Monoclonal Antibody Characterization of Two Distant Sites Required for Function of the Central Cell-binding Domain of Fibronectin in Cell Adhesion, Cell Migration, and Matrix Assembly

Toshihiko Nagai,*§ Naomi Yamakawa,*† Shin-ichi Aota,‡ Susan S. Yamada,† Steven K. Akiyama,*§ Kenneth Olden,*† and Kenneth M. Yamada*

*Howard University Cancer Center, Washington DC 20060; †Laboratory of Developmental Biology, National Institute of Dental Research, National Institutes of Health; and §Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract. Site-directed mutagenesis studies have suggested that additional peptide information in the central cell-binding domain of fibronectin besides the minimal Arg-Gly-Asp (RGD) sequence is required for its full adhesive activity. The nature of this second, synergistic site was analyzed further by protein chemical and immunological approaches using biological assays for adhesion, migration, and matrix assembly. Fragments derived from the cell-binding domain were coupled covalently to plates, and their specific molar activities in mediating BHK cell spreading were compared with that of intact fibronectin. A 37-kD fragment purified from chymotryptic digests of human plasma fibronectin had essentially the same specific molar activity as intact fibronectin. In contrast, other fragments such as an 11.5-kD fragment lacking NH₂-terminal sequences of the 37-kD fragment had only poor spreading activity on a molar basis. Furthermore, in competitive inhibition assays of fibronectin-mediated cell spreading, the 37-kD fragment was ∼325-fold more active than the GRGDS synthetic peptide on a molar basis. mAbs were produced using the 37-kD protein as an immunogen and their epitopes were characterized. Two separate mAbs, one binding close to the RGD site and the other to a site ∼15 kD distant from the RGD site, individually inhibited BHK cell spreading on fibronectin by >90%. In contrast, an antibody that bound between these two sites had minimal inhibitory activity. The antibodies found to be inhibitory in cell spreading assays for BHK cells also inhibited both fibronectin-mediated cell spreading and migration of human HT-1080 cells, functions which were also dependent on function of the α5β1 integrin (fibronectin receptor). Assembly of endogenously synthesized fibronectin into an extracellular matrix was not significantly inhibited by most of the anti-37-kD mAbs, but was strongly inhibited only by the antibodies binding close to the RGD site or the putative synergy site. These results indicate that a second site distant from the RGD site on fibronectin is crucial for its full biological activity in diverse functions dependent on the α5β1 fibronectin receptor. This site is mapped by mAbs closer to the RGD site than previously expected.

Cellular adhesion to specific extracellular matrix components is required for a variety of biological events including cell proliferation, embryonic development, wound healing, and disease pathogenesis. The adhesive glycoprotein fibronectin has been an especially important model system for deriving insights into the mechanisms of cell adhesion and migration (8, 9, 23, 36). Fibronectin is a multifunctional glycoprotein with multiple binding activities including those for heparin, collagen, fibrin, and cell surface receptors. These binding activities are contained in distinct, protease-resistant domains. The best characterized cell-binding domain of fibronectin occupies its central region. This central cell-binding domain of fibronectin mediates cell adhesion with a wide variety of cell types (1, 45, 46) through a (Gly)-Arg-Gly-Asp-(Ser) sequence. For example, synthetic peptides containing this sequence block fibronectin-mediated adhesion and binding to cell surface receptors (18, 39, 40, 54). Moreover, direct deletion of the Arg-Gly-Asp (RGD) sequence by site-directed mutagenesis causes a loss of most cell adhesion activity (38).

Although the Arg-Gly-Asp sequence is clearly of great importance as a cell adhesive signal, several major questions remain. In vitro mutagenesis and analysis of bacterially expressed fibronectin fusion proteins indicates that a second

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major region of the protein is required for adhesive function (4, 38). Studies comparing the biological activities of large and small fibronectin fusion fragments and synthetic peptides derived from fibronectin also indicate that most biological activity is lost when short fibronectin fragments and peptides are assayed even when the RGD sequence is retained (2, 49). Furthermore, the RGD sequence has been found in several adhesive proteins that have distinct functions and specificities and is also found in nonadhesive proteins (39, 45, 52). Consequently, further dissection of the molecular mechanisms of action of fibronectin is required to elucidate how a single molecule can mediate different types of adhesion in different cell types, how the widely distributed RGD peptide sequence can be used as part of specific receptor recognition systems, and how specific sequences in the molecule are used in the more complex processes of cell migration and organization of fibronectin into a three-dimensional fibrillar extracellular matrix (4, 8, 23, 33, 36, 53).

Our original finding of a putative synergistic site (38) that might provide binding affinity and specificity to the fibronectin RGD site has been clouded by several major uncertainties. Because the synergy site was identified using only amino-terminal deletion analysis, the final fusion proteins might have had an incorrect conformation in the region of the RGD recognition site due to the lack of a correct amino-terminal sequence, thus, artifactually producing losses of activity (see also ref. 27). Furthermore, no information was available on which fibronectin receptor(s) could interact with the synergy site and which types of processes involving fibronectin require this "synergy" region, e.g., whether more complex functions such as cell migration or fibronectin matrix assembly require this site. Since the publication of the work of Obara et al. (38), it has been reported that matrix assembly may require both the α5β1 fibronectin receptor and another, unidentified β, integrin (13).

