoclonal antibodies are described in Ref. 25. The concentrations of coated proteins were 5 µg/ml for FN, 58, and 83, and 40 µg/ml for 98, 97, and 106.

**FIG. 7.** Amino acid residue substitutions introduced into the synergistic region. Five amino acid residue substitutions were introduced between the 1373rd and 1380th residues of the PIR protein sequence databank entry FNHU. The amino acid substitutions were either conservative or represented the corresponding residues in the adjacent 8th type III repeat. For comparison, the carboxyl-terminal boundaries of the internal deletions shown in Figs. 4 and 5 are indicated by the arrows and the clone names. The binding of mAb 3B8 was abolished in mutation S2 and substantially decreased in S3 (Table I).

Experiments using human HT-1080 cells indicate that cell spreading that occurs on FN and mutant fusion proteins is dependent on the α5β1 FN receptor and that the removal of synergistic subregions decrease α5β1 FN receptor-dependent cell spreading. These results suggest that the synergy subre-
Table I

Mapping of the binding site of a monoclonal antibody directed against the FN synergy region

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Monoclonal Antibodies</th>
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<tr>
<td></td>
<td>3B6</td>
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<td></td>
<td>13G12</td>
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<td></td>
<td>12B4</td>
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<td>11E5</td>
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<td></td>
<td>16G3</td>
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<tr>
<td>S5</td>
<td>69 ± 9</td>
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<td>S4</td>
<td>88 ± 4</td>
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<tr>
<td>S3</td>
<td>59 ± 7</td>
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<tr>
<td>S2</td>
<td>0.3 ± 0.8</td>
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<tr>
<td>S1</td>
<td>90 ± 8</td>
</tr>
<tr>
<td>83</td>
<td>90 ± 4</td>
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<tr>
<td>106</td>
<td>−0.2 ± 2.2</td>
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Substitution of either of two single amino acid residues (S2 or S3) was sufficient to abolish or to weaken an epitope for the 3B6 monoclonal antibody; this antibody is thought to block function of this second, synergy site of fibronectin. The binding of other monoclonal antibodies was not affected by these point mutations, including even the binding of an antibody to an adjacent epitope located between this putative second site and the RGD site. These results precisely map the epitope of the interesting 3B6 monoclonal, and they further support the apparent importance of this particular region (subregion B in Fig. 4) in fibronectin adhesive function. None of the five point mutations tested in this region resulted in a loss of biological activity. This result indicates that at least some parts of the site involved in antibody binding are not additionally part of a critical receptor interaction site; it is not unusual for immunoglobulin molecules to block functions of polypeptide regions very close to, but not identical to, the exact antibody-binding residues in a protein. Further mutational analysis may define the key residues needed for biological function. In particular, it is not clear whether function of the synergistic subregions depend on short, essential peptide sequences or on longer stretches of polypeptide in particular conformation(s); in this regard, the finding of apparent step-like losses of B region activity in the internal deletion study (Fig. 4) may help answer this question.

During the final preparation of this paper, a report directly relevant to our studies was published by Kimizuka and coworkers (15). These authors also used site-directed mutagenesis to investigate the role of FN type III homology repeats in function of the cell-binding domain. Their final conclusions, however, differ substantially from ours: they interpreted their findings to suggest that “a second site is not present within the cell-binding domain” and that “the number and kinds of type III repeats and their correct alignment rather than the putative synergistic site decide the extent of the specific cell adhesive activity” (15). Nevertheless, careful examination of that paper shows significant areas of agreement, e.g. that deletion of the 9th type III repeat causes a 25-fold loss of activity (compared with our more than 20-fold loss), whereas deletion of earlier repeats had much less effect.