A relatively direct, independent approach to answering these questions is the development of specific mAbs that can inhibit fibronectin function. If they can be mapped to different points along the molecule, a series of inhibitory and noninhibitory mAbs can provide insights into which sites are important for function in the form of an independent test of the existence of a second synergy site. They can also provide tests of the role of this site in different biological processes.

In this study, we have characterized a "synergy" region in the central cell-binding domain that is distinct from the RGD site. Our approach has been to isolate and characterize the shortest fully active fragment of fibronectin generated by proteolysis. This protein fragment was then used as an immunogen to obtain mAbs clustered along this key region of fibronectin. These mAbs have then been used in the immunological mapping of sites in fibronectin that are critical for cell spreading and migration, as well as for assembly of a fibrillar fibronectin matrix.

**Materials and Methods**

**Purification of Fibronectin and Fragments**

Fibronectin was purified from fresh-frozen citrated human plasma (National Institutes of Health Blood Bank, Bethesda, MD) by gelatin-Sepharose affinity chromatography and citric acid elution (35). The 37-kD fragment was generated by digesting 130 mg fibronectin in 90 ml 0.15 M NaCl, 10 mM Tris-HCl, 0.02% sodium azide, pH 7.4, at 37°C for 20 h with 1.3 mg chymotrypsin (Worthington Biochemicals, Freehold, NJ). The digestion was terminated by adding PMSE to a final concentration of 1 mM. After dialysis against 50 mM Tris-HCl, pH 7.4, the crude digest was applied to a 27-ml bed volume gelatin-Sepharose affinity column. The flow-through fractions were immediately applied to a 44-ml bed volume heparin-Sepharose affinity column. Both affinity columns had been pre-equilibrated with 50 mM Tris-HCl, pH 7.4. The flow-through fractions from the heparin-Sepharose column were pooled and dialyzed against 3 mM sodium phosphate, 0.02% sodium azide, pH 5.8, and applied to a preparative Mitsubishi Toaitsu HCA hydroxyapatite HPLC column (Rainin, Woburn, MA). The column was eluted with a linear gradient of 3 to 120 mM sodium phosphate, pH 5.8, using an FPLC system (Pharmacia LKB Biotechnology, Piscataway, NJ). The material eluting between 95 and 115 mM sodium phosphate was pooled, dialyzed against 1 mM sodium phosphate, pH 6.8, and concentrated by applying to a 2-ml Bio-gel HTP hydroxyapatite column (BioRad Laboratories, Richmond, CA), eluting with 200 mM sodium phosphate, pH 6.8. The eluted material was further concentrated using a Centricon-10 microconcentrator (Amicon Corp., Danvers, MA), and applied to a Superose 12 column (Pharmacia LKB) and eluted with PBS+. Column fractions containing protein as judged by absorbance at 280 nm were analyzed using gel electrophoresis and Western blotting using mAb 333, which binds to fibronectin near the RGD site (2).

The 11.5-kD cell-binding fibronectin fragment was prepared as described using a mAb 333 affinity column (2). Gly-Arg-Gly-Asp-Ser (GRGDS) synthetic peptide was custom synthesized by Immuno-Dynamics, Inc. (La Jolla, CA) and was further purified by reversed phase HPLC and Dowex chromatography before use.

**NH2-terminal and COOH-terminal Amino Acid Sequencing**

Purified 37-kD fragment was precipitated with ethanol, washed several times with absolute ethanol, dried by SpeedVac (Savant Instruments, Inc., Hicksville, NY), redissolved in 50% acetic acid, and analyzed by automated gas phase sequencing (Applied Biosystems, Foster City, CA). This amino acid sequence analysis was performed in the Protein Analysis Laboratory of the Cancer Center of Wake Forest University supported in part by NIH grants CA-12197, RR-04869, and a grant from the North Carolina Biotechnology Center. To identify COOH-terminal amino acids, 1.5 nmol of purified 37-kD fragment in 100 μl 0.1 M pyridine-acetate-collidine buffer, pH 8.3, was digested by carboxypeptidase A (type 1-DFP; Sigma Chemical Co., St. Louis, MO) for 20 h at 37°C at a 5:1 molar ratio. The reaction was terminated with 50 μl acetic acid, and the solution was dried by SpeedVac and resolubilized in 100 μl 0.2 M citric acid (PH 2.2). Liberated amino acids were analyzed by HPLC using an AA-Pak column (Japan Spectroscopic Co., Ltd., Tokyo) with post-column o-phthalaldehydehydri- dation (with the help of Dr. Masafumi Kamada, Department of Biochemistry, Science University of Tokyo), and quantitated using external amino acid standards. Controls were carboxypeptidase incubation without sample, and substitution of met-enkephalin (Tyr-Gly-Gly-Phe-Met) as a positive control.

**Biological Assays**

Cell spreading assays were performed in 96-well microtiter plates using fibroblastic BHK cells as described previously (54). Substrates were prepared either by coating tissue culture plates (Costar Laboratories) with solutions of fibronectin or fragments as described (45) or by covalently coupling fibronectin or its fragments to CovAlink plates (Nunc, Naperville, IL). To prepare covalently coupled substrates, triplicate CovAlink wells were treated with 100 μl of 1 mg/ml bis(sulfosuccinimidyl) suberate cross-linking reagent (BS3; 1 Pierce Chemical Co., Rockford, IL) in PBS+ for 30 min at 4°C. After washing, the wells were incubated with 100 μl of various concentrations of fibronectin or fragments for 1 h at room temperature. Background spreading was minimized by first incubating the wells with 0.15 M glycine for 30 min, followed by a 30-min incubation with 1% heat-denatured BSA, prepared as described (54). The plates were extensively washed with PBS+ before use. One well of each triplicate set was used to assay cell spreading activity. The remaining two wells of each set were used to quantitate amounts of each protein covalently coupled in the wells by ELISA using 5 μg/ml mAb 333 as the first antibody in PBS+ supplemented with 0.15 M glycine and 0.1% BSA.

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1. Abbreviation used in this paper: BS3, bis(sulfosuccinimidyl) suberate.
Cell migration assays were performed using the procedure described previously (3, 50). Fibronectin matrix assay essays using WI-38 human embryonic lung fibroblasts were carried out as described (3). Fibronectin matrix assays were performed with fibronectin antibody. Fibronectin antibodies were photographed using identical exposure times for all treatment conditions with Kodak Tri-X film in a Zeiss Photomicroscope III using a 63×, 1.4 NA objective. Matrix assembly was quantitated by counting the number of fibrils observed to be crossing a straight line of arbitrary fixed length (100 µm on each field) drawn across randomly selected microscope fields.

ELISAs were carried out by incubating protein coated wells of either Immunolon-1, Costar, or Covadip microtiter plates with 100 µl of either hybridoma-conditioned medium or purified anti-fibronectin mAbs in 0.5% Tween-20 in PBS (incubation buffer) for 2 h at room temperature followed by washing with incubation buffer. In experiments where Immunolon-1 or Costar plates were used, HRP-conjugated, affinity purified anti-rat IgG (ICN Immunobiologicals, Costa Mesa, CA) at a 1:200 dilution in incubation buffer was used as the secondary antibody. After washing, peroxidase substrate in the form of 2 mM 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2.5 mM H2O2 in 0.1 M sodium acetate, 0.05 M NaH2PO4, 0.01% thimerosal was added and reaction product was quantitated at 405 nm. When Covadip plates were used, the secondary antibody was biotin-labeled anti-rat IgG (ICN Immunobiologicals) diluted 1/300 in incubation buffer. The wells were washed again with incubation buffer and 100 µl of a 1/200 dilution of HRP-conjugated avidin (ICN Immunobiologicals) was added for 1 h, followed by further washing and addition of peroxidase substrate.

Production and Characterization of mAbs against the 37-kD Fragment

Hybridomas secreting anti-37-kD fragment mAbs were produced by the fusion of Y3 rat myeloma cells with spleen cells from a Sprague-Dawley rat as described (15). The rat had been previously immunized with highly purified 37-kD fragment by intraperitoneal injection with complete Freund's adjuvant and boosted with the 37-kD fragment in incomplete Freund's adjuvant. Positive clones were identified by ELISA using 100 µl hybridoma conditioned medium as the first antibody. ELISA substrates were prepared by incubating 5 µg/ml 37-kD fragment with 96-well Costar tissue culture clusters and then blocking with 0.1% crystalline BSA. The positive hybridomas were subcloned twice by limiting dilution and isoyyped using a Rat MonoAb-Id/SP kit (Zymed Laboratories, Inc., San Francisco, CA). For the large-scale production of antibodies, the hybridomas were cultured in serum-free medium supplemented with BSA and ITS Premix (Collaborative Research, Bedford, MA) as described (2). mAbs were purified from hybridoma-conditioned serum-free medium by protein G affinity chromatography (Pierce Chemical Co.). All hybridomas selected for further experimentation were subcloned at least twice by limiting dilution.

Epitope mapping of mAbs was carried out by ELISA using fusion proteins expressed by the xgt11 system in E. coli based on recombinant DNA mutagenesis of fibronectin (38; footnote 2). After affinity purification using mAb 333-Sepharose columns (38), the fusion proteins or fibronectin fragments were coated onto Immunolon-1 plates (Dynatech Laboratories, Inc., Chantilly, VA) at 5-10 µg/ml in PBS+ for 1 h at room temperature, then subjected to ELISA using purified IgG at 50 µg/ml or, in some cases, hybridoma-conditioned medium containing 40 to 50 µg/ml IgG. mAb binding competition assays were carried out as described above for the standard ELISAs using fibronectin-coated Costar 96-well plates. The wells were precultivated with one of each of the unlabeled mAbs for 1 h. Then mAbs that had been directly labeled with HRP were added for 1 h. After extensive washing, binding of labeled mAb was quantitated as described for the standard ELISA. Purified mAbs were labeled directly with HRP using preactivated peroxidase in the form of ActiZyme-HRP (Zymed Laboratories, Inc.) according to the manufacturer's instructions. Affinity constants of mAb binding to fibronectin were determined by radioimmunoassay using the procedure of Franke. (14).