The major differences are as follows. (a) Kimizuka et al. (15) report that by far the largest losses of activity occurred with simple truncation of the sequence, and conversely that “cell adhesive activity increased as type III repeats were added to the NH2 terminus.” For example, their clone C-279, while still containing the 8th, 9th, and 10th type III repeats, showed more than a 10-fold loss of activity. In contrast, as shown in Fig. 2, our similar clone, San, showed an activity comparable to FN (less than 2-fold difference in activity from the original full-length clone, M57). We suggest that this and other discrepancies seen in the Kimizuka et al. study may be explained by their assay method requiring the adsorption of progressively shorter polypeptides to plastic substrates. Adsorption of small polypeptides to plastic can substantially suppress activity: previous studies showed that the CS1 region of fibronectin lacked activity if adsorbed to plastic (16), yet had high activity if conjugated to a carrier protein or used as a competitive inhibitor (17). In an earlier study using proteolytic fragments of the cell-binding domain of FN, the loss of estimated binding affinity of an 11.5-kDa fragment lacking the synergistic region but containing the RGDS sequence was independently established as being 10–100-fold as indicated by competitive inhibition assays (10) (for which Kimizuka et al. report >1000-fold losses of activity compared to FN). In fact, even short RGD peptides show a maximum of 100–200-fold losses of activity compared with intact FN (10, 11). Moreover, Pierschbacher et al. (18) were able to demonstrate cell spreading activity for the 11.5-kDa fragment, but they noted problems in accessibility to an anti-functional antibody when it was adsorbed to plastic. The presence of the large β-d-galactosidase “carrier” in all of our constructs may allow uniform adsorption to the substrate, avoiding possible conformational destabilization and denaturation of shorter constructs, although this hypothesis remains to be proven. The major discrepancy in overall conclusions of the two studies can be attributed to these differences in the quantification of effects of truncation on activity, since if this effect is excluded, other findings such as the 25-fold loss of activity by an internal deletion in the region of the 9th type III repeat they report would be consistent with our results, in which we were able to map the start of this major putative synergistic subregion to the center of the 9th repeat.

(b) Kimizuka et al. (15) also describe the interesting idea that losses of activity upon deletion of a single type III unit are inversely proportional to the distance of a type III deletion from the RGDS site. Our more extensive series of deletions within a shorter overall span may not fully agree with this conclusion, since the 5' truncations appeared to show a clear two-step transition, and since the A and B subregions could
be identified. Nevertheless, the loss of the B region did cause more of an effect than that of A. The discrepancy might be explained by the fact that we examined smaller increments, and were thus able to detect a more complex pattern.

(c) Kimizuka et al. (15) suggest that the greater activity attributable to the 9th type III repeat (which contains our B subregion) may be due to its higher content of basic amino acids, possibly interacting with non-integrin target(s) such as heparan sulfate proteoglycan on the cell surface. Although such an interaction is possible, it may not be important, since there is no inhibition of cell spreading by even high concentrations of heparin or heparan sulfate (10), and this study demonstrates that half of the 9th repeat can be successfully replaced by sequences from the 8th repeat.

d) Finally, the finding that moving the 9th type III repeat further away from the RGD sequence causes decreased activity (15) can also be interpreted as indicating that the spacing between the putative B synergistic subregion and the RGD sequence is important.

Besides the complex organization of the synergy region for the RGD site described in this paper, other studies have described additional sources of complexity in the interactions of certain cells with fibronectin. Heparin-binding domains of FN are required by certain cells for fully efficient formation of focal contacts with the substrate using the central cell-binding domain of FN. This process may involve a signaling process, since the amounts of these domains needed can be quite low (19, 20). Neurite extension can be influenced by polypeptide sequences associated with RGD in complex patterns that remain to be elucidated (21, 22). Neural crest derivatives such as melanoma cells can adhere and spread by using RGD-independent polypeptide sequences from the IIICS (V) region or from the high affinity heparin-binding domain of fibronectin (17, 23–24). Further characterizations and comparisons of these regions with the activity of the synergy region analyzed in detail in the present study should help to elucidate the unexpectedly complex mechanisms of cell adhesion.

REFERENCES