Other Methods

Protein concentrations were determined by the BCA protein assay (Pierce Chemical Co.) using BSA as a standard. SDS-PAGE was performed as described (29), using a 4% stacking gel and 10% resolving gel. Proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Keene NH) as described (7). The protein bands were localized either by staining with 2% Amido Black 10B or with mAb 333. Blots used for mAb detection of fibronectin fragments were blocked for 30 min with 5% nonfat dry milk solids in PBS+, and immunostaining was carried out using mAb 333 and HRP-conjugated, affinity-purified rabbit anti-rat IgG (ICN Immunobiologicals) as first and second antibodies, respectively. HRP activity was localized using 5 µg/ml 3,3'-diaminobenzidine, 0.01% H2O2 in 0.1 M Tris-HCl, pH 7.4.

### Results

**Purification and Characterization of a 37-kD Cell-binding Fragment**

Previous reports have described proteolytic cell-binding fragments derived from the central portion of fibronectin and containing the Arg-Gly-Asp cell recognition site ranging in size from 11.5 up to 120 kD (2, 19, 41, 17a, 46, 47, 57). Whereas the larger fragments bind to cells with similar affinities as the intact molecule, the small 11.5-kD fragment has been found to bind to cells with a markedly lower affinity (2). We have used a partial chymotrypsin digestion followed by chromatographic methods to generate a fibronectin cell-binding fragment that would be of an intermediate size between those of the fully active 75-kD fragment and the low affinity 11.5-kD fragment. As shown in Fig. 1 (lane 2), a homogeneous fragment with an estimated size of 37 kD was obtained after purification. This fragment was recognized by mAb 333, which binds to the previously described 11.5-kD cell-binding fragment and inhibits cell attachment to the Arg-Gly-Asp site (2. 21).

The sequence of the first 20 amino acids of the 37-kD fragment was found to be Thr-Val-Lys-Asp-Asp-Lys-Glu-Ser-Val-Arg-Gly-Asp site (2, 21).

Figure 1. Western blot analysis of chymotryptic digest of fibronectin and purified 37-kD cell-binding fragment. Fragments generated by treating human plasma fibronectin with chymotrypsin (lanes 1 and 3) or purified 37-kD fragment (lanes 2 and 4) were resolved by electrophoresis on a 10% polyacrylamide gel under nonreducing conditions followed by electrophoresis on a 10% polyacrylamide gel under nonreducing conditions followed by electroblotting to nitrocellulose. Fragments were stained with either amido black (lanes 1 and 2) or mAb 333 (which binds close to the RGD site of fibronectin) and HRP-labeled anti-rat IgG serum (lanes 3 and 4). The relative positions of molecular weight standards are indicated on the left.
sequence of this 37-kD fragment exactly matched that of a 34-kD fragment from bovine fibronectin isolated by Skorstengaard et al. (47) after extensive digestion with chymotrypsin. Analysis of carboxy-terminal amino acids by carboxypeptidase A treatment and amino acid analysis yielded 214 pmol Met and 174 pmol Gln from an initial 300 pmol of 37-kD fragment, indicating that the 37-kD human fragment has the same carboxy-terminal Gln-Met sequence as the bovine fragment (47). This fragment therefore starts at residue 1218 (Thr,128), lacks the ED-B domain, and terminates at the methionine at residue 1517 (using the published nomenclature omitting the ED-B sequence: refs. 28 and 47).1

Biological Activity of the 37-kD Fragment

The ability of solubilized 37-kD fragment to inhibit BHK fibroblastic cell spreading on fibronectin substrates was compared to that of the 11.5-kD fragment and synthetic GRGDS peptide. As shown in Fig. 2, the 37-kD fragment, the 11.5-kD fragment, and GRGDS peptide could all inhibit cell spreading on fibronectin with half-maximal inhibition occurring at concentrations of 47 μg/ml (1.2 × 10−6 M), 850 μg/ml (74 × 10−6 M) and 190 μg/ml (390 × 10−6 M), respectively. Thus, on a molar basis, the 37-kD fragment was 325-fold more active than the GRGDS peptide and 60-fold more active than the 11.5-kD fragment in this assay. These results indicate that information present in the 37-kD fragment, but lacking from the 11.5-kD fragment and synthetic peptide, is important for biological activity.

To compare directly the relative cell spreading activities of fibronectin and fragments, substrates were prepared by covalently coupling either intact fibronectin, the 37-kD fragment, or the 11.5-kD fragment to CovaLink activated polystyrene 96-well assay plates, and the relative amounts of each protein were measured by ELISA using duplicate parallel wells as described in Materials and Methods. The data, shown in Fig. 3, were plotted as the percentage of spread cells as a function of the relative amount of moles of fibronectin or fragment actually present on the substrate. This experiment showed that the 37-kD fragment had essentially the same cell spreading activity as fibronectin itself on a molar basis. The activity of the 11.5-kD fragment was very low, suggesting that the 11.5-kD fragment lacks information contained in the 37-kD fragment required for complete interaction with fibroblastic cells.

Characterization of mAbs Raised Against the 37-kD Fragment

Five hybridoma clones designated 11E5, 3B8, 13G12, 12B4, and 16G3 were selected for use in this study. All of these hybridoma clones secreted antibodies of the IgG subclass. The epitope of each mAb was analyzed by ELISA using a

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2. Using terminology based on the presently known full sequence (22, 36), the carboxy-terminal residue of the 37-kD fragment is Met608. This fragment contains the terminal 17 amino acids of the fibronectin repeating unit III-7, the entirety of III-9, III-10, and III-11, and eight amino acids of III-12. Its theoretical molecular size from the sequence of human plasma fibronectin in the absence of posttranslational modification would be 33 kD (28). For comparison, the 11.5-kD fragment extends from Ile109 to the same Met608 in the current full sequence nomenclature (36, 39, 41), and it consists primarily of unit III-11 (with the carboxy-terminal end of III-10 and the amino-terminal end of III-12).
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Table I. Epitope Mapping by ELISA

<table>
<thead>
<tr>
<th>mAb</th>
<th>Positive</th>
<th>Residue number</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>11E5</td>
<td>1217</td>
<td>1260</td>
<td></td>
</tr>
<tr>
<td>3B8</td>
<td>1355</td>
<td>1359</td>
<td></td>
</tr>
<tr>
<td>13G12</td>
<td>1359</td>
<td>1363</td>
<td></td>
</tr>
<tr>
<td>12B4</td>
<td>1363</td>
<td>1368</td>
<td></td>
</tr>
<tr>
<td>333</td>
<td>1384</td>
<td>1429</td>
<td></td>
</tr>
<tr>
<td>16G3</td>
<td>1429</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The epitopes for each of the mAbs generated in this study and for the previously described mAb 333 were mapped on the 37-kD fibronectin cell-binding fragment by ELISA using a variety of deletion mutants in the form of β-galactosidase fusion proteins expressed in the Agt11 bacterial expression system (38, footnote 2). The values in the table indicate the last amino acid residue present at the boundaries of the fibronectin sequence deletions from the amino terminus. For example, mAb 11E5 showed positive ELISA for deletions extending up to (but not including) residue 1234, but this mAb was negative for binding to adjacent deletion mutants, the closest of which retained residue 1260. The binding site for mAb 16G3 was the most carboxyl-terminal. The epitope for mAb 11E5 was also mapped for its carboxy-terminal boundary, which was residue 1329. All residue numbers are based on the numbering system for fibronectin of entry FNHU, accession number A03213, in the NBRF PIR (Protein Identification Resource) database, which omits the ED-B (Ellib) alternatively spliced domain and matches the notation of previous mutants (38).

Another method of mapping is based on numbering the 17 known type III repeats of fibronectin consecutively (ED-B would be numbered III-8 according to the nomenclature of ref. 36, p. 4). Although further mapping is needed to determine exact epitope boundaries, mAb 11E5 appears to bind to III-9; 3B8, 3G12, and 12B4 bind to portions of III-10 and possibly III-11; 16G3 recognizes III-11; and 333 binds III-10 and/or III-11.

Table II. Mutual Competition Binding Profiles of Anti-37-kD mAbs by ELISA

<table>
<thead>
<tr>
<th>Nonlabeled mAbs</th>
<th>Labeled mAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11E5</td>
</tr>
<tr>
<td>11E5</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>3B8</td>
<td>110 ± 6</td>
</tr>
<tr>
<td>13G12</td>
<td>88 ± 7</td>
</tr>
<tr>
<td>12B4</td>
<td>91 ± 7</td>
</tr>
<tr>
<td>16G3</td>
<td>116 ± 5</td>
</tr>
</tbody>
</table>

Competitive inhibition of binding between HRP-labeled mAbs and nonlabeled mAbs in all combinations was tested by ELISA. Aliquots of fibronectin coated onto Costar plates at a concentration of 5 μg/ml solution were preincubated with each of the nonlabeled mAbs (100 μl at 50 μg/ml) for 3 h at room temperature. Subsequently, 10 μl of HRP-labeled mAb (50 μg/ml) was added to each well and the antibodies were reincubated for 1 h at room temperature (nonlabeled mAbs labeled mAb 16G3 at 20 ng/ml). After washing, the activity of the HRP on the labeled mAbs bound to fibronectin was measured. Data were expressed as percentage of total absorbance observed without nonlabeled mAb (noncompeted control) for each mAb, which were as follows: 11E5, 1.86 ± 0.05 absorbance units; 3B8, 2.22 ± 0.12; 13G12, 1.99 ± 0.03; 12B4, 1.62 ± 0.01; and 16G3, 1.98 ± 0.06. All values represent the mean ± SEM of four assays.

Immunological Mapping of Two Separate Regions Required for Cell Adhesion

The biological activities of each mAb were examined in three different assays to evaluate fibronectin functions in cell spreading, cell migration, and matrix assembly. As shown in Fig. 4 (bottom), mAbs 3B8 and 16G3 were both found to inhibit BHK cell spreading, requiring only ~10 μg/ml for half-maximal inhibition. Similarly, half-maximal inhibition of HT-1080 fibrosarcoma cell spreading was also obtained with mAbs 3B8 and 16G3 at ~10 μg/ml. These two mAbs also inhibited BHK cell spreading on the 37-kD fragment itself, whereas even 100 μg/ml of each did not affect spreading on 5 μg/ml vitronectin (data not shown). In contrast to the inhibitory activity of mAbs 3B8 and 16G3 on fibronectin, mAbs 11E5, 13G12, and 12B4 had minimal inhibitory activity in this assay, even at concentrations of 100 μg/ml (Fig. 4). The inhibitory activity of mAb 16G3 can be explained by the proximity of its epitope to the Arg-Gly-Asp site. However, the inhibition by the mAb 3B8 can only be easily explained by its blocking of a second cell adhesion site within the 37-kD fragment. It is important to note that in this experiment, mAb 13G12 (and to a lesser extent 12B4), which bound to sites on the 37-kD fragment between those for the two inhibitory mAbs, can be considered negative controls that rule out possible steric effects of the inhibitory antibodies and that demonstrate separability of the two sites.

One possible trivial explanation of the cell spreading data is that the inhibitory mAbs bound to fibronectin with high affinity and the noninhibitory mAbs bound with low affinity. To rule out this possibility, the binding constant of each mAb for fibronectin was determined by radioimmunoassay. The inhibitory mAbs 3B8 and 16G3 were found to have estimated Kd values for fibronectin coated onto the plastic substrate of 1.3 × 10⁻⁷ and 1.0 × 10⁻⁷ M, respectively. The noninhibitory antibodies 11E5, 13G12, and 12B4 were found...
to have estimated $K_d$ values of $1.2 \times 10^{-9}$, $1.2 \times 10^{-10}$, and $1.6 \times 10^{-10}$ M, respectively. Thus, differences in the apparent affinity of the mAbs cannot account for the observed differences in biological activity, and the simplest interpretation of the data is that mAb 3B8 inhibits cell spreading by binding to a second, crucial cell-binding site ~15 kD distant from the RGD site in the NH₂-terminal direction on fibronectin.

**Immunological Mapping of Sites Required for Cell Migration**

Besides mediating adhesion, fibronectin can also promote migration by a variety of cell types. Fig. 5 shows that mAbs 3B8 and 16G3 at 200 μg/ml strongly inhibited cell migration by HT-1080 cells on fibronectin (72 and 84% inhibition, respectively; both values were statistically significant at the $p < 0.001$ level); half-maximal inhibition was observed at ~2 μg/ml of each of these two antibodies (data not shown). The inhibition of migration in this outgrowth assay was confirmed by time-lapse video microscopy: both mAb 3B8 and mAb 16G3 strongly inhibited the migration of HT-1080 cells as determined by following the paths of individual cells, whether they existed as single cells or as aggregates of cells in contact; although cells treated with these two mAbs remained attached to the fibronectin substrate, they moved back-and-forth randomly in place rather than undergoing directed translocation (data not shown). In outgrowth assays conducted in parallel to these anti-37-kD experiments, similarly high levels of inhibition of migration (75-100%) were obtained with mAb 16, a mAb that specifically inhibits function of the α5β1 fibronectin receptor in cell migration by this particular cell on fibronectin (data not shown; see also refs. 3 and 55).

In contrast, mAb 13G12 did not significantly inhibit migration (Fig. 5), even though the epitope for this mAb lies between those of the two highly active mAbs. mAb 12B4 reproducibly showed substantial but not complete inhibitory activity in these cell migration assays (60% inhibition, significant at the $p < 0.001$ level). Although minimal inhibitory activity was observed for this particular mAb in the BHK cell spreading assay for cell adhesion (see Fig. 4), significant activity was also found in parallel spreading assay conducted with the HT-1080 cells used for the cell migration assays (50% inhibition; Fig. 6).

**Immunological Mapping of Sites Required for Fibronectin Matrix Assembly**

Regions of the central cell-binding domain of fibronectin molecule required for its assembly into a fibrillar matrix were mapped by incubating human WI-38 fibroblasts with each mAb during the process of matrix assembly over a period of 24 h. As shown in Fig. 7, mAb 3B8 that binds to the putative synergy region inhibited strongly, and mAb 16G3 that binds close to the RGD site was also inhibitory, whereas the three other mAbs had little or no effect on this process. Direct quantitation of fibrillogenesis as shown in Fig. 8 showed that mAb 3B8 inhibited fibril formation by 75%, while mAb 16G3 inhibited by 63% at 200 μg/ml ($p < 0.001$ for both antibodies). Half-maximal inhibition of fibrillogenesis occurred at ~20 μg/ml for mAb 3B8 and at
Monoclonal Antibodies

40 µg/ml for mAb 16G3; similar patterns of inhibition were observed using a previously described immunoblotting protocol (3; data not shown). mAb 333, which also binds close to the RGD site (though not as closely as mAb 16G3), also inhibited matrix assembly by 73% (Fig. 8). There were only minimal amounts of inhibition by control mAbs that did not reach statistically significant levels, including by the mAbs located between the two active sites: mAb 13G12 showed 8% inhibition (p = 0.5), and mAb 12B4 showed only 15% inhibition (p = 0.3) in comparison with the untreated control (Fig. 8). As reported previously (3), mAb 16 and mAb 13 against the α and β subunits of the αβ fibronectin receptor also inhibited fibrillogenesis in this newer assay (Fig. 8; 75% inhibition). mAb 13 was the most effective of all mAbs inhibitors in this assay, inhibiting fibril formation by 86% (Fig. 8). These levels of inhibition can be compared with the 75% inhibition by 3B8. Interestingly, the maximal concentrations of the anti-αβ fibronectin receptor antibody and the antifibronectin synergy region antibody resulted in similar levels of inhibition (both inhibited 75 ± 5%), whereas the anti-β antibody mAb 13 showed additional inhibition (86 ± 4%).

**Discussion**

We have purified and characterized a 37-kD fragment of fibronectin that was found to retain cell adhesive activity similar to that of intact fibronectin. This human fibronectin fragment corresponds in structure to a protease-resistant fragment of bovine fibronectin sequenced by Skorstengaard et al. (47) containing the 9th through 11th type III repeats of fibronectin. Its resistance to proteolysis suggests that it possesses considerable structure, rather than existing as a linear polypeptide. Using this fragment as an immunogen, we have generated a panel of mAbs that bind to four distinct epitopes found in the 37-kD fragment. Applying these antibodies as functional probes, we find that two distinct regions of the central cell-binding domain are important for fibronectin-mediated cell spreading and migration, and also for the assembly of extracellular fibronectin fibrils. One region appears to involve the Arg-Gly-Asp site. The second region maps a substantial distance away from the RGD site, to a location estimated to be <15 kD of polypeptide sequence toward the amino terminus. This site, which is inhibited by...
mAb 3B8, is flanked by epitopes to which other mAbs bind with high affinity, yet binding to these flanking sites is not inhibitory. For example, mAb binding to an epitope estimated to be \( \geq 25 \text{kD} \) of polypeptide sequence away from RGD is not inhibitory, and an epitope with a boundary \( > 14 \text{kD} \) away from RGD is also not inhibitory in various biological assays.

These distances can be compared with rougher estimates from previous studies based on adhesive activity of several truncation mutants of fibronectin expressed in bacteria (38) or on partially inhibitory activities of mAbs (26). Our previous study showed a loss of activity after truncation of fibronectin sequences \( \geq 20 \text{kD} \) away (38). The mAb study mapped a partially active site 153–229 amino acid residues (17-25 kD) upstream from the RGD site (26). The finer mapping possible in the present study suggests that a particularly important region is located \( \sim 14-15 \text{kD} \) away from the RGD site, rather than the original rough estimates a greater distance away. This new estimate is of interest in that it moves the mapped site of this postulated second, synergy region away from the location of the alternatively spliced ED-B (EIIIA) site, reducing the likelihood that alternative splicing could directly affect the biological function of this region. Recent fine-structure mapping of the putative synergistic region by site-directed mutagenesis has confirmed that a major functional subregion corresponds to the binding site of mAb 3B8 (4a). A second, quantitatively less important subregion was mapped further toward the amino terminus (4a), corresponding to the originally described site of loss of activity (38) and possibly to the binding site of the partially active mAb described by Katayama et al. (26).

The 37-kD proteolytic fragment we describe is half the size of the smallest fragment previously reported to retain full biological activity (19). As such, it directly confirms the prediction from mutagenesis studies that at least 52% of the amino-terminal sequence of fibronectin is not necessary for cell adhesion. The sequences in fibronectin required for cell migration require further characterization. The Arg-Gly-Asp sequence of fibronectin and other molecules is thought to be required for the migration of certain embryonic and adult cells based on competitive inhibition studies (6, 10, 30, 48). More extensive studies of neural crest derivatives reveal complex requirements for sites besides the RGD sequence; these cells might use the CSI alternatively spliced sequence, the synergy region, and other sequences in fibronectin for migration or neurite extension (11, 21, 31, 37).

In this study, we examined cell migration in a system where the requirements for migration were simpler. HT-1080 cells were chosen because of their marked dependence on the \( \alpha_5 \beta_1 \) fibronectin receptor for migration (55). Under our present assay conditions, an antibody against either the \( \alpha_5 \) or \( \beta_1 \) subunits blocks migration. Conversely, antibodies against either subunit can substitute for the ligand and serve as substrates for migration (55). Using this cell line, we find by antifibronectin mAb analysis that both the synergy region and the RGD region are important for migration mediated by this particular receptor. A mAb that binds to fibronectin between these regions with high affinity (mAb 13G12) is not inhibitory. Consistent with the results, preliminary binding studies indicate that 3B8, the mAb that binds to the synergy region, inhibits the binding of \( \alpha_5 \beta_1 \) to a fibronectin cell-binding domain affinity column.

Extracellular matrix assembly is a complex process thought to be dependent on integrin receptors and on both an amino-terminal 70-kD domain and the central cell-binding domain of fibronectin (34, 36). Antibodies against the \( \alpha \) subunit of the classical fibronectin receptor \( \alpha_5 \beta_1 \) partially inhibit fibronectin matrix assembly (3), and transfection of this receptor into CHO cells substantially promotes matrix assembly (16). Nevertheless, at least one other \( \beta \) integrin appears to be involved in matrix assembly, since full inhibition is obtained only by blocking the functions of all \( \beta \) integrins (13). Our present results indicate that important sites are located in both the RGD region and the synergy region of fibronectin. mAbs against either of these sites strongly inhibit fibronectin matrix assembly. Interestingly, none of the antifibronectin mAbs alone inhibited matrix assembly to the same degree as the antiintegron \( \beta \) mAb, consistent with earlier findings that interactions of fibronectin with multiple \( \beta \) integrins are required for proper fibril formation (13, 42).

A number of integrin receptors have been reported to bind to fibronectin, including \( \alpha_5 \beta_1 \), \( \alpha_6 \beta_1 \), \( \alpha_5 \beta_1 \), and \( \alpha_5 \beta_1 \). Direct binding to the RGD motif is thought to explain the function of many of these fibronectin receptors (4, 5, 8, 12, 17, 20, 22, 23, 25, 36). It is interesting to compare the requirements for different receptors for differing cellular functions involving fibronectin; initial cell attachment to fibronectin often involves the classical \( \alpha_5 \beta_1 \) receptor. Cell spreading on fibronectin can require this receptor (55) or proceed quite well...
Figure 7. Inhibition of fibronectin extracellular matrix assembly by anti-37-kD mAbs. WI-38 human lung fibroblasts were cultured for 24 h with or without the indicated mAbs at 200 μg/ml, then fixed, permeabilized, and stained with FITC-labeled rabbit antihuman fibronectin antibody as described previously (3). These immunofluorescence micrographs were taken and printed using identical exposure times to permit direct comparisons. (a) Untreated control; (b) mAb 11E5; (c) mAb 3B8; (d) untreated control; (e) mAb 13G12; (f) mAb 12B4; (g) mAb 16G3; (h) control rat mAb 13H7. Bar, 20 μm.

Figure 8. Quantitation of the inhibition of fibronectin matrix assembly by mAbs. WI-38 fibroblasts were cultured for 24 h in the presence of 200 μg/ml of the indicated mAbs, and then stained with FITC-labeled antifibronectin antibody. Fibrils observed to cross a line of fixed length in random microscopic fields were counted for each experimental condition. Bars indicate the average of data from 10 random fields for each condition showing numbers of fibrils per 100 μm ± SEM.

in other cells such as WI-38 fibroblasts even if it is inhibited (4). Migration on fibronectin can be inhibited by antibodies to this receptor in HT1080 cells and others, yet WI-38 fibroblasts show no inhibition or even an augmentation of migration rates. Fibronectin matrix assembly is partially dependent on α5β1, yet may also require other β1 integrins (4, 13, 16, 42).

This complex picture is further complicated by the question of whether more than the RGD site is needed for various functions. Mutagenesis studies have implicated a second, synergistic site in cell adhesion to fibronectin by BHK cells (27, 38). Such studies are undermined by concerns about the effects of mutations on polypeptide conformation, which may help to explain the discrepancies between these investigations (compare refs. 27, 38, and footnote 2). This study provides independent, immunological evidence for the role of a second region in the central cell-binding domain of fibronectin that is involved not only in cell adhesion, but in at least two other major functions of the protein. The fact that this epitope located distant from the RGD site, but not one or two intervening epitopes, is implicated in three different processes involving cell interactions with fibronectin indicates that this region is a functional site that either binds directly to one or more key receptors, or must be in close
proximity. In contrast, one or two intervening fibronectin sequences are apparently not close in the folded protein. The slightly different profile of inhibition for cell migration as opposed to matrix assembly involving increased relative sensitivity of migration to 12B4 (Fig. 5) suggests the possibility of slightly different types of receptor interaction with this synergistic region of fibronectin.

An attractive but unproven hypothesis that could explain all of our immunological and mutagenesis results, is that the αβ, fibronectin receptor binds directly to both the RGD site and the synergistic region. Approaches to evaluate this hypothesis would include determination of the three-dimensional molecular structure of the entire fibronectin cell-binding domain by x-ray crystallography or nuclear magnetic resonance spectroscopy, as well as chemical crosslinking experiments. It is of interest that an x-ray inactivation study attempting to determine the minimal functional size of the cell-binding site of the fibronectin molecule estimated a polypeptide target size of 32 kD, rather than only the RGD tripeptide sequence (56). This target size of 32 kD is quite close to that of the 34–37-kD protease-resistant domain described by Skorstengaard et al. (47) and characterized biochemically and immunologically in the present paper.

Our characterization of the mAb 3B8 site does not exclude the possibility that other cell-binding sites in the fibronectin molecule can be important for cell adhesion. The cell-binding site located in the IICS region (21) is important for the adhesion of melanoma cells and other cells derived from the neural crest. The adhesion-promoting sites in the heparin-binding domain can also be important for certain cell types, especially for the formation of focal contacts and microfilament bundles (24, 32, 51). Although none of these other sites have been shown to interact with the high affinity αβ integrin fibronectin receptor as do the sites contained in the 37-kD fragment, it will be important to extend studies on fibronectin-mediated cell adhesion to determine if any or all of these various cell interaction sites on fibronectin can interact in an independent, cooperative, or synergistic fashion.

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